

## In Pursuance of Differentiation Inducers to Combat Cancer via Targeting of Abnormal Methylation Enzymes

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### Authors' contributions

This work was carried out in collaboration among authors. Author MCL designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author JHK assisted the study and performed artwork of the manuscript. Author JPF managed the analyses of the study and edited the final manuscript. All authors read and approved the final manuscript.

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### ABSTRACT

Cell differentiation agent-2 (CDA-2) was a promising hypomethylating agent approved by the Chinese FDA for the therapy of MDS in China. The active components of CDA-2 are differentiation inducers (DIs) and differentiation helper inducers (DHIs). DIs are chemicals capable of eliminating telomerase from abnormal MEs commonly found in human cancers. The major DI of CDA-2 is an organic acid without UV absorption. Without UV absorption as a guide, it was difficult to purify the DI of CDA-2 for identification. Thus, we pursued possible candidates to function as DIs in this study.

Cancer MEs become abnormal due to association with telomerase. Naturally we sought telomerase inhibitors as possible candidates of DIs. Prostaglandin E2 (PGE2) attracted our attention because it was implicated to involve in wound healing, which is a major biological mission

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of progenitor stem cells (PSCs) and cancer stem cells (CSCs). Eradication of CSCs has been a major focus of our studies. Besides, PGE2 fits the description of the major DI of CDA-2. Induction of terminal differentiation (TD) of HL-60 cells by NBT assay was employed to evaluate the activity of chemicals as DIs. Cell growth was based on cell numbers. All-trans retinoic acid (ATRA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) are two well known DIs. ATRA displayed a wide active dosage range from 0.2 to 4.5  $\mu\text{M}$  with a maximum of inducing 89% NBT+ cells at 3  $\mu\text{M}$ . TPA displayed a narrow active dosage range from 0.2 to 0.6 nM with a maximum of inducing 84% NBT+ cells at 0.4 nM. BIBR1532 and bodine were the two telomerase inhibitors studied. Both were found active as DIs. BIBR1532 was active in the dosage range from 30 to 75  $\mu\text{M}$  with a maximum of inducing 86% at 63  $\mu\text{M}$ . Bodine was active in the dosage range from 60 to 98  $\mu\text{M}$  with a maximum of inducing 80% at 98  $\mu\text{M}$ . PGE2 was active in the dosage range from 20 to 70  $\mu\text{M}$  with a maximum of inducing 80% at 56  $\mu\text{M}$ . DIs at dosages not active as DIs could function as effective DHIs to other DIs.  $\text{RI}_{0.5}$  of BIBR1532, boldine and PGE2 as DHIs were 2.02  $\mu\text{M}$ , 3.11  $\mu\text{M}$ , and 0.92  $\mu\text{M}$ , respectively. DIs alone, no matter how effective, could not induce NBT+ cells to reach 100%. 95% (89% plus 6% of blank) was the highest value achieved by ATRA. Incomplete induction of TD was the reason for frequent recurrence when ATRA was used alone in the therapy of acute promyelocytic leukemia (APL). A combination of ATRA and a DHI could induce NBT+ cells to reach 100% to avoid recurrence.

*Keywords: Differentiation therapy; differentiation inducers; differentiation helper; inducers; methylation enzymes.*

## 1. INTRODUCTION

MEs are a ternary enzyme complex consisting of methionine adenosyltransferase (MAT)-methyltransferase (MT)-S-adenosylhomocysteine hydrolase (SAHH) [1]. These enzymes function as a switch to turn stem cells on to replication in response to growth factors, and to turn replicating cells off to TD when growth factors are no longer available. The association of telomerase with MEs converts these enzymes to become exceptionally stable and active so that hypomethylation of nucleic acids necessary for the cell to undergo TD cannot take place [2]. The association of telomerase with MEs alters kinetic properties of MAT-SAHH isozyme pair. The tumor isozyme pair display  $K_m$  values 7-fold higher than the normal isozyme pair [1,3,4]. The increased  $K_m$  values of the tumor isozyme pair suggest that the tumor MEs hold a higher level of S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy). The increased pool sizes of AdoMet and AdoHcy are important to maintain the stability, and therefore, the activity of tumor MEs. According to Prudova et al. [5], the binding of AdoMet to a protein protected the protein against protease degradation. Chiba et al. [6] showed when cancer cells were induced to undergo TD, the pool sizes of AdoMet and AdoHcy shrunk greatly. Therefore, the association of telomerase with MEs is an important issue in the promotion of malignant growth.

Progenitor stem cells (PSCs) and embryonic stem cells (ESCs) express telomerase. MEs of these primitive stem cells are abnormal like cancer cells. However, these primitive stem cells are able to carry out differentiation programs, relying on Ten-eleven translocation 1 (TET 1) enzyme to achieve DNA hypomethylation [7-9]. TET 1 is silenced in cancer cells [10-12]. Thus, the differentiation inducing capability of cancer cells is totally lost. PSCs and CSCs are very much alike based on cell features and biological missions. It is very likely that CSCs are originated from PSCs. The evolution of cancer clearly showed to start from cells expressing abnormal MEs in preneoplastic hyperplastic nodules or PSCs, then to advance to CSCs, and finally to acquire activated oncogenes or inactivated suppressor genes to become faster growing cancer cells [3,13]. The evolution of CSCs from PSCs can be easily achieved if PSCs are allowed to buildup, since these cells have abnormally active MEs. Here comes the important function of chemo-surveillance to prevent the buildup of PSCs [14]. Human body produces metabolites active as DIs and DHIs which play an important surveillance role to direct TD of cells with abnormal MEs. CDA-2 was a preparation of urinary DIs and DHIs [15], which has been approved by the Chinese FAD for the therapy of myelodysplastic syndrome (MDS). The major DI of CDA-2 was an organic acid without UV absorption. Without UV absorption as a guide, it was difficult to purify the DI of CDA-2

for identification. Thus, we pursued possible candidates to function as DIs.

Cancer MEs become abnormal due to association with telomerase. Naturally we sought telomerase inhibitors as candidates of DIs [16,17]. PGE2 attracted our attention because it was implicated to involve in wound healing [18], which is a major biological mission of PSCs and CSCs. Eradication of CSC has been a major focus of our studies. Besides, PGE2 fits the description of the major DI of CDA-2.

We have previously carried out extensive studies on DHIs [15,19-22]. The discovery of effective DIs will enable us to formulate good differentiation agents for the prevention and therapy of cancer.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals and Reagents

Chemicals and cell culture supplies were purchased from Sigma, St. Louise, MO. 35x10 mm cell culture dishes were purchased from CytoOne, USA Scientific.com. HL-60 cells were purchased from ATCC, Manassan, VI.

HL-60 cells 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 RPMI1640 medium to replace ISCOVE's medium. HL-60 cells grow better from frozen cells in ISCOVE's medium than in RPMI1640 medium. Our previous studies were all carried out on HL-60 cells maintained in RPMI 1640. Therefore, we changed the medium to RPMI1640 whence cells were ready for experiments. Cells were subcultured every 3 to 4 days at an initial concentration of 5 to 10x10<sup>4</sup> cells/ml. The incubator was supplied with 5% CO<sub>2</sub>.

### 2.2 NBT Assay

NBT assay was conducted as previously described [22]. Each 35x10 mm culture dish contained 2 ml of RPMI1640 culture medium. HL-60 cells at an initial concentration of 5-10x10<sup>4</sup> cells/ml were incubated with or without drugs for 4 days. Drugs were dissolved in methanol. The stock solution of TPA was 1 mg/ml DMSO. The working solutions were methanol diluted solutions. The total volume of methanol applied was limited to 2.5%. After incubation, cell numbers were counted using a hemocytometer. Approximately 2.5x10<sup>5</sup> cells were sedimented at

600xg for 5 min. The cell pellet was suspended in 2 drops from a Pasture pipet of NBT reagent consisting 1 mg NBT and 5 µg TPA 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 Pasture pipet of 4% paraformaldehyde in HBSS. NBT+ cells were counted under microscope using a hemocytometer.

### 2.3 Determination of Potency of DHIs

The potency of DHIs was assessed by the reductive index as previously described [22]. Cell culture dishes were divided into several sets of 5 dishes containing ATRA of different concentrations to induce between 0 to 60% NBT+ cells. One set had ATRA alone as control to yield effective dosage<sub>50</sub> (ED<sub>50</sub>) of ATRA. Other sets had different concentrations of DHIs together with ATRA concentrations matching the control set. After incubation at 37°C for 96 h, cell numbers from each dish were counted using a hemocytometer, and an aliquot was withdrawn for NBT assay as above described. NBT+ cells in the control dishes without drug were 5-7%. In the presence of different DHIs alone, NBT+ cells in general were below 12%. The respective control value was subtracted from each experimental value to yield the actual ED value. ED<sub>50</sub> value, 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 (RI) is defined as the ED<sub>50</sub> in the presence of DHI divided by the ED<sub>50</sub> value of ATRA alone. The value is inversely related to the effectiveness of DHI agent.

## 3. RESULTS

ATRA and TPA are well known DIs. As shown in Fig. 1 and 2, both ATRA and TPA were very good DIs at very low dosages, particularly TPA. ATRA displayed a wide active dosage range from 0.2 to 4.5 µM with a maximum of inducing 89% NBT+ cells at 3 µM. TPA displayed a rather narrow active range from 0.2 to 0.6 nM with a maximum of inducing 84% NBT+ cells at 0.4 nM.

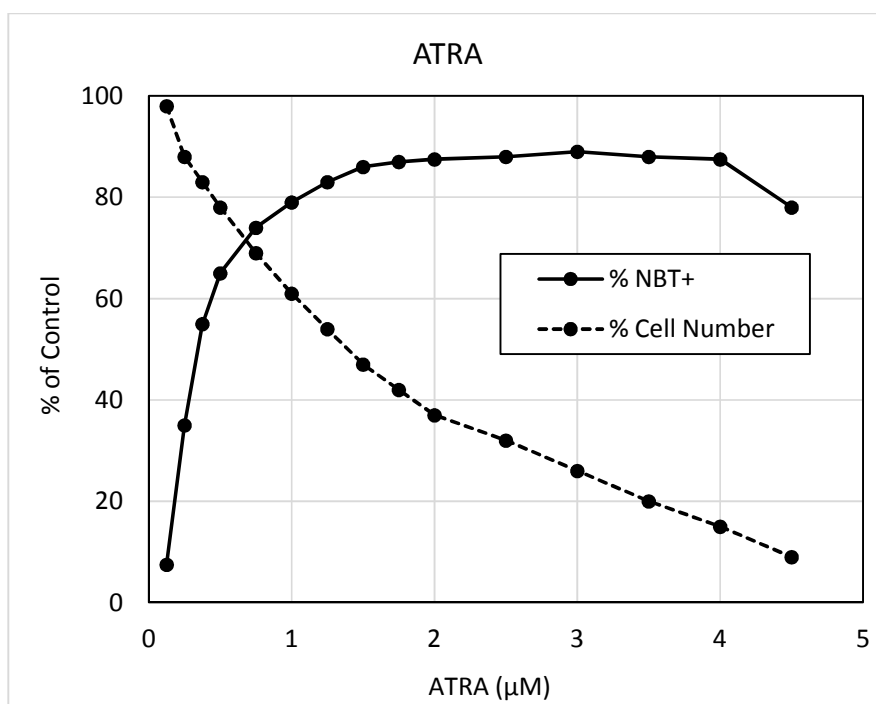
Since BIBR1532 was found a very good telomerase inhibitor [16], we picked this drug as the first telomerase inhibitor to study. It was active in the dosage range from 30 to 75 µM with a respectable maximum activity of inducing 86% NBT+ cells at 63 µM as shown in Fig. 3. Boldine is a natural aporphine alkaloid richly found in the boldo tree (*Peumus Boldus*). It was found to inhibit telomerase enzyme activity as well as its

gene expression [17]. As shown in Fig. 4, it was not as effective as BIBR1532, but was active in the dosage range from 60 to 98  $\mu\text{M}$  with a maximum of inducing 80% NBT+ cells at 98  $\mu\text{M}$ . PGE2 was a slightly better DI than the previous two telomerase inhibitors as shown in Fig. 5. The active dosage range was from 20 to 70  $\mu\text{M}$  with a maximum of inducing 80% NBT+ cells at 56  $\mu\text{M}$ .

ED<sub>25</sub>, ED<sub>50</sub>, and ED<sub>75</sub> data of DIs are useful in the consideration of CDA formulations as previously described [15]. These data are presented in Table 1.

Our previous studies indicated that the combination of two different DIs resulted in

additive effect, whereas the combination of a DI and a DHI resulted in a synergistic effect [15]. We were surprised to find that a DI could function as a DHI when used at concentrations below active dosages as DIs. Thus, low concentrations BIBR1532, boldine, and PGE2 were all found to potentiate the activity of ATRA as presented in Table 2. Because of the activity as DHI, a combination of two DIs could also reach a complete induction of 100% NBT+ cells. This was a feature that could not be accomplished by DIs as a single agent. The combinations of low dosage of PGE2 acting as a DHI with either ATRA, TPA, or BIBR1532 acting as DIs were able to achieve 100% induction of NBT+ cells as presented in Table 3.

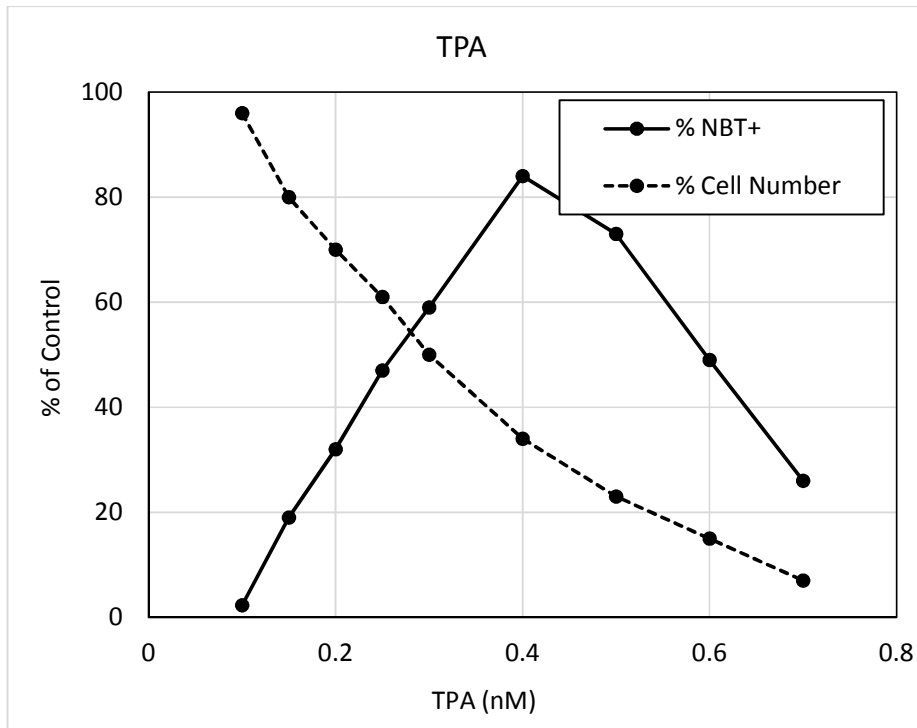


**Fig. 1. Effectiveness of ATRA on the induction of TD of HL-60 cells. HL-60 cells used for the study of the effectiveness of DIs were maintained between 2-8 months from frozen cells. The  $N_4/N_0$  ratios were between 4-6. ATRA was dissolved in methanol to apply to the medium not to exceed 2.5% of the volume. Cell culture and NBT assay were conducted as described in Materials and Methods. Data represent the average of two determinations**

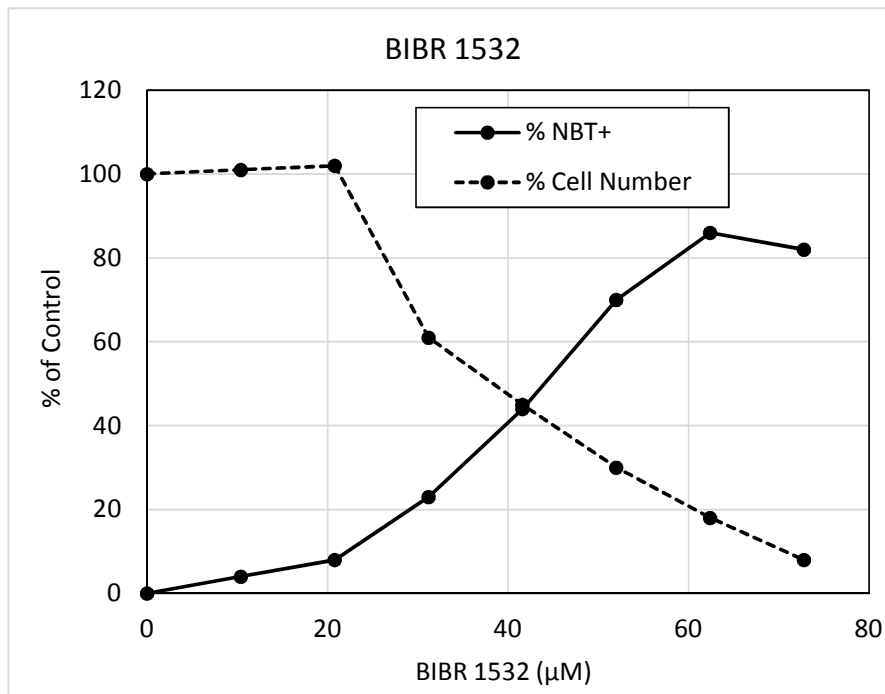
**Table 1. ED<sub>25</sub>, ED<sub>50</sub>, and ED<sub>75</sub> of DIs**

DIs	ED <sub>25</sub>	ED <sub>50</sub>	ED <sub>75</sub>
ATRA	0.18±0.01 $\mu\text{M}$	0.36±0.05 $\mu\text{M}$	0.75±0.07 $\mu\text{M}$
TPA	0.17±0.02 nM	0.26±0.03 nM	0.36±0.06 nM
BIBR1532	32.3±2.6 $\mu\text{M}$	43.7±4.7 $\mu\text{M}$	55.1±3.8 $\mu\text{M}$
Boldine	60.1±3.5 $\mu\text{M}$	78.8±6.5 $\mu\text{M}$	94.2±9.1 $\mu\text{M}$
PGE2	20.6±1.7 $\mu\text{M}$	32.0±2.2 $\mu\text{M}$	46.5±3.3 $\mu\text{M}$

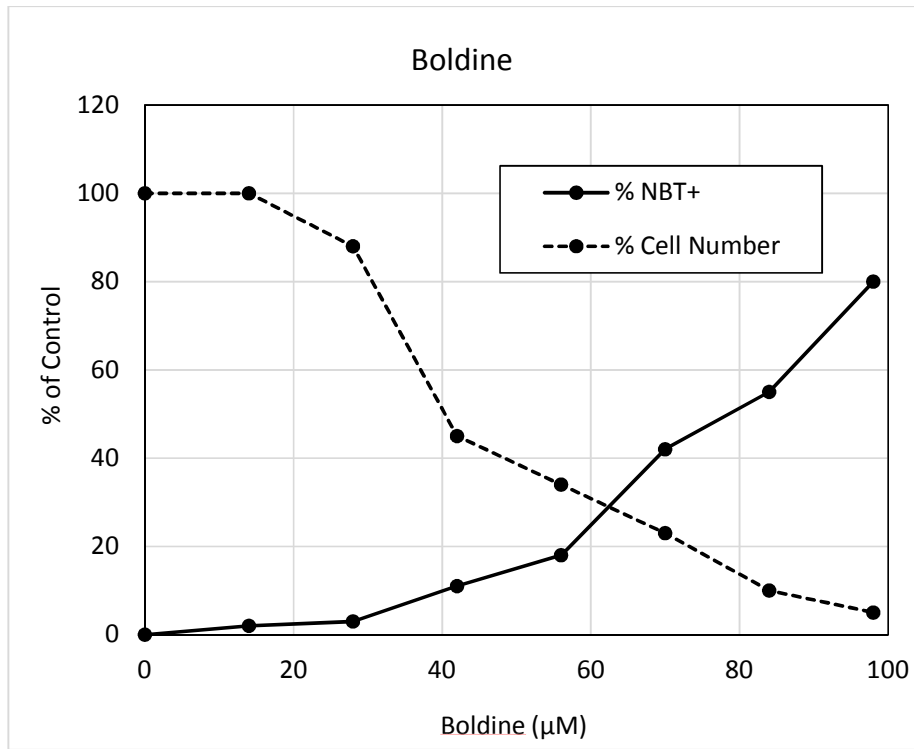
Data of ED<sub>25</sub>, ED<sub>50</sub>, and ED<sub>75</sub> are obtained from Fig. 1 through 5. The data presented in Table 1 represent the average of at least two determinations expressed as mean±S.D



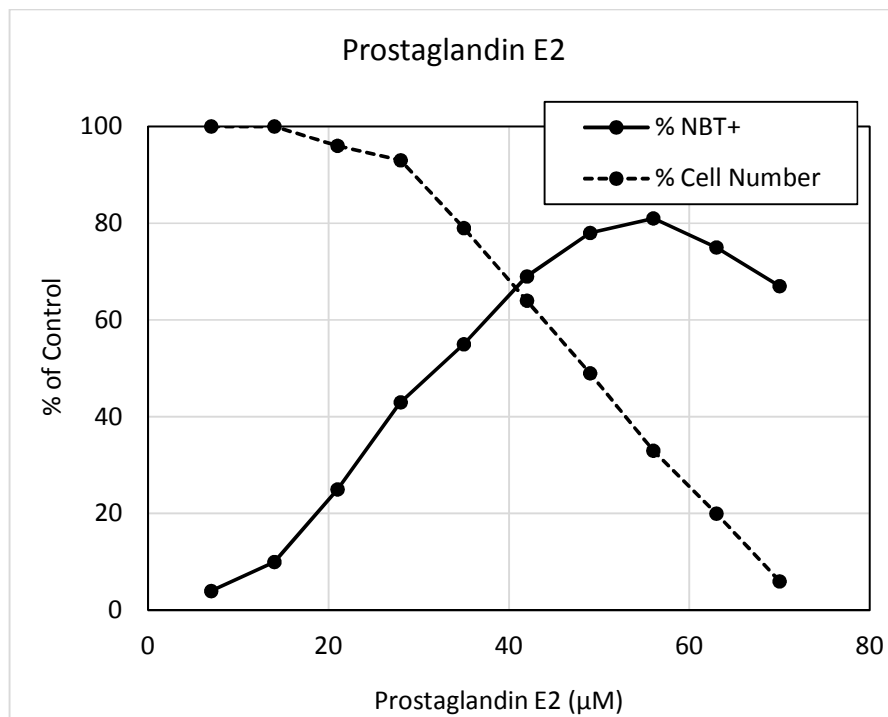
**Fig. 2.** Effectiveness of TPA on the induction of TD of HL-60 cells. Experiments were conducted as described in Fig. 1. Working solutions of TPA were diluted methanol solutions from the stock solution of 1 mg TPA/ml DMSO. Data represent the average of two determinations



**Fig. 3.** Effectiveness of BIBR1532 on the induction of TD of HL-60 cells. Experiments were conducted as described in Fig. 1. Data represent the average of three determinations



**Fig. 4.** Effectiveness of boldine on the induction of TD of HL-60 cells. Experiments were conducted as described in Fig. 1. Data represent the average of two determinations



**Fig. 5.** Effectiveness of PGE2 on the induction of TD of HL-60 cells. Experiments were conducted as described in Fig. 1. Data represent the average of four determinations

**Table 2. Dis as DHIs when combined with ATRA**

Dis as DHIs	RI <sub>0.5</sub>
BIBR1532	2.02±0.16 µM
Boldine	3.11±0.12 µM
PGE2	0.92±0.05 µM

*Cell culture, NBT assay, and determination of reductive indices were conducted as described in Materials and Methods. Data are the average of two or more determinations expressed as mean±S.D*

**Table 3. Combination effect of two dis to induce 100% NBT+ Cells**

Addition	% Cell Number	% NBT+ Cells
Control	100	5.3±0.4
1. ATRA, 0.3 µM	84±3.3	50.2±2.2
2. TPA, 0.25 nM	61±2.9	51.5±3.4
3. BIBR, 42 µM	45±2.1	50.5±3.5
4.PGE2, 1.92 µM	91±6.4	9.1±0.3
1+4	28±1.2	100
2+4	22±0.9	100
3+4	18±0.5	100

*Experiments were conducted as described in Table 1. Data are the average of two determinations expressed as mean±S.D*

#### 4. DISCUSSION AND CONCLUSION

MDS is a classic disease to demonstrate the evolution of CSCs from PSCs [13]. CDA-2 is a preparation of natural metabolites active as Dis and DHIs, which was approved by the Chinese FDA in the fall of 2017 for the therapy of MDS [15]. MDS is a disease attributable entirely to CSCs [23], which can be a disease for the evaluation of candidate drugs against CSCs. The active components of CDA-2 are the basis of surveillance chemicals that protect healthy people from becoming cancer patients. Apparently, chemo-surveillance [14] is functioning very well as there are far more healthy people than cancer patients, considering the evolution of CSCs from PSCs is a very simple matter because PSCs have abnormally active MEs that can easily silence TET 1 to convert PSCs to CSCs. Obviously, destabilization of abnormal MEs by means of Dis and DHIs is the nature's choice to combat cancer. A stroke to destabilize abnormal MEs terminates persistent replication of cancer cells, and thus negates the contribution of oncogenes and suppressor genes to drive cell cycle progression. This is a wonderful way to keep cancer away. The identification of active components of CDA-2 is very important for the formulation of drugs effective against cancer in general and CSCs in particular. We have carried out extensive studies on DHIs of CDA-2 [19-22]. Our studies on Dis of CDA-2 were not as successful, because the most important DI of CDA-2 did not have UV absorption [15]. Without

UV absorption as a guide, it was difficult to purify the active Dis.

Telomerase inhibitors as Dis are highly recommended, because these inhibitors are selective against telomerase which is only expressed in cancer cells and a few normal stem cells, and effective against all cancer cells by passing receptor requirement. We tested two telomerase inhibitors, both turned out positive. Available telomerase inhibitors should be investigated to select better telomerase inhibitors as Dis.

ATRA is an excellent DI effective at very low dosages with a wide active dosage range. It was approved as a standard care for the treatment of APL [24,25]. Differentiation induced by ATRA is mediated through retinoic acid receptor (RAR) to activate 2',5'-oligoadenylate synthetase [26]. Not all cancer cells express RAR. Primitive stem cells do express RAR. It is a good DI for those cancers expressing RAR.

TPA is the most impressive DI, effective in nM quantities. But it is also a potent tumor promoter, a totally unacceptable side effect as a cancer drug.

PGE2 is a hormone-like substance that acts through multiple membrane receptors to activate adenylylase-protein kinase A signaling to incite a wide range of body functions [27]. One of the side effects of PGE2 is unacceptable tumor promotion like TPA. Besides, it is not a stable

molecule. It is quickly inactivated by PGE2 dehydrogenase [27]. PGE2 definitely is not worthy of consideration as a DI. If its stable metabolites retain DI activity and without unacceptable tumor promoting activity, those metabolites are certainly good candidates as DIs. One of such metabolites, namely 16, 16-dimethyl PGE2, has the potential as an acceptable DI, which has been shown to display effective inhibitory activity against spontaneous evolution of cancer [28].

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

It is not applicable.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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