

Benzodithiophenes Induce Differentiation and Apoptosis in Human Leukemia Cells

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Abstract

All-trans retinoic acid (ATRA) induces clinical remission in patients with t(15;17) acute promyelocytic leukemia (APL) carrying leukemogenic promyelocytic leukemia-retinoic acid receptor α (PML-RAR α) fusion protein by overcoming PML-RAR α transcriptional repression and inducing myeloid differentiation. To identify more potent chemical differentiation inducers, a screening assay was developed utilizing an ATRA-insensitive NB4 cell line (NB4-c) in which differentiation could be measured after 48 hours when primed with ATRA followed by other potential inducers. Over 300 cytostatic agents selected from the National Cancer Institute library were screened using this established method. Three compounds, NSC656243, NSC625748, and NSC144168, were identified to amplify ATRA-induced differentiation with acceptable cytotoxicity in NB4-c cells. In the absence of ATRA, these compounds also induced HL-60 and murine erythroleukemia cells to undergo partial differentiation. NSC656243, a benzodithiophene compound, was selected for further studies to examine the underlying mechanism of action. The differentiation effect of NSC656243 was associated with enhanced ATRA-mediated up-regulation of cell cycle regulatory proteins p21^{waf1} and p27^{kip1}, retinoblastoma dephosphorylation, expression of *RIG-E* and *RIG-G*, and myelomonocytic differentiation-specific down-regulation of the *myeloperoxidase (MPO)* gene. Moreover, at 2- to 3-fold higher concentrations than those used to synergize with ATRA, NSC656243 induced apoptosis in NB4-c cells by reactive oxygen species-mediated pathways. The dual effects of benzodithiophenes (i.e., differentiation and apoptosis induction) support further development of these compounds as therapeutic agents for leukemia. (Cancer Res 2005; 65(17): 7847-55)

Introduction

Treatment with *all-trans* retinoic acid (ATRA), a normal metabolite of vitamin A, in combination with chemotherapy, results in clinical remission in acute promyelocytic leukemia (APL) patients with t(15;17) translocation that encodes the promyelocytic leukemia-retinoic acid receptor α (PML-RAR α) leukemogenic fusion protein (1–3). PML-RAR α is a dominant repressor of normal RAR α function and blocks granulocytic differentiation (4, 5). It has

been found that PML-RAR α binds to the RAR α nuclear receptor corepressors SMRT, N-CoR, and histone deacetylase more tightly than wild-type RAR α and, therefore, cannot be dissociated by physiologic ATRA concentrations (6–9). However, pharmacologic ATRA concentrations dissociate the corepressors from PML-RAR α and overcome PML-RAR α transcriptional repression (7, 8). Inhibitors of histone deacetylase, such as trichostatin A and butyrates, synergize with ATRA-mediated differentiation in APL cells; phenylbutyrate, in combination with ATRA treatment, induced clinical remission in one patient with APL in ATRA-resistant relapse (10, 11). However, preclinical research predicts that high (mmol/L) concentrations with currently used inhibitors of histone deacetylase, such as phenylbutyrate or valproic acid, will be required to reach a therapeutic effect (12–14). Therefore, identification of more potent agents to enhance ATRA-induced differentiation is needed. Previously, we reported that APL t(15;17) NB4 cells are induced to differentiate by ATRA but not by nonretinoid inducers (15). However, ATRA-induced differentiation is amplified and more rapid when combined with various nonretinoid chemical differentiation inducers (15). This synergism is termed superinduction of differentiation and considered a desirable therapeutic goal. A rapid (24–48 hours) assay was established to screen for compounds that enhance ATRA-induced differentiation. Three hundred and seventy-one cytostatic agents were selected from the National Cancer Institute (NCI) library and screened. Three compounds, NSC656243 (4,8-dioxo-benzo[1,2-b:5,4-b']dithiophene-2-carboxylic acid), NSC625748 (3,6-bis[1-azetidiny]-1,2,4,5-tetrazine), and NSC144168 (3-bromomethyl-2[1H]-quinoxalinone), at low μ mol/L concentrations, were identified to enhance ATRA-induced differentiation of NB4-c cells. Moreover, these agents were also able to partially induce HL-60 and murine erythroleukemia cell differentiation in the absence of ATRA priming. We further evaluated NSC656243, a benzodithiophene (16, 17), which was the most potent differentiation inducer and induced apoptosis at 2- to 3-fold higher concentrations.

Materials and Methods

Cell culture. Parental NB4 cells (kindly provided by Dr. M. Lanotte) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and slowly became ATRA differentiation insensitive (NB4-c cells). Thereafter, aliquots of NB4-c cells were frozen and thawed as needed for ATRA priming experiments (15). HL-60 cells (obtained from Dr. Robert Gallagher, Montefiore Medical Center, Bronx, NY) were maintained in RPMI 1640 supplemented with 10% FBS (18). Mouse erythroleukemia line DS-19 (obtained from Dr. Paul Marks, Memorial Sloan-Kettering Cancer Center, NY) were maintained in DMEM medium with 15% FBS (19). HL-60/Bcl-2 and HL-60/neo cells transfected with Bcl-2 expression vector or vector alone were obtained from Dr. Michael Cleary (Stanford University School of Medicine, Stanford, CA) and were cultured as described (20). All cell lines

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were passaged in late log phase; for each experiment, there was preseeding to maintain log phase.

Screening assay for differentiation inducers. Compounds were selected for study from the NCI library of compounds previously evaluated in the 60–cancer cell line program. Three hundred and seventy-one cytostatic compounds, identified as those that maintained multilog activity between 30% and 60% growth inhibition in the 48-hour NCI screening assay, were selected on the basis of stability, solubility, and availability. NB4-c cells were primed with ATRA (0.5 $\mu\text{mol/L}$ for 1 hour), washed, resuspended, and distributed into 96-well plates at 60,000 cells per well. Negative and positive controls were NB4-c cells treated with 0.5 $\mu\text{mol/L}$ ATRA alone or ATRA + 2 mmol/L hexamethylene bisacetamide (HMBA), respectively, as described previously (15). Compounds to be tested were added at the estimated 50% growth inhibitory concentration (GI_{50}) or at a 10-fold dilution of the GI_{50} concentration. The plates were incubated at 37°C for 48 hours; during this time, they were checked for gross contamination and the presence of precipitated drug. After incubation, they were centrifuged at 1,700 rpm for 10 minutes, the drug-containing media was aspirated, and differentiation induction was assayed by adding 200 μL of media containing 20 μL per stock nitroblue tetrazolium (NBT) and 1 $\mu\text{L/mL}$ tetradecanoylphorbol acetate and incubating for 25 minutes. Following centrifugation, the NBT media was removed and 200 μL of 10% SDS/0.4 NH_4Cl warmed to 70°C were added to each well. The plates were read on a Bio-Tek reader using 750 nm filters. The plates were then placed in the –70°C freezer. To assay toxicity, the plates were removed and placed into a 60°C oven for 2 hours. Fifty microliters of propidium iodide (PI) were added and incubated in the dark for 2 hours. PI was able to penetrate intact cells as a result of freezing. The plates were read on a Millipore fluorescence reader with filter set at EX530/25 and EM 620-40. The association between induction of differentiation and growth inhibitory effects of the compounds was calculated using the ratio of NBT/PI measured in cells treated with drug + ATRA and compared with that of cells treated with ATRA alone. A test compound was considered effective if at $\mu\text{mol/L}$ concentration, the enhancement of ATRA differentiation was equal or superior to that induced by HMBA, and growth inhibition was <65%. Several of these compounds, including HMBA, were submitted for blind, independent assessment of growth inhibition, cytotoxicity, and differentiation in a 5-day assay by using regular methods described below. The integrity of this rapid screening assay was confirmed because the same lead compounds were identified using more complete 5-day differentiation assay.

Nonscreening assessment of toxicity and differentiation. The percentage of viable cells after treatment was determined by trypan blue exclusion. Myeloid differentiation of HL-60 and NB4-c cells was determined

by NBT reduction assay as described before (21). Erythroid differentiation of murine erythroleukemia cells was determined by benzidine staining assay for hemoglobin as described before (22).

Western blot analysis. Protein extracts (50 μg), prepared with radio-immunoprecipitation assay lysis buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, 1 mmol/L phenyl-methylsulfonyl fluoride, 100 mmol/L leupeptin, and 2 $\mu\text{g/mL}$ aprotinin (pH 8.0)], were separated on a 8% or 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were stained with 0.2% Ponceau S red to assure equal protein loading and transfer. After blocking with 5% nonfat milk, the membranes were incubated with antibodies to p27^{kip}, p21^{waf1}, retinoblastoma, Bcl-2, and poly(ADP-ribose) polymerase (PARP; Oncogene, Co., San Diego, CA) proteins. The immuno-complex was visualized by chemiluminescence.

Northern blot analysis. Total RNA was isolated with RNazol B (Bioctax) from 10^6 cells. Twenty micrograms RNA was sized fractionated on a 1.2% agarose-2.2 mol/L formaldehyde gel, transferred to hybrid-N+ membrane (Amersham, Piscataway, NJ) in 20 \times SSC, and UV cross-linked (Stratalinker). *RIG-E* and *RIG-G* cDNAs were cloned as in our previous reports (23, 24). *Myeloperoxidase (MPO)* cDNA was provided by Dr. P. Koeffler (UCLA School of Medicine, Los Angeles, CA; ref. 25). *Glyceraldehyde-3-phosphate dehydrogenase* cDNA was obtained from Ambion (Austin, TX). The probes were labeled with [$\alpha^{32}\text{P}$]dCTP by random priming to a specific activity of 0.5 to 1×10^9 cpm/ μg . The membranes were prehybridized for 4 hours at 42°C in 50% formamide, 6 \times saline-sodium phosphate-EDTA, 5 \times Denhardt's reagent, and 0.2 mg/mL ssDNA, and hybridized with radio-labeled probe.

Apoptosis analysis. Apoptotic cells stained with acridine orange and ethidium bromide were assessed by fluorescence microscopy. Briefly, 1 μL of stock solution containing 100 $\mu\text{g/mL}$ acridine orange and 100 $\mu\text{g/mL}$ ethidium bromide were added to 25 μL of cell suspension. Total cells, as well as apoptotic cells that showed nuclear shrinkage, blebbing, and apoptotic bodies, were counted. DNA fragmentation analysis was done as previously described (26).

Results

Identification of compounds that enhance differentiation of all-trans retinoic acid-primed NB4-c cells. Based on our previous report (15), we developed a rapid screening assay to assess the effectiveness of compounds to enhance ATRA-induced differentiation in NB4-c cells. NB4-c cells, primed for

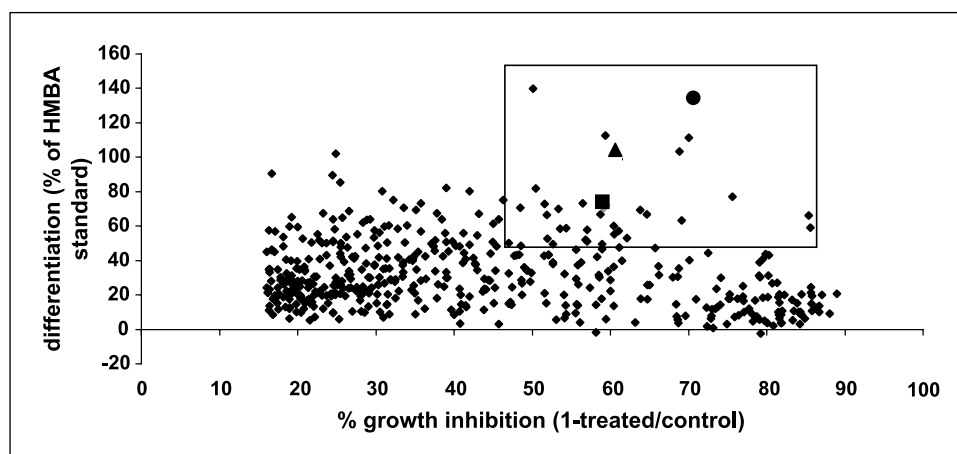
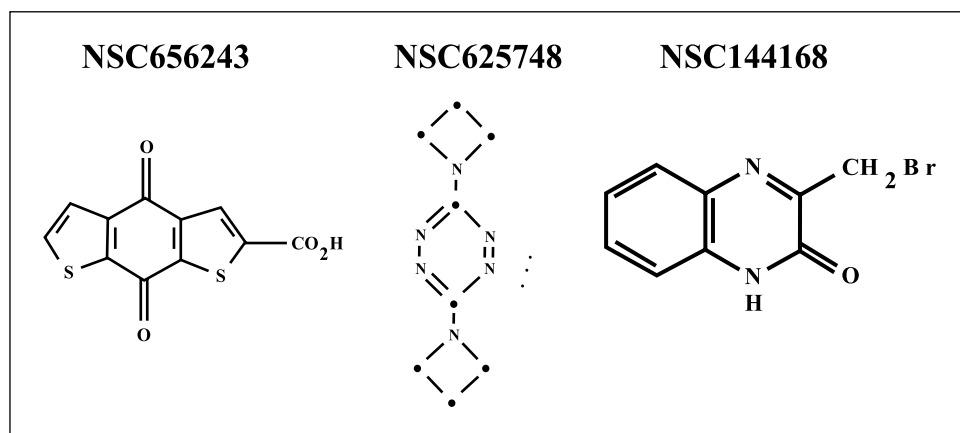


Figure 1. Correlation between growth inhibition and differentiation induction in 371 unique agents. Differentiation induction by these compounds was measured by development of NBT color. Growth inhibition was measured by PI staining in freeze-thawed NB4-c cells primed with ATRA followed by 48 hours treatment with each compound as described in Materials and Methods. Extent of differentiation was expressed as a ratio of the production of NBT color of NB4-c cells primed with ATRA following 48 hours drug treatment as a fraction of surviving cells measured by PI (NBT/PI) compared with NBT/PI from NB4-c cells primed with ATRA and treated with HMBA, the positive control. ● (large symbol), NSC656243; ■, NSC625748; ▲, HMBA. The compounds in the square were selected for confirmation of differentiation with a more detailed NBT assay as described in Materials and Methods.

Figure 2. Chemical structures of NSC656243, NSC625748, and NSC144168, which can enhance ATRA-induced differentiation in NB4-c cells.



1 hour with 0.5 $\mu\text{mol/L}$ ATRA and washed, show minimal differentiation induction (<10%) as measured by the NBT assay after 48 hours in culture. A 4-fold induction of differentiation was shown upon the addition of 2 mmol/L HMBA to the ATRA-primed NB4-c cells (15). A subset of compounds (371) were selected from the NCI compound library, based on a cytostatic profile, defined as maintenance of multilog activity between 30% and 60% growth inhibition in the NCI 60 cell line, 48-hour screening assay. These were compared with HMBA as a positive control and evaluated for enhancement of ATRA-induced cell differentiation. No correlation was found between extent of toxicity and degree of enhanced differentiation among the treatment by these compounds because most compounds were inactive in this screening assay (<20%) regardless of toxicity (Fig. 1). Several compounds enhanced ATRA-induced cell differentiation with 40% to 70% growth inhibition but without significant cytotoxicity (Fig. 1); however, those selected for further study reflect those without restriction and with a replenishable supply.

To validate the rapid screening assay, we selected 20 compounds for a blinded, more extensive 3- and 5-day study of drug-induced differentiation by NBT assay, and cytotoxicity using trypan blue exclusion. The differentiation enhancers identified in the initial screening assay were confirmed in these more detailed studies, thus validating the screening assay. Three compounds, NSC656243, NSC635748, and NSC144168 (structures are shown in Fig. 2), exhibited similar or greater differentiation than that by the positive control, HMBA (Table 1). This differentiation-enhancing effect was not associated with significant cell death as measured by trypan blue (data not shown). These active compounds were significantly more potent than HMBA, requiring $\mu\text{mol/L}$ rather than the 2 mmol/L concentration needed for HMBA. Differentiation induction in NB4-c cells by NSC656243 was comparable with HMBA and most effective at 5 $\mu\text{mol/L}$, 635748 at 150 $\mu\text{mol/L}$, and 144168 at 20 $\mu\text{mol/L}$. There was minimal differentiation induction in the absence of ATRA priming and it is consistent with reports that ATRA is needed for differentiation induction of NB4 cells (15). HMBA is a powerful differentiation inducer of mouse erythroleukemia and human HL-60 leukemia cells at concentration of 2 to 4 mmol/L (27, 28). The three selected compounds are also capable of inducing partial murine erythroleukemia and HL-60 cell differentiation with minimal toxicity at lower concentrations than that required for HMBA (Table 1).

Dose-dependent effect of NSC656243, a benzodithiophene, on NB4-c, HL-60, and murine erythroleukemia cell differentiation.

Because NSC656243, a benzodithiophene compound, enhanced ATRA-primed NB4-c cells to undergo differentiation at 400-fold lower concentrations than that of HMBA and 3- to 30-fold lower than that of NSC144168 and NSC625748 (Table 1), it was selected for further study. The dose-dependent effect of NSC656243 on induction of differentiation and cytotoxicity was studied in the three cell lines. As shown in Fig. 3A, NSC656243 at a concentration of below 5 $\mu\text{mol/L}$ did not inhibit cell growth but induced <10% differentiation in NB4-c cells. When the cells were primed with 0.5 $\mu\text{mol/L}$ ATRA followed by 1 to 5 $\mu\text{mol/L}$ NSC656243, minimal cell growth inhibition was found without cell death (>90% of the cells alive), and 80% NBT-positive cells were detected in this sequential treatment (Fig. 3B). Similarly, NSC656243 also enhanced the induction of differentiation in ATRA-primed parental NB4 cells. Only 15.3% of NBT-positive cells were obtained in parental NB4 cells primed with 0.1 $\mu\text{mol/L}$

Table 1. The concentrations of NSC compounds used for differentiation induction in NB4-c, HL-60, and murine erythroleukemia cells

	NSC625748	NSC656243	NSC144168	HMBA
Concentrations ($\mu\text{mol/L}$)				
NB4-c				
+ATRA	150	5	20	2,000
-ATRA	—	—	—	—
HL-60	400	5	14	2,000
Murine erythroleukemia	500	20	10	4,000
Percentage differentiation				
NB4-c				
+ATRA	60	83	73	81
-ATRA	3	8	6	3
HL-60	50-63	25-30	58	44
Murine erythroleukemia	97	40-50	55	91

NOTE: Percentage differentiation: benzidine positive after 6-day treatment in murine erythroleukemia cells, and NBT positive after 5-day treatment in HL-60 and 3-day treatment in NB4-c.

ATRA without NSC656243 treatment and 64.4% of parental NBT-positive cells was obtained in NB4 cells primed with 0.1 $\mu\text{mol/L}$ ATRA followed with 5 $\mu\text{mol/L}$ NSC656243 for 3 days. NSC656243 induced 30% of HL-60 cells to undergo differentiation at 6 $\mu\text{mol/L}$ with 20% growth inhibition (Fig. 3C). Unlike NB4-c and HL-60 cells, murine erythroleukemia cells were less sensitive to NSC656243-induced growth inhibition. NSC656243 at 20 $\mu\text{mol/L}$ induced 40% of murine erythroleukemia cells to undergo erythroid differentiation with 20% cell growth inhibition without cell death (Fig. 3D).

NSC656243 enhances all-trans retinoic acid-dependent gene induction. The effect of NSC656243 on ATRA-up-regulated gene expression was studied in NB4-c cells after cotreatment with ATRA (0.5 $\mu\text{mol/L}$) in combination with NSC656243 (5 $\mu\text{mol/L}$), because enhancement of differentiation was similar to ATRA priming followed by NSC656243 (29). As shown in Fig. 4, the known ATRA-responsive genes *RIG-E* and *RIG-G* were weakly induced by treatment with ATRA alone and not by NSC656243 alone. A synergistic induction was found with the combined treatment. *MPO* is a down-regulated gene during ATRA induction of differentiated NB4 cells (25). In NB4-c cells, ATRA or NSC656243 alone weakly down-regulated *MPO* expression, but the down-regulation was synergized by the combination treatments (Fig. 4). The cell cycle- and differentiation-related proteins (p21^{waf1}, p27^{KIP1}, and retinoblastoma) were evaluated in NB4-c cells after ATRA plus NSC656243 treatment and were weakly induced by

treatment with ATRA or NSC656243 alone, whereas strong sustained induction was found with their combined treatment (Fig. 5). The response to the drug combination was also associated with retinoblastoma hypophosphorylation (Fig. 5). According to these data, it seems that ATRA-inducible gene expression is minimal by NSC656243 alone but is enhanced by combinatorial treatment in NB4-c cells. Thus, ATRA, but not nonretinoid chemical inducers, plays a critical role in the differentiation program of NB4 cells as reported (15).

NSC656243 induces apoptosis in NB4-c and HL-60 cells. NSC656243, at 2-fold higher concentrations than the optimal concentration used to enhance ATRA differentiation, induced NB4-c and HL-60 cells to undergo cell death with the morphologic features of apoptosis. The induction of apoptosis is time and dose dependent (Fig. 6). NSC656243 at 5 $\mu\text{mol/L}$ concentration, which induced 83% differentiation of NB4-c cells in combination with ATRA (Table 1), induced <10% of NB4-c cell to undergo apoptosis. However, NSC656243 at 10 $\mu\text{mol/L}$ induced 70% apoptosis in NB4-c cells after 48 hours treatment. HL-60 cells were less sensitive than NB4-c cells to NSC656243-induced apoptosis. NSC656243 at 10 $\mu\text{mol/L}$ only induced 10% apoptosis in HL-60 cells, whereas 20 $\mu\text{mol/L}$ induced nearly the same amount of apoptosis in both NB4-c and HL-60 cells. A time-dependent study indicated that NSC656243 at 10 $\mu\text{mol/L}$ induced 15%, 58%, and 82% of apoptotic NB4-c cells after 1, 2, and 3 days treatment. However, at the same concentrations,

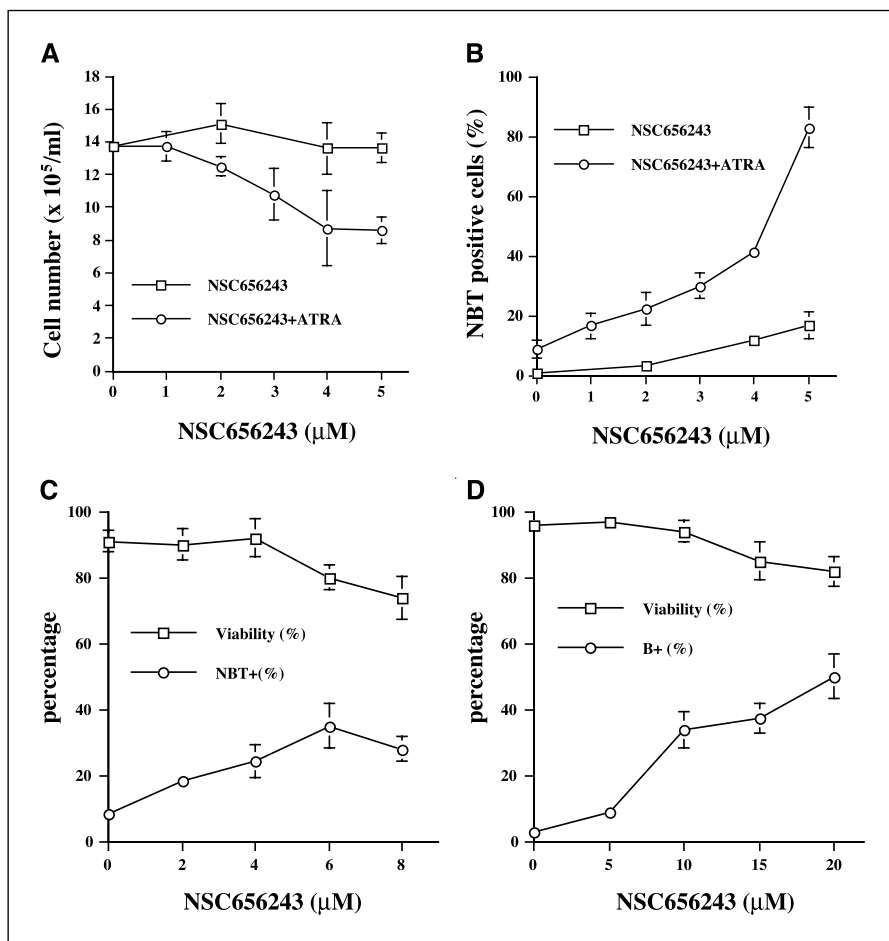


Figure 3. NSC656243 synergized ATRA-induced differentiation in NB4-c cells, HL-60, and murine erythroleukemia cells. **A**, growth inhibition in NB4-c cells. **B**, differentiation of NB4-c cells as determined by NBT reduction. NB4-c cells were treated with NSC656243 at indicated concentrations alone or combined with ATRA (0.5 $\mu\text{mol/L}$) for 3 days. **C**, cytotoxicity and differentiation of HL-60 cells (3-day treatment). **D**, cytotoxicity and differentiation of murine erythroleukemia cells (5-day treatment). Cell growth was measured by measuring cell number after staining with trypan blue and differentiated cells were measured by NBT reduction ability (NBT positive; NB4-c and HL-60 cells) or benzidine staining (B+; murine erythroleukemia cells).

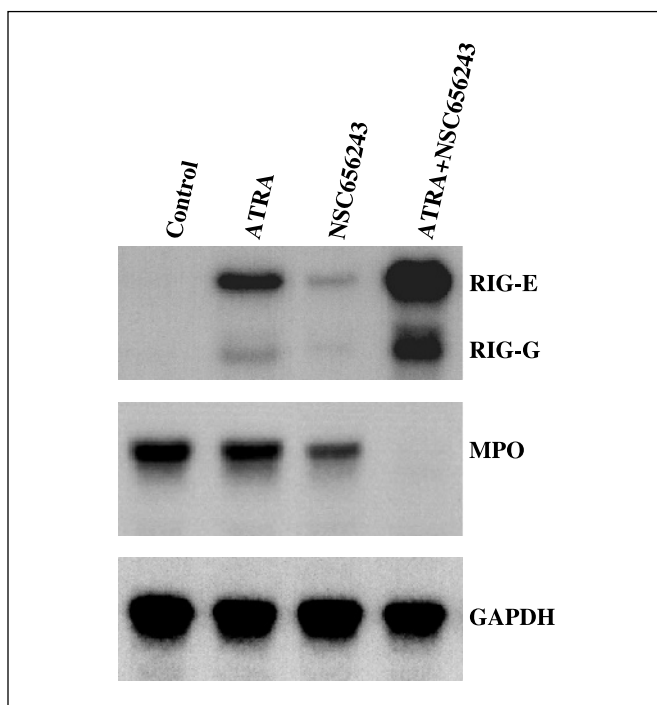


Figure 4. The effect of NSC656243 on ATRA-dependent gene expression in NB4-c cells. NB4-c cells were treated with 0.5 $\mu\text{mol/L}$ ATRA alone, 5 $\mu\text{mol/L}$ NSC656243 alone, or their combination for 3 days. Northern blot analysis was used to analyze the gene expression.

NSC656243 induced 5%, 12%, and 25% apoptotic HL-60 cells after 1, 2, and 3 days treatment (Fig. 6). NSC656243-induced apoptosis in NB4-c cells was confirmed by assay of DNA fragmentation and activation of caspase-3 as detected by PARP cleavage. Bcl-2 protein levels were not influenced by NSC656243 treatment (Fig. 7).

Antioxidants and Bcl-2 inhibited NSC656243-induced apoptosis. NB4-c cells were reported to be sensitive to apoptosis induced by agents that produce reactive oxygen species (30). NSC656243 contains a quinone structure that predicts an ability to produce reactive oxygen species. Antioxidants were used to evaluate the role of reactive oxygen species in NSC656243-induced apoptosis. *N*-acetylcysteine blocked NSC656243-induced apoptosis in NB4-c cells (Fig. 8A). Similarly, pyrrolidine dithiocarbamate, another antioxidant, inhibited NSC656243-induced apoptosis in NB4-c cells (Fig. 8B). To test the role of Bcl-2 in NSC656243-induced apoptosis, HL-60/Bcl-2 cells with forced Bcl-2 overexpression (Fig. 8D) were used to compare the NSC656243-induced apoptosis with vector alone-transfected cells. NSC656243 at 20 $\mu\text{mol/L}$ induced 70% apoptosis of HL-60/neo cells, but only 20% apoptosis of HL-60/Bcl-2 cells after 2 days treatment (Fig. 8C).

Benzodithiophene structural relationship to differentiation. Derivatives of NSC656243 with modifications of carboxylic acid group and ketone group were used to determine benzodithiophene moieties required for enhancement of differentiation and/or apoptosis. Replacement of the acidic group with an aldehyde (NSC656240, 2-hydroxymethyl-4,8-dihydrobenzo[1,2-*b*:5,4-*b'*]dithiophene-4,8-dione) or methyl group (NSC656238, 2-methyl-4,8-dihydrobenzo[1,2-*b*:5,4-*b'*]dithiophene-4,8-dione) significantly decreased the amount required to induce maximal

enhancement on ATRA-induced differentiation of NB4-c cells as measured by the NBT assay (Fig. 9). NSC682994 (4,8-dibutanoylbenzo[1,2-*b*:4,5-*b'*]dithiophene), a benzodithiophene modified to have an open quinone structure, was the most efficient ATRA differentiation enhancer with maximal effective concentration of 10 nmol/L.

Discussion

APL t(15;17) with PML-RAR α fusion protein is the only human cancer that has successfully undergone complete remission through induction of differentiation (31). PML-RAR α is a dominant-negative receptor blocking RAR α -mediated hematopoietic cell differentiation (32). Transcriptionally repressed RAR α involves binding of nuclear corepressors, adaptor proteins, and recruitment of histone deacetylase. Physiologic ATRA binding to RAR α releases the corepressors and leads to recruitment of coactivator proteins and activates gene transcription (7, 8). PML-RAR α , the APL oncogenic protein, binds the nuclear corepressor SMRT and N-CoR tightly through a nuclear corepressor (CoR) binding site located in the RAR α moiety and requires pharmacologic ATRA concentrations to dissociate nuclear corepressors from PML-RAR α protein and initiate transcription (33–36). This action has been thought to be the mechanism whereby pharmacologic ATRA concentrations induce APL cell differentiation. Theoretically, agents that facilitate the release or block the activity of corepressors will enhance ATRA-mediated gene induction and cell differentiation. Indeed, it has been found that inhibitors of histone deacetylase, such as sodium butyrate and trichostatin A, synergize ATRA-mediated differentiation by overcoming PML-RAR α transcriptional repression in APL cells and in one APL patient in relapse and resistant to ATRA treatment was successfully treated by the addition of sodium phenylbutyrate (10–13). Thus, the combination of ATRA with inhibitors of histone deacetylase may provide a powerful treatment for APL. However, the high therapeutic concentrations

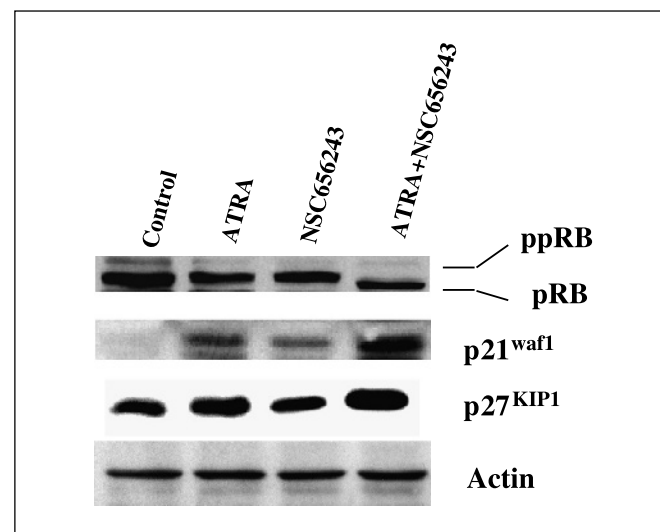


Figure 5. The effect of ATRA in combination with NSC656243 on p21^{waf1}, p27^{KIP1}, and retinoblastoma protein levels in NB4-c cells. The cells were treated with 0.5 $\mu\text{mol/L}$ ATRA alone, 5 $\mu\text{mol/L}$ NSC656243 alone, or their combination for 3 days. Western blot was used to analyze retinoblastoma, p21^{waf1}, and p27^{KIP1} protein levels.

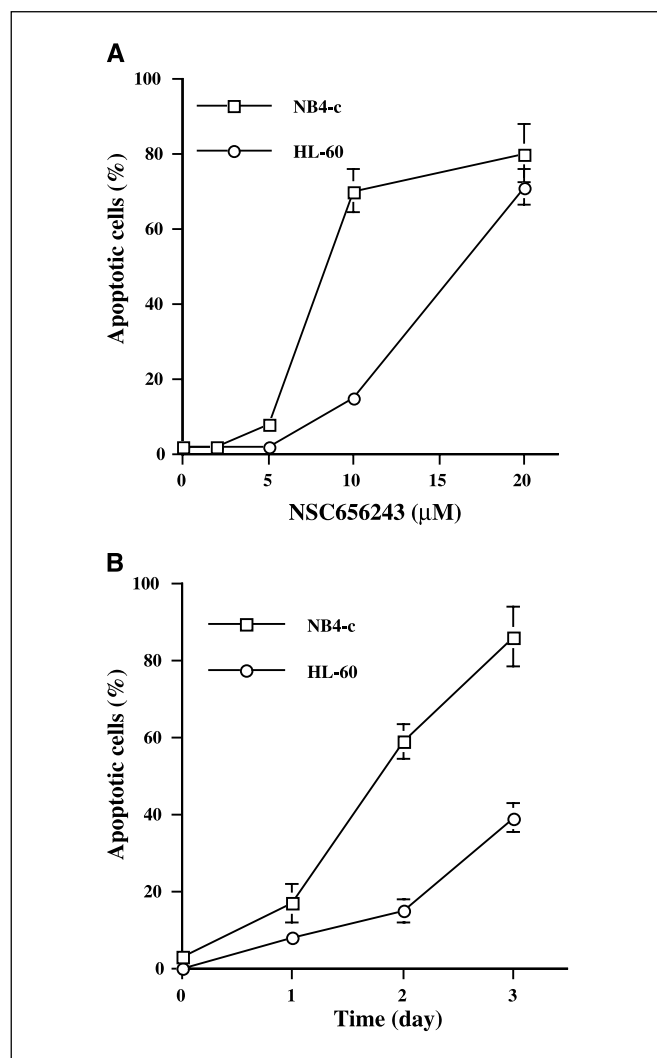


Figure 6. Dose- and time-dependent induction of apoptosis in NB4-c and HL-60 cells by NSC656243. *A*, dose-dependent effect. Cells were treated for 2 days with the indicated concentrations of NSC656243; *B*, time-dependent effect. Cells were treated with NSC656243 (10 μmol/L) for the indicated times. Apoptotic cells were detected by using fluorescence microscopy after staining with acridine orange and PI as described in Materials and Methods.

of these agents may limit their therapeutic effect *in vivo* and new agents that can enhance/synergize ATRA-induced differentiation need to be identified. NSC656243, NSC625748, and NSC144168 enhanced ATRA-triggered differentiation at or below μmol/L concentrations with modest cytotoxicity (Table 1), thus qualifying as potential agents for combinatorial treatment of APL with ATRA.

Enhanced induction of differentiation occurs in NB4-c cells treated with butyrate or HMBA after ATRA priming (8, 15). In contrast, differentiation is not enhanced in NB4-c cells primed with butyrate or HMBA then followed by ATRA (15). These observations support the critical role of ATRA in overcoming PML-RARα transcriptional repression. Butyrate, but not HMBA, induces global histone acetylation; however, both agents enhance ATRA-induced gene transcription and differentiation (37). In contrast to butyrate, but similar to HMBA, the compounds selected through the screen do not affect global histone acetylation (38). Consistent with this, NSC656243 does not

inhibit purified histone deacetylase activity, which is inhibited by butyrate using an *in vitro* assay (38). This suggests that NSC656243 enhances ATRA-mediated cell differentiation by a histone deacetylase inhibition-independent pathway. However, we have not excluded site-specific gene promoter histone acetylation modulated by NSC656243. Because NSC656243 could induce partial differentiation in HL-60 and murine erythroleukemia cells, which do not contain the PML-RARα translocation (Table 1 and Fig. 3), the enhanced effect of NSC656243 on ATRA-mediated differentiation in NB4-c cells may be by a pathway unrelated to PML-RARα transcriptional repression. NSC656243 alone induces 10% NB4-c cell differentiation, weakly increases p21^{waf1} protein, and decreases *MPO* mRNA levels (Figs. 4 and 5). The partial differentiation effect observed may result from enhancement by NSC656243 on physiologic factors, such as ATRA or granulocyte colony-stimulating factor, triggered cell differentiation.

When the NSC656243 concentration is doubled, marked cytotoxicity with features of apoptosis is observed (Figs. 6 and 7).

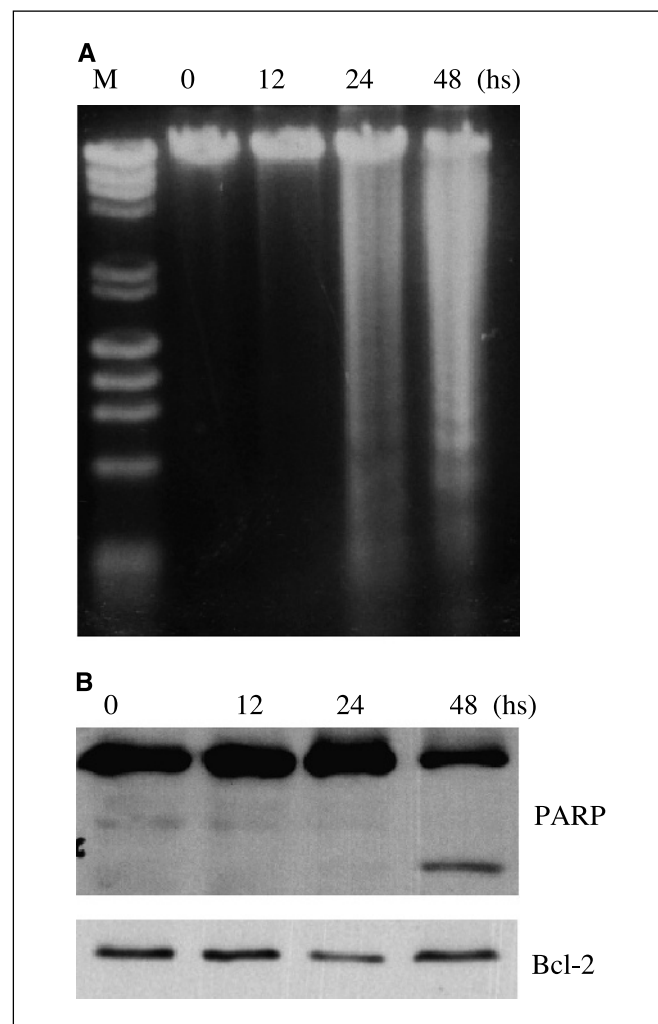
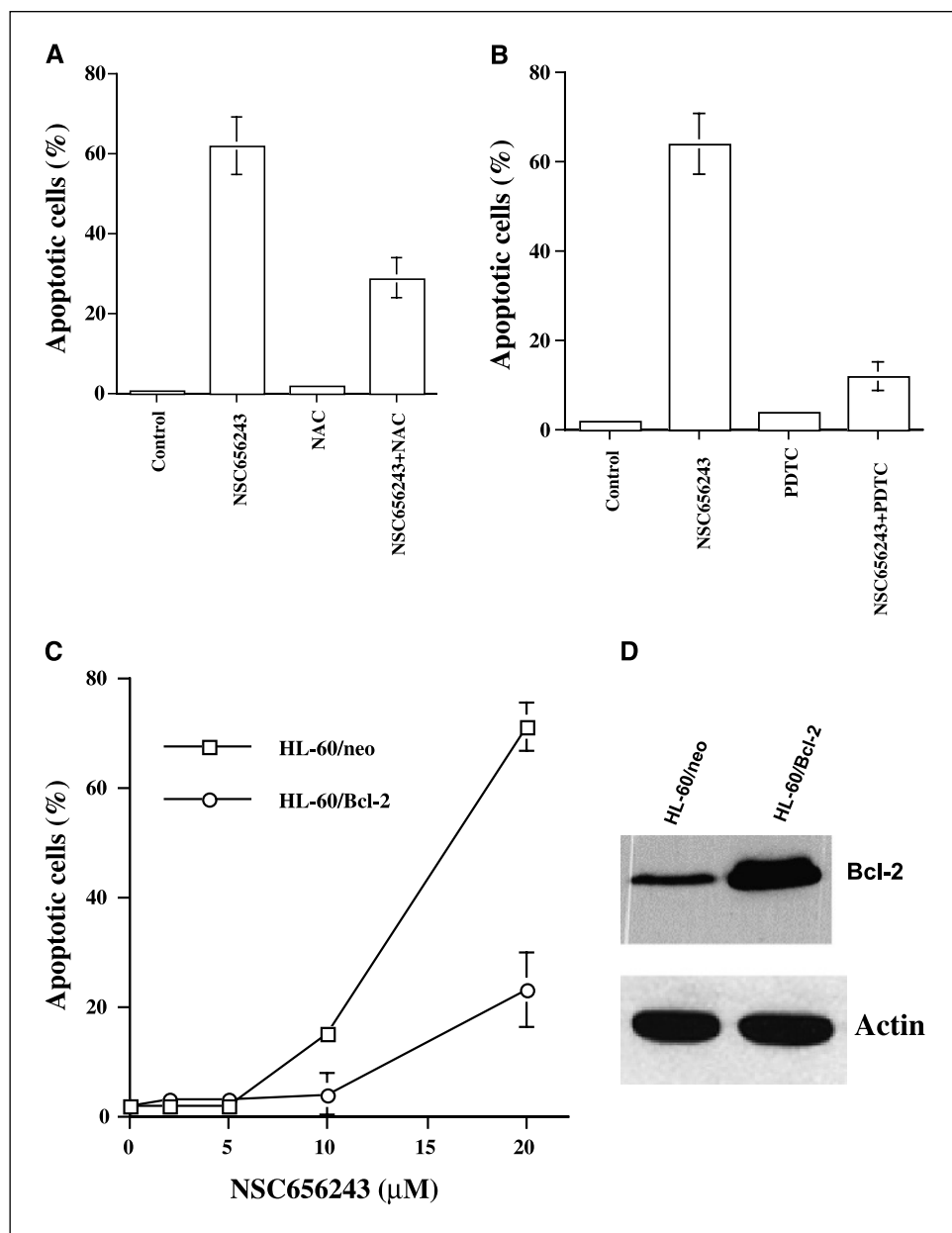


Figure 7. DNA fragmentation and PARP cleavage in NB4-c cells after treatment by NSC656243. *A*, DNA fragmentation. *B*, PARP and Bcl-2 protein levels. NB4-c cells were treated with NSC656243 (10 μmol/L) for the indicated times. DNA was isolated for fragmentation assay and protein was isolated for testing PARP and Bcl-2 levels by Western blot analysis as described in Materials and Methods.

Figure 8. Antioxidants and Bcl-2 inhibit NSC656243-induced apoptosis in NB4-c cells. *N*-acetylcysteine (NAC; 10 mmol/L; A) and pyrrolidine dithiocarbamate (PDTC; 20 μ mol/L) inhibited NSC656243 (10 μ mol/L) induced apoptosis in NB4-c cells (B). The cells were treated for 2 days. HL-60/Bcl-2 cells with overexpressed Bcl-2 protein (D) inhibited NSC656243-induced apoptosis (C). HL-60/neo and HL-60/Bcl-2 cells were treated at the indicated concentrations of NSC656243 for 2 days (C).



Moreover, NB4-c cells are more sensitive than HL-60 cells to NSC656243-induced apoptosis (Fig. 6). This is similar to arsenic trioxide (As_2O_3)-induced apoptosis in both cell lines (30). The finding of PARP cleavage indicates that activation of caspase-3 is involved in NSC656243-induced apoptosis (Fig. 7). Antioxidants, such as *N*-acetylcysteine and pyrrolidine dithiocarbamate, block NSC656243-induced apoptosis (Fig. 8), suggesting that reactive oxygen species production may mediate the process. NSC656243 and derivatives synergize or induce reactive oxygen species-mediated apoptosis in several solid tumor cell lines, including melanoma (39) and pancreatic cancer (40). Forced expression of Bcl-2, an inhibitor of reactive oxygen species-mediated apoptosis (41), decreased extent of apoptosis in NSC656243-treated HL-60/Bcl-2 cells (Fig. 8).

NSC656243 is a benzodithiophene that contains a quinone structure (Fig. 9; refs. 16, 17). A derivative with a reduced quinone

structure (NSC682994) at 10 nmol/L enhances ATRA-induced differentiation in NB4-c cells, making it the most potent derivative. However, it requires severalfold higher concentrations to induce apoptosis (data not shown). Thus, an intact quinone structure seems to be required for induction of apoptosis but not for induction of differentiation. ATRA is required for benzodithiophene-enhanced differentiation but not apoptosis of NB4-c cells (data not shown). Therefore, it seems that benzodithiophene enhancement of differentiation and induction of apoptosis occurs by a concentration-dependent activation of at least two independent pathways.

In summary, we find that several compounds from the NCI, Developmental Therapeutics repository, NSC656243, NSC625748, and NSC144168, induce leukemia cell differentiation by enhancing ATRA-mediated gene transcription at low concentrations and induce apoptosis at higher concentrations, which seems to

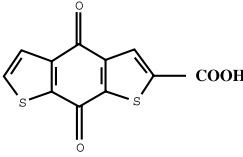
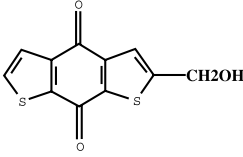
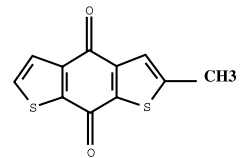
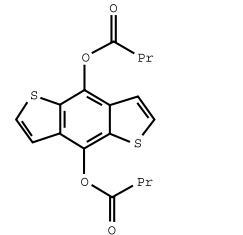
STRUCTURE	NUMBER	DOSE (μM)	NBT+ (%)
	NSC656243	5-7	53
	NSC656240	0.05	46
	NSC656238	0.05	50
	NSC682994	0.01	50

Figure 9. The enhancement of derivatives of NSC656243 on ATRA-induced cell differentiation of NB4-c cells. NB4-c cells were primed with 0.5 $\mu\text{mol/L}$ ATRA following indicated drugs at the indicated concentrations for 48 hours. NBT reduction was used to measure differentiation induction as described in Materials and Methods.

involve reactive oxygen species production. The sensitivity of APL cells to the dual effects of benzodithiophenes (i.e., differentiation at low concentrations and apoptosis induction at higher concentrations) implies that benzodithiophenes might be potent agents for treatment of leukemia alone or in combination with ATRA.

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References

- Huang ME, Ye YC, Chen SR, et al. Use of *all-trans* retinoic acid in the treatment of acute promyelocytic leukemia. *Blood* 1988;72:567-72.
- Castaigne S, Chomienne C, Daniel MT, et al. *All-trans* retinoic acid as a differentiation therapy for acute promyelocytic leukemia. I. Clinical results. *Blood* 1990; 76:1704-9.
- Warrell RP Jr, Frankel SR, Miller WH Jr, et al. Differentiation therapy of acute promyelocytic leukemia with tretinoin (*all-trans*-retinoic acid). *N Engl J Med* 1991;324:1385-93.
- de The H, Lavau C, Marchio A, Chomienne C, Degos L, Dejean A. The PML-RAR α fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. *Cell* 1991;66:675-84.
- Kakizuka A, Miller WH Jr, Umesono K, et al. Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR α with a novel putative transcription factor, PML. *Cell* 1991; 66:663-74.
- Perez A, Kastner P, Sethi S, Lutz Y, Reibel C, Chambon P. PMLRAR homodimers: distinct DNA binding properties and heteromeric interactions with RXR. *EMBO J* 1993;12:3171-82.
- Lin RJ, Nagy L, Inoue S, Shao W, Miller WH Jr, Evans RM. Role of the histone deacetylase complex in acute promyelocytic leukaemia. *Nature* 1998;391: 811-4.
- Guidez F, Ivins S, Zhu J, Soderstrom M, Waxman S, Zelent A. Reduced retinoic acid-sensitivities of nuclear receptor corepressor binding to PML- and PLZF-RAR α underlie molecular pathogenesis and treatment of acute promyelocytic leukemia. *Blood* 1998;91:2634-42.
- He LZ, Guidez F, Tribioli C, et al. Distinct interactions of PML-RAR α and PLZF-RAR α with co-repressors determine differential responses to RA in APL. *Nat Genet* 1998;18:126-35.
- Kosugi H, Towatari M, Hatano S, et al. Histone deacetylase inhibitors are the potent inducer/enhancer of differentiation in acute myeloid leukemia: a new approach to anti-leukemia therapy. *Leukemia* 1999; 13:1316-24.
- Warrell RP Jr, He LZ, Richon V, Calleja E, Pandolfi PP. Therapeutic targeting of transcription in acute promyelocytic leukemia by use of an inhibitor of histone deacetylase. *J Natl Cancer Inst* 1998;90:1621-5.
- Kitamura K, Hoshi S, Koike M, Kiyoi H, Saito H, Naoe T. Histone deacetylase inhibitor but not arsenic trioxide differentiates acute promyelocytic leukaemia cells with t(11;17) in combination with *all-trans* retinoic acid. *Br J Haematol* 2000;108:696-702.
- Gottlicher M, Minucci S, Zhu P, et al. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO J* 2001; 20:6969-78.
- Claus R, Lubbert M. Epigenetic targets in hematopoietic malignancies. *Oncogene* 2003;22:6489-96.
- Chen A, Licht JD, Wu Y, Hellinger N, Scher W, Waxman S. Retinoic acid is required for and potentiates differentiation of acute promyelocytic leukemia cells by nonretinoid agents. *Blood* 1994;84:2122-9.
- Chao YH, Kuo SC, Wu CH, et al. Synthesis and cytotoxicity of 2-acetyl-4,8-dihydrobenzodithiophene-4,8-dione derivatives. *J Med Chem* 1998;41:4658-61.

17. Chao YH, Kuo SC, Ku K, et al. Synthesis and cytotoxicity of methyl-4,8-dihydrobenzo[1,2-b:5,4-b']dithiophene-4,8-dione derivatives. *Bioorg Med Chem* 1999;7:1025-31.
18. Li YP, Andersen J, Zelent A, et al. RAR α /RAR α 2-PML mRNA expression in acute promyelocytic leukemia cells: a molecular and laboratory-clinical correlative study. *Blood* 1997;90:306-12.
19. Marks PA, Rifkind RA. Hexamethylene bisacetamide-induced differentiation of transformed cells: molecular and cellular effects and therapeutic application. *Int J Cell Cloning* 1988;6:230-40.
20. Naumovski L, Cleary ML. Bcl2 inhibits apoptosis associated with terminal differentiation of HL-60 myeloid leukemia cells. *Blood* 1994;83:2261-7.
21. Jing Y, Wang L, Xia L, et al. Combined effect of *all-trans* retinoic acid and arsenic trioxide in acute promyelocytic leukemia cells *in vitro* and *in vivo*. *Blood* 2001;97:264-9.
22. Waxman S, Scher BM, Hellinger N, Scher W. Combination cytotoxic-differentiation therapy of mouse erythroleukemia cells with 5-fluorouracil and hexamethylene bisacetamide. *Cancer Res* 1990;50:3878-87.
23. Yu M, Tong JH, Mao M, et al. Cloning of a gene (RIG-G) associated with retinoic acid-induced differentiation of acute promyelocytic leukemia cells and representing a new member of a family of interferon-stimulated genes. *Proc Natl Acad Sci U S A* 1997;94:7406-11.
24. Mao M, Yu M, Tong JH, et al. RIG-E, a human homolog of the murine Ly-6 family, is induced by retinoic acid during the differentiation of acute promyelocytic leukemia cell. *Proc Natl Acad Sci U S A* 1996;93:5910-4.
25. Koefler HP, Ranyard J, Pertcheck M. Myeloperoxidase: its structure and expression during myeloid differentiation. *Blood* 1985;65:484-91.
26. Dai J, Weinberg RS, Waxman S, Jing Y. Malignant cells can be sensitized to undergo growth inhibition and apoptosis by arsenic trioxide through modulation of the glutathione redox system. *Blood* 1999;93:268-77.
27. Breslow R, Jursic B, Yan ZF, et al. Potent cytodifferentiating agents related to hexamethylene bisacetamide. *Proc Natl Acad Sci U S A* 1991;88:5542-6.
28. Marks PA, Michaeli J, Jackson J, Richon VM, Rifkind RA. Induced differentiation of murine erythroleukemia cells (MELC) by polar compounds: marked increased sensitivity of vincristine resistant MELC. *Prog Clin Biol Res* 1989;316B:171-81.
29. Waxman S, Zelent A, Melnick A, et al. Development of novel agents targeted to overcome site-specific transcriptional repression of leukemia [abstract]. *Blood* 1999;94:61A.
30. Jing Y, Dai J, Chalmers-Redman RM, Tatton WG, Waxman S. Arsenic trioxide selectively induces acute promyelocytic leukemia cell apoptosis via a hydrogen peroxide-dependent pathway. *Blood* 1999;94:2102-11.
31. Tallman MS. Acute promyelocytic leukemia as a paradigm for targeted therapy. *Semin Hematol* 2004;41:27-32.
32. Jing Y. The PML-RAR α fusion protein and targeted therapy for acute promyelocytic leukemia. *Leuk Lymphoma* 2004;45:639-48.
33. Zhu J, Lallemand-Breitenbach V, de The H. Pathways of retinoic acid- or arsenic trioxide-induced PML/RAR α catabolism, role of oncogene degradation in disease remission. *Oncogene* 2001;20:7257-65.
34. de The H, Chelbi-Alix MK. APL, a model disease for cancer therapies? *Oncogene* 2001;20:7136-9.
35. Moe-Behrens GH, Pandolfi PP. Targeting aberrant transcriptional repression in acute myeloid leukemia. *Rev Clin Exp Hematol* 2003;7:139-59.
36. Rego EM, Pandolfi PP. Reciprocal products of chromosomal translocations in human cancer pathogenesis: key players or innocent bystanders? *Trends Mol Med* 2002;8:396-405.
37. Jing Y, Xia L, Waxman S. Targeted removal of PML-RAR α protein is required prior to inhibition of histone deacetylase for overcoming *all-trans* retinoic acid differentiation resistance in acute promyelocytic leukemia. *Blood* 2002;100:1008-13.
38. Xu K, Chung D, Glasow A, et al. Dithiophenes potentiate differentiation of APL by lowering the threshold for ligand mediated co-repressor/co-activator exchange with RAR α and enhancing changes in ATRA regulated gene [abstract]. *Blood* 2003;102:859A.
39. Lebedeva IV, Su ZZ, Sarkar D, et al. Melanoma differentiation associated gene-7, mda-7/interleukin-24, induces apoptosis in prostate cancer cells by promoting mitochondrial dysfunction and inducing reactive oxygen species. *Cancer Res* 2003;63:8138-44.
40. Lebedeva IV, Su ZZ, Sarkar D, et al. Induction of reactive oxygen species renders mutant and wild-type K-ras pancreatic carcinoma cells susceptible to Ad.mda-7-induced apoptosis. *Oncogene* 2005;24:585-96.
41. Hockenbery DM, Oltvai ZN, Yin XM, Millman CL, Korsmeyer SJ. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* 1993;75:241-51.