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Research Article

Potentiation of ATRA Activity in HL-60 Cells by Targeting Methylation Enzymes

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Abstract

All Trans Retinoic Acid (ATRA) is a Differentiation Inducer (DI) of Acute Promyelocytic Leukemia (APL) with proven clinical utility. Its benefits for other types of Acute Myelocytic Leukemia (AML) have been limited. In APL, ATRA targets the PML-RARA (promyelocytic leukemia/retinoic acid receptor-alpha)/DNA methyltransferase (DNMT)/Histone Deacetylase (HDAC) complex, facilitating its degradation, leading to loss of gene silencing and Terminal Differentiation (TD). In other forms of AML, ATRA targeting of WT RARA as a single agent fails to modulate the epigenetic changes blocking differentiation. However, when combined with agents that inhibit DNMT, such as 5-azacytidine, ATRA shows improved *in vitro* and clinical activity against AML. We previously demonstrated that targeting the methylation enzyme complex (MMS), consisting of Methionine Adenosyltransferase (MAT), Methyltransferase (MT) and S-Adenosylhomocysteine Hydrolase (SAHH), induced differentiation in the AML M2 HL-60 cell line model. Inhibitors of the ternary methylation enzyme complex act as Differentiation Helper Inducers (DHIs). While they are unable to induce significant terminal differentiation by themselves, they potentiate the action of DI's. We report here that DHIs that destabilize SAHH potentiate the capacity of ATRA to induce terminal differentiation in both sensitive and resistant HL-60 cells *in vitro*.

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We also evaluated Tyrosine Kinase Inhibitors (TKIs) that interfere with the production of the stabilizing factor of SAHH, and steroid analogs that compete with the endogenous steroid stabilizing factor of SAHH. While 72% of early passage (sensitive) HL-60 cells demonstrated induction of Terminal Differentiation (TD) after exposure to 1μM concentrations of ATRA, only 43% of late passage (resistant) cells showed TD. When ATRA was combined with TKI imatinib mesylate or with the steroid analogs resveratrol or β-sitosterol, agents with no innate capacity to induce differentiation, late passage HL-60 cell TD increased significantly to 98%, 99% and 94% of cells, respectively. Only modest improvements in TD percentages were seen for combinations of ATRA with cytotoxic chemotherapy agents: ATRA plus topotecan: 76%; ATRA plus oxaliplatin: 63%; ATRA plus paclitaxel: 59%. Combining ATRA with agents that interfere with maintenance of the methyl group pool potentiated its effects on an AML M2 cell line, and potentially in other forms of AML.

Keywords: APL; ATRA; Differentiation; Differentiation inducer; Differentiation helper inducer; DNA methyltransferase; Methylation enzymes; Steroid analogues; Tyrosine kinase inhibitors

Introduction

Methylation enzymes play a critical role in the regulation of cellular replication and differentiation, and can be dysregulated in cancer, including AML [1,2]. DNA methylation controls the expression of tissue specific genes, and pre-r RNA ribose methylation controls the production of ribosome's, which in turn dictate the commitment of cells to initiate replication [3-5]. Agents that interfere with methylation pathways and DNMT can induce cancer cell differentiation [1].

Biological generation of donor methyl groups that support DNA methylation is mediated by the ternary MMS enzyme complex consisting of MAT-MT-SAHH [6]. In the monomeric state the individual enzymes undergo rapid inactivation, while their engagement in the ternary enzyme complex promotes their stability and function. Monomeric SAHH is the most unstable, followed by MT and then MAT. Stability corresponds to their molecular size [6]. SAHH requires a stabilizing factor to assume a configuration favorable for the formation of a dimeric enzyme complex with MT, which can then form a ternary enzyme complex with MAT. In steroid hormone target tissues, such as prostate and breast, steroid hormones act to stabilize SAHH [6]. SAHH in other tissues also requires stabilizing factors similar to steroid hormones. In normal cells, SAHH is the dominant factor regulating the stability and activity of methylation enzymes. Therapeutic targeting to block stabilizing factor function can disrupt the ternary complex, leading to depletion of methyl group pools and cellular differentiation.

In cancer cells, MMS associates with telomerase (hTERT), altering the regulation and kinetic properties of the ternary enzyme complex [7,8]. K_m values of the normal MAT (MAT^L) and hTERT-associated MAT (MAT^{LT}) are 3 μM and 20 μM methionine, respectively. Those of $SAHH^L$ and $SAHH^{LT}$ are 0.3 μM and 2 μM adenosine, respectively. The increased K_m value of the cancer MAT^{LT} also suggests that methylation enzymes of cancer cells have elevated levels of bound

S-adenosylmethionine (AdoMet), which may exercise a positive influence on the stability of ternary methylation enzymes. Binding of AdoMet by β-cystathionase has been shown to protect that enzyme against protease digestion [9]. These findings suggest that the increased K_m value for MAT^{LT} in malignant cells may contribute to MMS complex stability and down-stream DNA methylation and gene silencing. Consistent with this model, it was reported that the pool size of AdoMet and S-adenosylhomocysteine (AdoHcy) was diminished in cancer cells undergoing drug-induced terminal differentiation [10]. DNA methylation maintains cell cycle transit, while incomplete methylation diverts replicating cells into terminal differentiation [11]. Therefore, factors affecting the integrity of ternary methylation enzymes are critical for cell-cycle regulation and differentiation.

ATRA, the standard therapy for APL, produces excellent initial therapeutic outcomes, with up to 90% of cases showing complete response [12]. However, remissions can be short-lived and relapse with resistance to further treatment occurs. This shortcoming, due to incomplete induction of terminal differentiation by ATRA alone, can be remedied by its use in combination therapy with drugs such as Arsenic Trioxide (ATO) [13], which is an effective DHI. Thus, a combination of DI and DHI is essential to make a perfect drug for cancer therapy.

We previously found that inhibitors of MAT and MT could potentiate ATRA induced TD of HL-60 cells at dosages not appreciably affecting the growth and differentiation of HL-60 cells [14-16]. We report here that Signal Transduction Inhibitors (STIs), polyphenols and steroids used in combination with ATRA were capable of dramatically potentiating TD of both ATRA sensitive and resistant HL-60 cells. These agents may act in part by preventing the production of, or by antagonizing stabilizing factor of SAHH, leading to decreased methyl pool generation.

Materials and Methods

Chemicals and reagents

Chemicals and cell culture supplies used in this study were purchased from Sigma, St. Louis, MO, unless otherwise indicated. 35x10 mm cell culture dishes were from CytoOne, USA Scientific. Com. Imatinib mesylate crystal was a gift from Dr. H. Nguyen of Novartis Corp., East Hanover, NJ. Sunitinib malate capsules were from Pfizer Labs, NY, NY. Pazopanib tablets were from GlaxoSmithKline, RTP, NC. Metformin tablets were from Zydus Pharm, Pennington, NJ.

Culture of HL-60 cells

HL-60 cells were purchased from ATCC, Manassas, VI, which were initially maintained in ISCOVE's modified medium, supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin-50 µg/ml streptomycin for a few generations, and then transferred to RPMI 1640 medium to replace ISCOVE's modified medium. Cells were subcultured every 3 to 4 days at an initial concentration of 5-10 x 10^4 cells/ml.

Nitroblue Tetrazolium (NBT) assay

NBT assay was conducted according to Blair et al., [17]. Each 35x10 mm cell culture dish contained 2 ml of RPMI culture medium. HL-60 cells at an initial concentration of 5-10 x 10^4 cells were incubated with or without drugs for 5 or 3 days depending on the cell passages. Approximated 2.5 x 10^5 cells were precipitated at 600xg for 5 min. The cell pellet was suspended in 3 drops from a Pasteur pipet of NBT reagent consisting of 1 mg NBT and 5 µg phorbol-12-myristate 13-acetate per ml Hank Balanced Salt Solution (HBSS), and incubated at 37°C for 30 min. The reaction was terminated by the addition of a drop from a Pasteur pipet of 4% paraformaldehyde in HBSS. NBT+ cells were counted using a hemacytometer.

Determination of potency of DHIs

The potency of DHIs was assessed by the Reductive Index (RI) as previously described [15]. Cell culture dishes were divided into several sets of 5 dishes containing ATRA of different concentrations to induce between 0 to 60% NBT+. One set had ATRA alone as control to yield ED_{50} of ATRA. Other sets had different concentrations of DHIs together with ATRA concentrations matching the control set. After incubation at 37°C for 72 h, cell numbers from each dish were determined, and an aliquot was withdrawn for NBT assay as above described. NBT+ cells in the control dishes without any drug were always below 1%. In the presence of different DHIs alone, NBT+ cells in general were below 10%. The respective control value was subtracted from each experimental value to yield the actual ED value. ED_{50} values, defined as the dosages that induced 50% NBT+ cells, were estimated from plots of NBT+ values versus concentrations of ATRA in the absence and presence of DHIs. The reductive index is defined as the ED_{50} value in the presence of DHI divided by the ED_{50} value of ATRA alone. This value is inversely related to the effectiveness of the DHI agent.

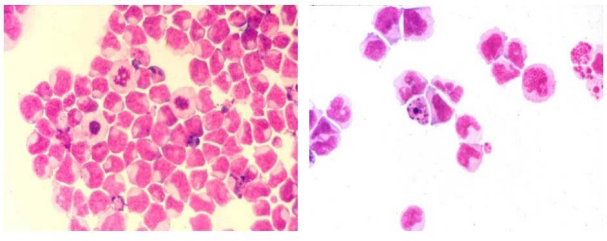
Results

Responsiveness of Early and Late Passage HL-60 Cells to the Induction of TD by ATRA

Early passage HL-60 cells (passaged *in vitro* for ≤ 3 months) replicated very slowly. They took ~5 days for 2 doublings, which was adequate to complete the differentiation process. In contrast, late passage cells cultured continuously for ≥ one year required only three days of incubation to undergo more than 2 doublings. The NBT assay was conducted on the 5th day of the early passage cells carried for < 3 months, and on the 3rd day of the late passage cells. Morphology of control HL-60 cells and TD cells induced with 1 µM ATRA for 72 h is shown in figure 1. The control cells typically show enlarged nuclei with very thin cytoplasm, whereas TD cells have more visible cytoplasm and shrunk nuclei. Approximately 15% of early passage HL-60 cells underwent spontaneous differentiation, i.e. NBT+, as shown in table 1. The early passage cells were more sensitive to induction of TD by ATRA. As the doubling time of HL-60 cells was reduced after multiple *in vitro* passages, these cells gradually lost the ability to undergo spontaneous differentiation and were less sensitive to the induction of TD by ATRA. The induction of TD of the early passage cells by ATRA was 95% at the peak concentration of 6 µM, which gradually declined as *in vitro* passages went on, and was reduced to only 33% by continuous *in vitro* passages for 5 years. Dosages above 6 µM did not improve the extent of TD.

In vitro passages	ATRA, µm	Final to initial cell concentration		% Cell growth	% NBT±
		Ratio $N_3/N0$	N_5/N_0		
0-3 Months	0		3.7 ± 0.29	100	15 ± 3.86
	0.5			61 ± 2.12	51 ± 3.39
	1			39 ± 4.36	72 ± 4.11
	6			28 ± 3.33	95 ± 4.43
	8			23 ± 2.12	88 ± 6.20
12 ± 1 Months	0	4.5 ± 0.72		100	2 ± 1.90
	0.5			72 ± 5.51	29 ± 2.24
	1			48 ± 7.44	55 ± 3.91
	6			40 ± 4.28	90 ± 5.18
	8			35 ± 3.85	83 ± 5.58
24 ± 2 Months	0	6.9 ± 1.24		100	0
	0.5			65 ± 4.72	16 ± 1.95
	1			54 ± 5.50	43 ± 3.43
	6			40 ± 3.77	82 ± 5.62
	8			35 ± 1.98	70 ± 4.85
36 ± 2 Months	0	9.5 ± 2.72		100	0
	0.5			79 ± 5.88	5 ± 2.38
	1			53 ± 3.42	15 ± 6.21
	6			47 ± 4.21	71 ± 5.15
	8			43 ± 2.26	60 ± 6.34
48 ± 2 Months	0	11.8 ± 3.41		100	0
	0.5			77 ± 6.33	3 ± 2.12
	1			55 ± 5.15	9 ± 4.75
	6			48 ± 6.77	55 ± 7.48
	8			40 ± 3.10	42 ± 3.33
60 ± 2 Months	0	13.8 ± 4.28		100	0
	0.5			83 ± 6.82	1 ± 0.51
	1			55 ± 4.45	5 ± 2.27
	6			47 ± 5.10	33 ± 4.38
	8			42 ± 2.78	20 ± 2.63

Table 1: Effectiveness of RA on the Induction of Terminal Differentiation of HL-60 Cells of Different Passages *In Vitro*.



A B

Figure 1: Hematoxylin & eosin staining of the HL60 cells before (A) and after (B) ATRA induced differentiation. The HL60 cells stained with hematoxylin (5 min) & eosin (5 min) at room temperature.

Improved Induction of TD by Imatinib, resveratrol and β-sitosterol

Imatinib mesylate was effective at promoting TD of the early passage cells, but not the late passage cells as shown in table 2. Resveratrol and β-sitosterol promoted TD of the early passage cells to a similar degree as imatinib, and were also ineffective at promoting TD of the late passage cells. It has been reported that resveratrol is an effective inhibitor of growth signals [18,19], and that β-sitosterol is capable of modulating the growth of estrogen-responsive cancer cells [20]. The effect of resveratrol and β-sitosterol to potentiate induction of TD of early passage cells may be attributable to its capacity to potentiate the endogenous DI. These three agents dramatically potentiated the ATRA induced TD for both early and the late passage HL-60 cells, as shown in table 3. The extent of NBT+ cells approached 100%.

Improved Induction of TD by Cytotoxic Drugs

Cytotoxic drugs were partially effective at potentiating induction of TD, as shown in table 4. The potentiation was, however, not as dramatic as growth inhibitors or steroid analogs. In addition, the effectiveness to potentiate TD by cytotoxic drugs fell in narrow dosage ranges. Higher doses caused significant inhibition of cell growth that interfered with the terminal differentiation process.

Growth inhibitors	*In vitro* passages of HL-60 cells			
	0-3 Months		24 ± 2 Months	
	% Cell growth	% NBT+	% Cell growth	% NBT+
Imatinib mesylate, µM				
8	90 ± 5.33	41.5 ± 3.21	98 ± 4.43	0
16	78 ± 2.02	56.2 ± 5.66	90 ± 4.95	0
24	54 ± 3.55	63.9 ± 4.11	88 ± 5.05	0
32	43 ± 3.03	78.1 ± 6.72	80 ± 3.52	0
Resveratrol, µM				
0.8	93 ± 6.82	34.3 ± 3.15	95 ± 3.63	0
1.6	56 ± 5.66	55.6 ± 5.16	66 ± 5.35	0
2.4	37 ± 3.07	72.8 ± 6.22	49 ± 3.63	0
3.2	24 ± 2.21	89.1 ± 7.75	35 ± 2.15	0
β-Sitosterol, µM				
1.3	80 ± 6.30	29.8 ± 1.08	93 ± 5.25	0
2.6	48 ± 2.83	53.6 ± 3.82	78 ± 4.49	0
3.9	28 ± 1.01	67.8 ± 6.45	55 ± 2.43	0
5.2	20 ± 0.82	88.5 ± 7.55	42 ± 2.66	0

Table 2: Effectiveness of Growth Inhibitors to Promote Terminal Differentiation of HL-60 Cells.

Cell incubation and NBT assay were conducted as described in table l. Imatinib mesylate, resveratrol, and β-sitosterol were dissolved in methanol. The volume applied was not to exceed 2.5%. Data are the average of at least two determinations expressed as mean ± S.D.

Additions	*In vitro* passages of HL-60 cells			
	0-3 Months		24 ± 2 Months	
	% Cell growth	% NBT+	% Cell growth	% NBT+
None	100	14.8 ± i.83	100	0
1. ATRA, 0.25 µM	85 ± 4.24	26.7 ± 1.40		
2. ATRA, 1 µM			54 ± 5.57	42.6 ± 4.35
3. Imatinib, 16 µM	78 ± 2.12	56.2 ± 5.66	90 ± 4.95	0
4. Resveratrol, 1.6 µM	56 ± 5.66	55.6 ± 5.16	66 ± 5.35	0
5. β-Sitosterol, 2.6 µM	48 ± 2.83	53.6 ± 3.82	78 ± 4.49	0
1 + 3	39 ± 2.48	99.8 ± 0.28		
2 + 3			44 ± 5.66	98.4 ± 0.64
1 + 4	18 ± 1.90	99.5 ± 0.71		
2 + 4			14 ± 1.83	99.1 ± 0.85
1 + 5	20 ± 2.07	97.8 ± 2.40		
2 + 5			22 ± 2.49	93.8 ± 2.35
2.6	48 ± 2.83	53.6 ± 3.82	78 ± 4.49	0
3.9	28 ± 1.01	67.8 ± 6.45	55 ± 2.43	0
5.2	20 ± 0.82	88.5 ± 7.55	42 ± 2.66	0

Table 3: Dramatic Improvement on the Induction of ATRA-Mediated Terminal Differentiation by TKIs.

Cell incubation and NBT assay were conducted as described in table 1. Data are the average of ≥ two determinations expressed as mean ± S.D.

Relative Potency of Growth Inhibitors or Steroid Analogs as DHIs: Dosages Needed to Achieve RI of 0.5

We previously defined DHIs as inhibitors of individual enzymes of MMS that don't induce terminal differentiation as singe agents, but when applied in combination with a DI such as ATRA, can potentiate induction of terminal differentiation [14,21]. The potency of DHIs was determined by the RI (see materials and methods) [15]. Briefly, ED_{50} values for TD in the absence and in the presence of a DHI were obtained from plots of NBT+ values versus ATRA concentrations. RI values were calculated from these data according to the following formula: RI = ED_{50} of ATRA in the presence of a DHI/ED_{50} of ATRA alone. Dosages of various growth inhibitors needed to achieve a RI of 0.5 are listed in table 5 and table 6.

Additions	*In vitro* passages of HL-60 cells			
	0-3 Months		24 ± 2 Months	
	% Cell growth	% NBT+	% Cell growth	% NBT+
None	100	14.8 ± 1.83	100	0
1. ATRA, 0.25 μM	85 ± 4.24	26.7 ± 1.40		
2. ATRA, 1 μM			54 ± 5.57	42.6 ± 4.35
3. Topotecan, 25 nM	62 ± 5.13	35.3 ± 1.70	62 ± 2.83	0
4. Oxaliplatin, 0.63 μM	77 ± 4.95	37.9 ± 0.35	88 ± 7.61	0
5. Paclitaxel, 0.25 μM	74 ± 4.12	28.2 ± 0.59	78 ± 4.53	0
1+3	25 ± 4.24	80.5 ± 5.09		
2+3			34 ± 2.31	76.1 ± 6.08
1+4	32 ± 2.12	66.4 ± 5.80		
2+4			33 ± 4.24	62.9 ± 4.03
1+5	23 ± 3.07	55.7 ± 3.48		
2+5			27 ± 3.34	58.5 ± 5.14

Table 4: Improvement on the ATRA-Induced Terminal Differentiation by Cytotoxic Drugs.

Cell culture and NBT assay were conducted as described in table 1. Topotecan and oxaliplatin were dissolved in Milli Q water and filtered through 0.2 μm membrane filters to sterilize the solutions. Paclitaxel was dissolved in methanol to apply to the medium not to exceed 2.5% of the volume. Data are the average of ≥ two determinations expressed as mean ± S.D.

STIs are in general small molecule inhibitors of protein tyrosine kinases. Aberrant activation of tyrosine kinases secondary to mutations produce excess growth signals that result in the production of stabilizing factors for SAHH. We tested a variety of TKIs with ATRA, including sunitinib malate, a multikinase inhibitor, berberine, a potent inhibitor of EGFR-MEK-ERK signaling pathway, pazopanib, a potent inhibitor of the vascular endothelial growth factor, and imatinib mesylate, which targets Bcr-Abl in Philadelphia chromosome positive chronic myeloid leukemia and CD117 (cKIT) in gastrointestinal stromal tumors [22-25]. We also evaluated metformin, a well-known oral hypoglycemic agent, in combination with ATRTA based on its capacity to inhibit mTOR [26,27]. Among TKIs studied, sunitinib malate had the most potent activity as a DHI. More selective TKIs, such as pazopanib and imatinib mesylate, were less active (Table 5). Broad spectrum TKIs may be better candidates as DHIs.

Among non-specific growth inhibitors studied, ATO and $CoCl_2$ had impressive activity as DHIs (Table 5). ATO, although quite toxic, required very low dosages to function as a DHI. $CoCl_2$, an agent that up regulates hypoxia inducible factor, induced HL-60cell attachment. When cells became attached to the culture dish, cell growth was greatly diminished. The induction of cell attachment may enable $CoCl_2$ to act as a DHI. Sodium selenite is linked to an array of health benefits, including prevention of cancer. However, its cancer fighting potential has never been well characterized. Its activity as a DHI was limited. As listed in table 5, we found that several polyphenols had impressive activity as DHI's. Many of these polyphenols are present in foods that are regularly consumed. The activity of polyphenols may be attributable to inhibition of signaling pathways [18, 23,28-31].

Steroid Analogs as Differentiation Helper Inducers

Two factors critical for the stability of ternary cancer methylation enzymes are the steroid factors produced in response to growth signals for the stabilization of SAHH, and the association of telomerase with MAT. We do not know the identity of the stabilizing factor of the SAHH of HL-60 cells. It is very likely a steroid derivative, since dihydrotestasterone is the stabilizing factor of prostate SAHH [6]. We therefore examined a series of steroidal compounds to determine their capacities to potentiate ATRA activity (Table 6). Pregnenolone, derived from cholesterol, is the precursor for all steroid hormones, showed moderate DHI activity. Vitamin D can also be synthesized from cholesterol, which has a similar basic ring structure as steroid hormones. Vitamin D3 and dexamethasone were the two most active analogs. This implies that the actual stabilizing factor of SAHH of HL-60 cells may be structurally close to these two agents. It was apparent that biologically active steroids were better DHIs than the inactive pregnenolone precursor, which had the least activity among the steroid analogs studied. Biologically active steroids may not be good candidates for clinical consideration as DHIs simply because their biological activity may complicate cancer therapy. On the other hand, biologically inactive steroids such as pregnenolone, pregnenolone sulfate, guggulsterone, and β-sitosterol are good candidates for drug development as differentiation inducing agents.

Discussion

Our findings suggest that agents that target the methylation enzyme complex given in combination with ATRA can promote HL-60 cell differentiation. While differentiation therapy for solid tumors has not led to wide spread benefits, it has made significant headway for the therapy of hematological cancers [6,8,19,24-26]. Unfortunately, acute leukemia's are made up of rapidly replicating cancer stem cells that develop resistance to differentiation therapy as well as chemotherapy [32,33]. Cancer stem cells are less responsive to cytotoxic chemotherapy because they are driven in part by signal transduction pathways that block apoptosis and they over express drug efflux pumps [34]. The option for the eradication of cancer stem cells is very limited. The ideal therapeutic agents must be small molecules that are relatively non-toxic and that bypass drug efflux pumps to reach

adequate intracellular concentrations to trigger cancer stem cells to undergo differentiation. Induction of differentiation is, therefore, an attractive strategy to eradicate cancer stem cells.

Growth inhibitors	Dosages needed to achieve reductive index of 0.5 in µM
Signal transduction inhibitors	
Sunitinib malate (Sutent)	0.28 ± 0.04
Berberine	1.62 ± 0.17
Pazopanib (Votrient)	10.1 ± 0.14
Imatinib mesylate (Gleevec)	11.9 ± 2.40
Metformin	46.9 ± 3.17
Growth inhibitors:	
As_2O_3	0.28 ± 0.11
$CoCl_2$	0.62 ± 0.04
Selenite	19.7 ± 1.56
Polyphenols	
Tannic acid	0.37 ± 0.02
Epigallocatechin gallate	0.62 ± 0.04
Resveratrol	1.16 ± 0.21
Curcumin	1.24 ± 0.16
Kuromanin	1.43 ± 0.17
Coumestrol	1.95 ± 0.22
Genistein	2.16 ± 0.35
Pterostilbene	2.19 ± 0.27
Pyrogallol	3.18 ± 0.28
Silibinin	3.80 ± 0.31
Caffeic acid	3.87 ± 0.23
Ellagic acid	4.45 ± 0.02
Gallic acid	5.35 ± 0.12
Ferulic acid	7.41 ± 0.15
Phloroglucinol	38.8 ± 6.20

Table 5: Relative Potency of Growth Inhibitors as Differentiation Helper Inducers: Dosages Needed to Achieve Reductive Index of 0.5.

Cell culture and NBT assay were conducted as described in table 1. HL-60 cells used for the determination of reductive indices of various DHIs were maintained *in vitro* between 3 to 4 years. The N_3/N_0 ratios were between 9.5 and 11.8. Berberine, imatinib mesylate and all polyphenols were dissolved in methanol to apply to the medium not to exceed 2.5% of the volume. Sunitinib was poured out of the capsule to be extracted with HBSS in a Dounce homogenizer. Pazopanib and metformin was extracted with HBSS in a Dounce homogenizer. Insoluble materials were removed by centrifugation at 1200xg for 10 min. $CoCl_2$ was dissolved in HBSS. As_2O_3 and selenite was suspended in HBSS with phenol red indicator. 1 N NaOH was added drop wise until pink color persisted. All aqueous preparations were sterilized by passing through 0.2 µm membrane filters. Reductive indices were obtained as described in Materials and Methods. Dosages of DHIs needed to achieve reductive index of 0.5 were obtained from the plots of reductive indices versus concentrations. Data are the average of ≥ two determinations expressed as mean ± S.D.

Our study showed dramatic improvement in the induction of TD after combined therapy with ATRA with TKIs or steroid analogs. This is thought to occur as a result of blockade of growth signals and methyl group transfer associated with gene silencing (Figure 2). These approaches may offer clinical utility for the treatment of ATRA-resistant clones. As shown in table 1, HL-60 cells gradually became resistant to ATRA-induced TD during prolonged propagation *in vitro*. Gene silencing secondary to abnormal methylation enzyme activity is thought to play a role in the development of ATRA resistance in AML [2]. Our data suggest that a multi agent approach combining agents that disrupt DNMT activity in conjunction with targeting RARA can produce favorable therapeutic results. The use of ATRA alone could stimulate at most 48% of resistant HL-60 cells to undergo TD, whereas ATRA in combination with DHI agents led to TD in almost 100% of the resistant cells.

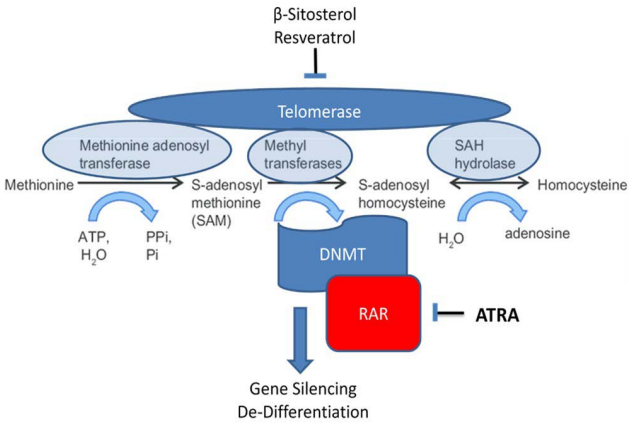


Figure 2: Mechanism of action of ATRA in combination with agents that disrupt the telomerase/methyltransferase complex.

Steroid analogs	Dosages needed to achieve reductive index of 0.5, µM
Vitamin D_3	0.61 ± 0.11
Dexamethasone	0.75 ± 0.20
Testosterone	1.55 ± 0.13
Gugulsterone	1.59 ± 0.16
β-Sitosterol	1.72 ± 0.02
Dehydroepiandrosterone	1.79 ± 0.24
Dihydrotestosterone	2.10 ± 0.20
Prenisolone	2.22 ± 0.12
Estradiol	2.45 ± 0.02
Progesterone	3.55 ± 0.18
Hydrocortisone	4.59 ± 0.23
Pregnenolone	7.16 ± 1.13
Pregnenolone sulfate	7.35 ± 1.06

Table 6: Relative Potency of Steroid Analogs as Differentiation Helper Inducers: Dosages Needed to Achieve Reductive of 0.5.

Cell culture, NBT assay, and determination of reductive indices were conducted as described in table 5. Data are the average of ≥ two determinations expressed as mean ± S.D.

ATRA is an indirect DI that targets the RARA receptor to promote differentiation. The responsible direct DI is very likely oligoisoadenylate. Activation of oligoisoadenylate synthetase is a common feature of many DIs not targeting abnormal methylation enzymes directly [35]. We speculate that early passage HL-60 cells express low levels of oligoisoadenylate, enabling these cells to undergo

spontaneous differentiation, while expression of this enzyme is totally lost in the late passage cells, preventing their capacity to carry out spontaneous differentiation.

Hypoxic growth *in vivo* is another element that could contribute to ATRA resistance. It has been shown that cancer stem cells able to undergo differentiation in normoxia were unable to undergo differentiation in hypoxia [36,37]. Hypoxia influences the switch between differentiation and stemness [36]. The enhanced expression of telomerase in hypoxia may be responsible for the blockade of differentiation. Cancer stem cells already express an unusually high level of telomerase [38], which has to be down-regulated for differentiation to take place [39]. Hypoxia Inducible Factor (HIF), stabilized under hypoxic conditions, is a transcription cofactor that up regulates telomerase, leading to stabilization of the MMS complex [40]. Resveratrol and topotecan treatment lead to decreased HIF under hypoxic conditions [41,42]. Consequently, resveratrol and topotecan may be candidates for targeting cancer stem cells. They were both found to promote TD in late passage HL-60 cells. Taken together, these data support the notion that ATRA in combination with DHI's may be of value in the treatment of non-M3AML.

Conflict of Interest

The authors have no conflicts of interest nor has this paper been published or is being considered for publication of any part of this manuscript elsewhere.

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References

1. Lyko F (2018) The DNA methyltransferase family: a versatile toolkit for epigenetic regulation. Nat Rev Genet 19: 81-92.
2. Figueroa ME, Lugthart S, Li Y, Erpelinck-Verschueren C, Deng X, et al. (2010) DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia. Cancer Cell 17: 13-27.
3. Liau MC, Hunt ME, Hurlbert RB (1976) Role of ribosomal RNA methylases in the regulation of ribosome production. Biochemistry 15: 3158-3164.
4. Bernstein KA, Bleichert F, Beach JM, Cross FR, Baserga SJ (2007) Ribosome Biogenesis is Sensed at the Start Cell Cycle Checkpoint. Mol Cell Biol 18: 953-964.
5. Jones PA, Issa JP, Baylin S (2016) Targeting the cancer epigenome for therapy. Nat Rev Genet 17: 630-641.
6. Liau MC, Chang CF, Saunders GF, Tsai YH (1981) S-Adenosylhomocysteine hydrolases as the primary target enzymes in androgen regulation of methylation complexes. Arch Biochem Biophys 208: 261-272.
7. Liau MC, Zhu PZ, Chiou GCY (2010) Identification of the tumor factor of abnormal cancer methylation enzymes as the catalytic subunit of telomerase. Clinical Oncology and Cancer Research 7: 86-96.
8. Liau MC, Chang CF, Becker FF (1979) Alteration of S-adenosylmethionine synthetase during chemical hepatocarcinogenesis and in resulting carcinomas. Cancer Res 39: 2113-2119.
9. Prudova A, Baumann Z, Braun A, Vitvitsky V, Lu SC, et al. (2006) S-Adenosylmethionine stabilizes β-cystathionine synthase and modulates redox capacity. Proc Natl Acad Sci U S A 103: 6489-6494.
10. Chiba P, Wallner L, Kaizer E (1988) S-Adenosylmethionine metabolism in HL-60 cells: effect of cell cycle and differentiation. Biochim Biophy Acta 971: 38-45.
11. Liau MC, Lee SS, Burzynski SR (1989) Hypomethylation of nucleic acids: a key to the induction of terminal differentiation. Intl J Exp Clin Chemother 2: 187-199.
12. Hu J, Liu YF, Wu CF, Xu F, Shen ZX, et al (2009) Long term therapy and safety of all-trans retinoid acid/arsenic trioxide-based therapy in newly diagnosed acute promyelocytic leukemia. Proc Natl Acad Sci USA 106: 3342-3347.
13. Degos L, Wang ZY (2001) All trans retinoic acid in acute promyelocytic leukemia. Oncogene 20: 7140-7145.
14. Liau MC, Liau CP, Burzynski SR (1992) Potentiation of induced terminal differentiation by phenylacetic acid and related chemicals. Int J Exp Clin Chemother 5: 9-17.
15. Liau MC, Huang LJ, Lee JH, Chen SC (1998) Development of differentiation helper inducers for differentiation therapy of cancer. Chin Pharm J 50: 289-303.
16. Liau MC, Liau CP (2002) Methyltransferase inhibitors as excellent differentiation helper inducers for differentiation therapy of cancer. Bull Chin Cancer 11: 166-168.
17. Blair OC, Carbone R, Sartorelli AC (1985) Differentiation of HL-60 promyelocytic leukemia cells monitored by flow cytometric measurement of nitro blue tetrazolium (NBT) reduction. Cytometry 6: 54-61.
18. Liu M, Wilk SA, Wang A, Zhou L, Wang RH, et al. (2010) Resveratrol inhibits mTOR signaling by promoting the interaction between mTOR and DEPTOR. J Biol Chem 285: 36387-36394.
19. Kundu JK, Surth YJ (2008) Cancer chemoprevention and therapeutic potential of resveratrol: mechanism perspectives. Cancer Lett 269: 243-261.
20. Ju YH, Clausen LM, Allred KF, Almada AL, Helferich WG (2004) beta-Sitosterol, beta-Sitosterol Glucoside, and a Mixture of beta-Sitosterol and beta-Sitosterol Glucoside Modulate the Growth of Estrogen-Responsive Breast Cancer Cells *In Vitro* and in Ovariectomized Athymic Mice. J Nutr 134: 1145-1151.
21. Liau MC, Lee SS, Burzynski SR (1990) Modulation of cancer methylation complex isozymes as a decisive factor in the induction of terminal differentiation mediated by Antineoplaston A5. Intl J Tiss React 12: 27-36.
22. Demetri GD, van Oosterom AT, Garrett CR, Blackstein ME, Shah MH, et al. (2006) Efficacy and safety of sunitinib in patient with advanced gastrointestinal stroma tumor after failure of imatinib: a randomized controlled trial. Lancet 368: 1329-1338.
23. Liu G, Xu X, Zhao M, Wei Z, Li X, et al. (2015) Berberine induces senescence of human glioblastoma cells by down regulating the EGFR-MEK-ERK signaling pathway. Mol Cancer Ther 14: 355-363.
24. Harris PA, Boloor A, Cheung M, Kumar R, Crosby RM, et al. (2008) Discovery of 5-[[4-[(2,3-dimethyl-2H-indazol-6-yl)methylamino]-2-pyrimidinyl]amino]-2-methyl-benzenesulfonamide (Pazopanib), a novel and potent vascular endothelial growth factor receptor inhibitor. J Med Chem 15: 4632-4640.
25. DiNitto JP, Wu JC (2011) Molecular mechanisms of drug resistance in tyrosine kinases cAbl and cKit. Critical Reviews in Biochemistry and Molecular Biology 46: 295-309.
26. Sahra IB, Regazzeti C, Robert G, Laurent K, Marchand-Brustel YL, et al. (2011) Metformin, independent of AMPK, induces mTOR inhibition and cell cycle arrest through REDD1. Cancer Res 71: 4366-4372.
27. Rocha GZ, Dias MM, Ropelle ER, Osório-Costa F, Rossato FA, et al. (2011) Metformin amplifies chemotherapy induced AMPK activation and antitumor growth. Clin Cancer Res 17: 3993-4005.

28. Liao S, Xia J, Chen Z, Zhang S, Ahmad A, et al. (2011) Inhibitory effect of curcumin on oral carcinoma CAL-27 cells via suppression of Notch-1 and NF-kB signaling pathways. J Cell Biochem 112: 1055-1065.

29. Moon SS, Kim MO, Xhoi YH, Lee HG, Kim ND, et al. (2008) Gossypol suppresses telomerase activity in human leukemia cells via regulation hTERT. FEBS Lett 582: 3367-3373.

30. Yang G, Fu Y, Malakhova M, Kurinov I, Zhu F, et al. (2014) Caffeic acid directly targets ERK1/2 to attenuate solar UV-induced skin carcinogenesis. Cancer Prev Res 7: 1056-1066.

31. Kim PK, Suh Y, Yoo KC, Cui YH, Hwang E, et al. (2015) Phloroglucinol suppresses metastatic ability of breast cancer cells by inhibition of epithelial-messenchymal cell transition. Cancer Sci 106: 94-101.

32. van Gils N, Verhagen HJMP, Smit L (2017) Reprogramming acute myeloid leukemia into sensitivity for retinoic-acid-driven differentiation. Exp Hematol 52: 12-23.

33. Phi LTH, Sari IN, Yang YG, Lee SH, Jun N, et al. (2018) Cancer stem cells (CSCs) in drug resistance and their therapeutic implications in cancer treatment. Stem Cells International 2018: 1-16.

34. McCracken MN, George BM, Kao KS, Marjon KD, Raveh T, et al. (2016) Normal and Neoplastic Stem Cells. Cold Spring Harb Symp Quant Biol 81: 1-9.

35. Liau MC (2004) Abnormal methylation enzymes: a selective molecular target for differentiation therapy of cancer. Chin Pharm J 56: 57-67.

36. Fruehauf JP, Liau MC (2011) Therapeutic targeting of HIF to reverse the cancer stem cell phenotype and epithelial-mesenchymal transition. Therapy 8: 737-740.

37. Kung AL, Zablludoff SD, Fraces DS, Freedman SJ, Tanner EA, et al. (2004) Small molecule blockade of transcriptional activation of hypoxia inducible factor pathway. Cancer Cell 6: 33-43.

38. Shay JN, Wright NE (2010) Telomeres and telomerase in normal and cancer stem cells. FEBS Lett 584: 3819-3825.

39. Reichman TW, Albanell J, Wang X, Moore MA, Studzinski GP (1997) Downregulation of telomerase activity in HL60 cells by differentiating agents is accompanied by increased expression of telomerase-associated protein. J Cell Biochem 67: 13-23.

40. Anderson CJ, Hoare SF, Ashcroft M, Bilsland AE, Keith WN (2006) Hypoxic regulation of telomerase gene expression by transcriptional and post-transcriptional mechanisms. Oncogene 25: 61-69.

41. Sun Y, Wang H, Liu M, Lin F, Hua J (2015) Resveratrol abrogates the effects of hypoxia on cell proliferation, invasion and EMT in osteosarcoma cells through downregulation of the HIF-1α protein. Mol Med Rep 11: 1975-1981.

42. Rapisarda A, Shoemaker RH, Melillo G (2004) Targeting topoisomerase I to inhibit hypoxia inducible factor 1. Cell Cycle 3: 172-175.

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