

# Combined effect of (-)-epigallocatechin-3-gallate and all-trans retinoic acid in *FLT3*-mutated cell lines

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Received October 14, 2019; Accepted May 5, 2020

DOI: 10.3892/br.2020.1332

**Abstract.** Patients diagnosed with acute promyelocytic leukemia were treated with Vesanoid® [all-trans retinoic acid (ATRA)]. ATRA promotes the maturation and differentiation of leukemia cells and is therefore capable of reducing the symptoms of leukemia by preventing aggregation of myeloid cells. However, the clinical applications of ATRA are limited by its side effects, including acute retinoid resistance, hypertriglyceridemia, mucocutaneous dryness, nausea, brief recovery time relapse and drug resistance. Therefore, combinations of ATRA and other anticancer drugs are being investigated to overcome these limitations. In our previous study it was shown that in leukemia cells, (-)-epigallocatechin-3-gallate (EGCG) reduced cell proliferation and induced apoptotic cell death. In the present study, an *in vitro* evaluation of the effects of the combination of EGCG and ATRA on *FLT3*-mutated cell lines was performed using the isobologram method. The results showed that there was an additive effect in leukemic cells when treated with a combination of ATRA and EGCG. Thus, it was concluded that the cytotoxic effects of EGCG were improved by ATRA.

## Introduction

FMS-like tyrosine kinase 3 (*FLT3*) belongs to the Receptor Tyrosine Kinase subclass III family, which serves a vital role in differentiation, proliferation and apoptosis of myeloid cells (1). The most frequently observed *FLT3* mutations are internal tandem duplications (*FLT3/ITD*) in the juxtamembrane

domain, which occur in 15-35% of patients with acute myeloid leukemia (AML) (2), and mutations in the tyrosine kinase activation loop are observed in 5-10% of AML patients (3). Patients with AML with *FLT3-ITD* have higher relapse rates (4) and consequently less favorable disease-free and overall survival rates (5), particularly in AMLs with a larger ITD sizes (6), higher allelic burden (7) or multiple ITDs (8). Therefore, inhibition of *FLT3* has become a potential therapeutic choice, and clinical trials of inhibitors of *FLT3* in AML have been going on for a decade (9). To date, there have been >20 small molecule inhibitors against *FLT3* which have been investigated; some of which have been examined in clinical trials (10). These include midostaurin (PKC412), sorafenib (BAY 43-9006), sunitinib (SU11248), tandutinib (MLN518), lestaurtinib (CEP-701), KW-2449, AKN-032, AC220, ABT-869 and Quizartinib (AC220) (11,12). The majority of these inhibitors are structurally heterocyclic compounds that inhibit *FLT3* activity by competing with adenosine triphosphate (ATP) to bind to the tyrosine kinase domain ATP-binding pocket (13). Functionally, these inhibitors may be general multikinase inhibitors. Their clinical activities appear to be mediated by *FLT3* inhibition, so their activity is restrained to AML carrying *FLT3-ITDs*, and associated with the inhibition of *FLT3* phosphorylation and its downstream signaling effectors (14).

Patients diagnosed with acute promyelocytic leukemia (a subtype of AML) are treated with Vesanoid® [all-trans retinoic acid (ATRA)]. ATRA promotes the maturation and differentiation of leukemia cells and is therefore capable of reducing the symptoms of leukemia by preventing aggregation of myeloid cells (15). Furthermore, ATRA has been shown to arrest cell growth, induce cell differentiation and induce cell death of various types of cancer cells *in vitro* (16). Nonetheless, the clinical applications of ATRA are limited by its side effects, including acute retinoid resistance, hypertriglyceridemia, mucocutaneous dryness, nausea, brief recovery time relapse and drug resistance (17). Additionally, due to its low plasma concentrations, its medical applications are further reduced. Therefore, combinations of ATRA and other anticancer drugs were investigated to overcome these limitations (18). A previous study showed that ATRA can increase the cytotoxic effects of protein kinase C 412 in AML cell populations with genetic *FLT3* abnormalities (19).

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*Abbreviations:* AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; ATRA, all-trans retinoic acid; EGCG, (-)-epigallocatechin-3-gallate; *FLT3*, FMS-like tyrosine kinase 3

*Key words:* ATRA, EGCG, green tea, isobologram, leukemia

Green tea (from *Camellia sinensis*) has been utilized as a Traditional Chinese Medicine for millennia. The primary active polyphenolic compounds of green tea are catechins [epicatechin, epigallocatechin and (-)-epigallocatechin-3-gallate (EGCG)]. Among these catechins, EGCG is the foremost viable catechin that can reduce the proliferation of cells and induce apoptosis in cancer cells (20). It has been shown that EGCG inhibits cancer growth, including lung (21), prostate (22), colon (23), skin (24) and breast cancer (25).

In previous reports, EGCG (26) and ATRA (19) demonstrated an anti-proliferative effect on AML cells with FLT3 mutations. In the present study, an *in vitro* investigation was performed to assess the effect of the combination of EGCG and ATRA on *FLT3*-mutated cell lines.

## Materials and methods

**Cell lines and cell culture.** Experiments were performed using four human leukemia cell lines: MOLM-14, MOLM-13, KOCL-48 and MV4-11 (26). These above cells were grown in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA) supplemented with 10% heat-inactivated FBS (Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and 0.1 mg/ml streptomycin (cat. no. P4333; Sigma-Aldrich; Merck KGaA) in a humidified incubator with 5% CO<sub>2</sub> at 37°C.

**Reagents.** EGCG (>97% purified powder) was generously gifted by Dr Yukihiko Hara (Tea Solutions, Hara Office Inc.) and ATRA was purchased from FUJIFILM Wako Pure Chemical Corporation. The reagents were dissolved in DMSO. Control cells were cultured with an equivalent concentration of DMSO as the maximum reagent dose. Throughout all the experiments, DMSO concentration did not exceed 0.1%, and thus should have had any effect on cytotoxicity (27).

**Cell proliferation assay.** Cell proliferation assays were performed using a trypan blue dye exclusion assay as described previously (26,28).

**Isobologram.** The dose-response interaction between ATRA and EGCG in the four cell lines were evaluated at the IC<sub>50</sub> doses using an isobologram of Steel and Peckham as described previously (19,29,30).

**Statistical analysis.** Data for isobologram were analyzed as described previously (26,31). The observed data were compared with the predicted minimum and maximum values for the combined effect. If minimum predicted value ≤ observed data ≤ maximum predicted data, the combined effect was additive. However if the mean of the observed data was higher than the maximum predicted data or lower than the minimum data, the combined effect was considered synergistic or antagonistic, respectively. To compare the three groups (observed, predicted minimum and predicted maximum data), a Friedman tests followed by a post hoc Nemenyi comparisons test was used.

To determine whether antagonism or synergism truly existed, a Wilcoxon signed-ranks test was used to compare the observed data with the predicted maximum or minimum data for an additive effect; the data were not normally distributed. P<0.05 suggested the combined effect was

considered significant. P≥0.05 suggested the combined the effect was regarded as being additive to antagonistic or additive to synergistic. Statistical analysis was performed using R version 4.0.0 (32). All experiments were performed at least three times.

The IC<sub>50</sub> values were calculated using linear approximation of the percentage of survival vs. the concentration of the drug and was performed using GraphPad Prism version 8.4.0 (GraphPad Software, Inc.).

## Results

ATRA has been shown to suppress cellular proliferation by inducing apoptosis (19), and EGCG is considered to be an FLT3-inhibitor which suppresses cell proliferation by disrupting a FLT3-Hsp90 interaction in *FLT3*-mutated cell lines (26). The aim of the present study was to determine whether a combination of the two reagents increased the effect of these drugs on suppression of cell growth in *FLT3*-mutated cell lines. The cytotoxic interaction of two reagents were examined by isobologram.

In MOLM-13 cells, one of the data points fell in the area of sub-additivity (Fig. 1A) but the results in Table I show that the mean value of the observed data (0.587) was smaller than that of the predicted maximum data (0.627) and larger than that of the predicted minimum data (0.328). Therefore, the combination of EGCG and ATRA was regarded as additive in MOLM-13 cells.

The results showed that the combination of ATRA and EGCG had an additive cytotoxic effect on MOLM-13 cells compared with each agent alone. For example, the IC<sub>50</sub> of ATRA alone in MOLM-13 cells was 0.0192±0.0054 μM; however, the IC<sub>50</sub> of ATRA was significantly reduced to 0.0008±0.0008 μM (P<0.01) following combined treatment with EGCG (15 μM) (Table II).

The results in Fig. 1B showed that almost all the data points in the KOCL-48 fell in the area of sub-additivity, suggesting that the combined effect of ATRA-EGCG was antagonistic, as the mean of the observed data (0.875) was significantly larger than both the predicted minimum (0.463) and maximum values (0.272) (Table I; P=0.0031). Nemenyi post-hoc tests were performed, and the results showed there was a significant difference between the observed data and the predicted minimum data (P=0.0026), but not between the observed data and the predicted maximum data (P=0.1455). To determine whether the condition of antagonism truly existed, a Wilcoxon signed-ranks test was used for comparing the observed data with the predicted maximum data for an additive effect (Fig. 2A). The results showed that the probability value was significant (P=0.0312) suggesting that the observed data were significantly higher than the predicted maximum data (Table I), indicative of an antagonistic effect of simultaneous exposure to the combined treatment in KOCL-48 cells.

Some data points fell on the border of additivity, whereas other data points fell in the area of sub-additivity in MOLM-14 and MV4-11 cells and were thus considered additive/antagonism (Fig. 1C and D; MOLM-14, P=0.0057; MV4-11, P=0.0009). Nemenyi post-hoc test results showed that there were significant differences between the observed

Table I. Mean values of the observed data and the estimated minimum and maximum values of combined treatment with ATRA and EGCG.

Cell line	n	Ob. data	Predicted values for the additive effect		P-value <sup>a</sup>	P-value <sup>b</sup>		P-value <sup>c</sup> , Ob./Max	Effect
			Minimum	Maximum		Ob./Min	Ob./Max		
MOLM-13	5	0.587	0.328	0.627					Additive
KOCL-48	6	0.875	0.272	0.463	0.0031	0.0026	0.1455	0.0312	Antagonism
MV4-11	7	0.676	0.301	0.593	0.0009	0.0005	0.1472	0.0156	Antagonism
MOLM-14	6	0.69	0.43	0.616	0.0057	0.0043	0.4804	0.0625	Additive to antagonistic

<sup>a</sup>Friedman test; <sup>b</sup>Nemenyi post hoc test; <sup>c</sup>Wilcoxon signed-rank test. Ob., observed; ATRA, all-trans retinoic acid; EGCG, (-)-epigallocatechin-3-gallate.

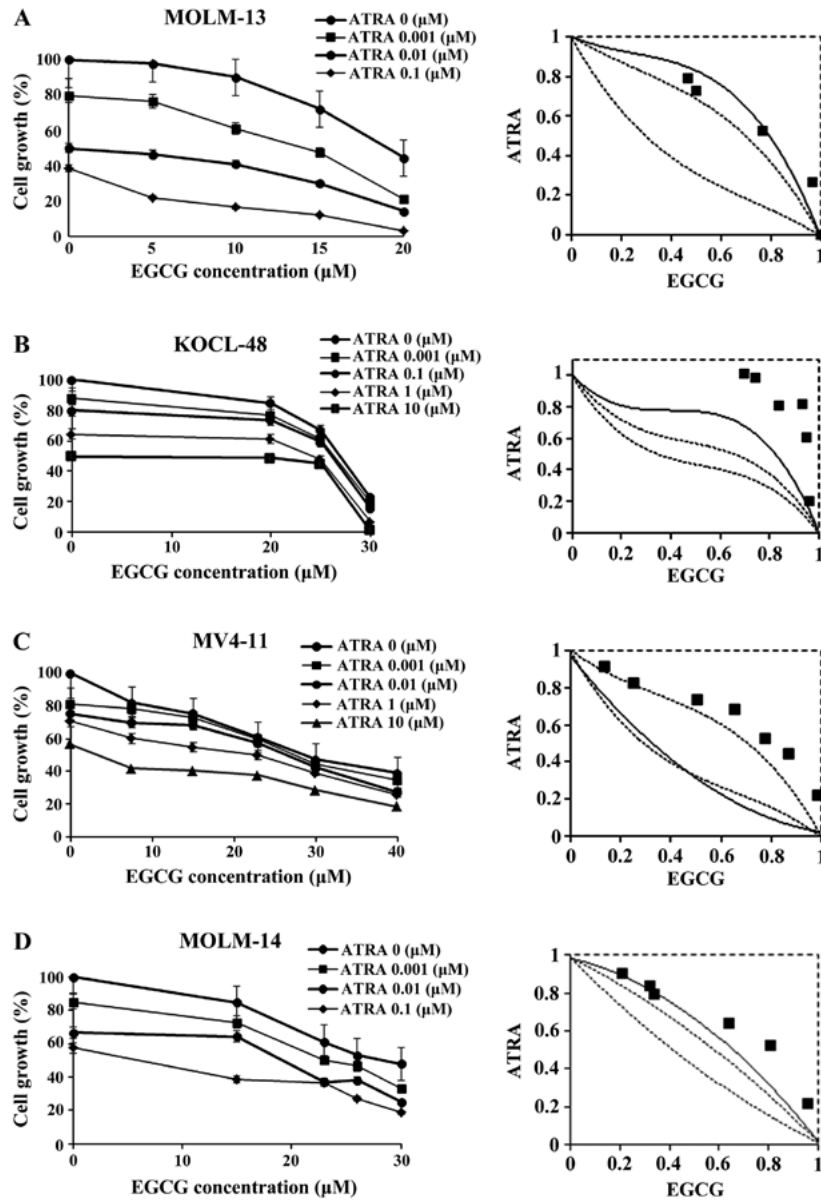


Figure 1. Isobolographs of simultaneous treatment with ATRA and EGCG. Isobolographs of the various treatments in (A) MOLM-13, (B) KOCL-48, (C) MV4-11 and (D) MOLM-14 cells. Each data point represents the mean  $\pm$  standard deviation of at least three separate experiments in isobolograms. Treatment with a combination of ATRA and EGCG had an additive effect on MOLM-13 cells and an antagonistic effect on MOLM-14, MV4-11 and KOCL-48 cells. ATRA, all-trans retinoic acid; EGCG, (-)-epigallocatechin-3-gallate.

Table II. IC<sub>50</sub> values of ATRA, EGCG and ATRA-EGCG combined on leukemia cells.

IC <sub>50</sub> value	Cell line			
	MOLM-13, $\mu$ M	MOLM-14, $\mu$ M	MV4-11, $\mu$ M	KOCL-48, $\mu$ M
ATRA	0.0192±0.0054	0.0867±0.0242	3.4933±0.2031	11.3421±1.0055
EGCG	18.8333±0.2902	26.7251±0.2554	26.7531±1.2773	26.6055±0.3783
ATRA+EGCG (5 $\mu$ M)	0.0088±0.0092	-	-	-
ATRA+EGCG (10 $\mu$ M)	0.0031±0.0009	-	-	-
ATRA+EGCG (15 $\mu$ M)	0.0008±0.0008	0.0259±0.01111	0.5683±0.0355	-
ATRA+EGCG (20 $\mu$ M)	-	-	-	15.2145±1.0054
ATRA+EGCG (23 $\mu$ M)	-	0.0015±0.0027	0.0317±0.0630	-
ATRA+EGCG (25 $\mu$ M)	-	-	-	0.9861±0.1845
ATRA+EGCG (26 $\mu$ M)	-	0.0011±0.0020	-	-

ATRA, all-trans retinoic acid; EGCG, (-)-epigallocatechin-3-gallate.

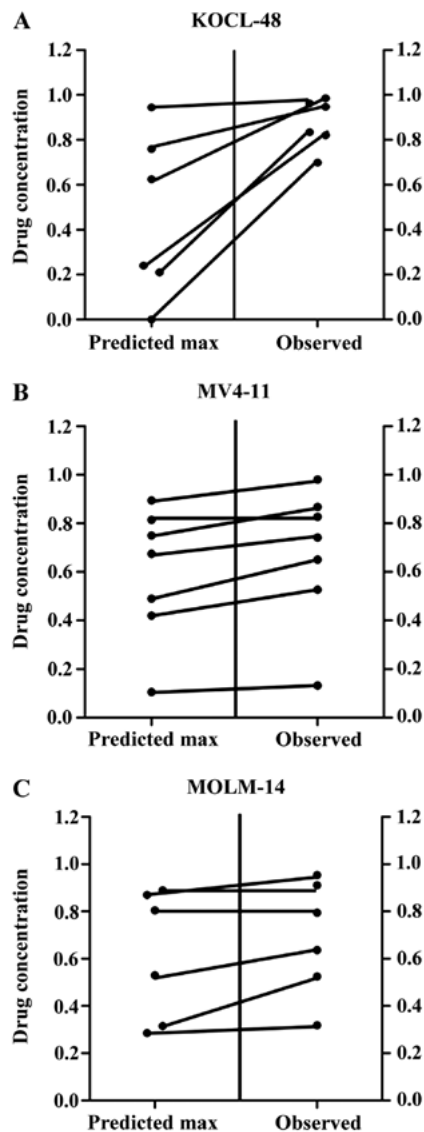


Figure 2. Comparison of the predicted maximum data for an additive effect with the observed data obtained from simultaneous exposure to all-trans retinoic acid and (-)-epigallocatechin-3-gallate. Predicted max and observed effects of combined treatment in (A) KOCL-18, (B) MV4-11 and (C) MOLM-14 cells.

data and the predicted minimum data ( $P < 0.05$ ), but not between the observed data and the predicted maximum data in MOLM-14 and MV4-11 (Table I). However, the mean value of the observed data (MV4-11, 0.676) was significantly higher than the predicted maximum value (MV4-11, 0.593;  $P < 0.0156$ ; Table I; Fig. 2B) suggesting a true antagonistic effect of the ATRA-EGCG combination in MV4-11 cells. In contrast, the  $P$ -value was 0.0625 suggesting an additive to antagonistic effect in MOLM-14 cells (Table I; Fig. 2C).

## Discussion

ATRA has been used as a major treatment intervention for patients with APL and functions by inhibiting vascular endothelial growth factor, which is crucial for angiogenesis (33). However, the duration of remission that is induced and maintained by ATRA therapy alone is short-lived, and ATRA alone fails to induce a second remission in the majority of patients following relapse (34). In order to address these issues, it may be necessary to enhance the efficacy of ATRA during the first treatment regimen. In general, AML is the result of at least two combined pathophysiological problems, including the acquisition of chromosomal rearrangements and multiple gene mutations which confer a proliferative, survival advantage and/or impaired hematopoietic differentiation (35). Therefore, administration of a therapy designed to address just one pathophysiological pathway is likely insufficient for a favorable response. In addition, administration of anticancer drugs may also result in severe cytotoxic side effects, restricting the window of doses which can be administered, thus limiting the potential efficacy of these therapeutic approaches (36). Through enhancing the effectiveness of cancer chemotherapy, the use of different combinations of anticancer drugs may overcome these limitations (36). The majority of anticancer drugs have distinct molecular mechanisms by which they exert their effects, and are thus associated with specific cytotoxic side-effects. Furthermore, for each drug there is an upper limit of concentration which can be used to achieve effective inhibition of tumor-cell proliferation whilst minimizing the extent of damage to healthy cells. A balance of a cocktail of anticancer

drugs may therefore maximize the beneficial effects, reducing the dose of each individual drug required and thus reducing the associated cytotoxic side effects of each individual drug (37).

The mechanism of the combined effect of ATRA and EGCG has only been extensively studied on APL and melanoma. ATRA enhances the antitumor activity of EGCG by upregulating the expression of 67-laminin receptor through retinoic acid receptor (38). EGCG has also been shown to support ATRA-induced neutrophil differentiation via death-associated protein kinase 2 (39). Another study found that ATRA combined with EGCG augmented cell differentiation in APL cells by enhancing the expression of phosphatase and tensin homolog to regulate the phosphatidylinositol 3-kinase PI3K/Akt/mTOR signaling pathway (40). However, to the best of our knowledge, there are no studies reporting on the combined treatment of ATRA and EGCG in AML cell lines carrying a *FLT3* mutation. Thus, the aim of the present study was to determine the impact of a combination of ATRA and EGCG on *FLT3*-mutated AML cell lines. A limitation of the present study is the fact that APL cell lines were not used to evaluate the effects of the combined treatment.

A previous study found that the side effects associated with ATRA treatment were correlated with the dose given (17). Therefore, combined treatment with ATRA and EGCG may maximize the therapeutic efficacy and mitigate the cytotoxic side effects.

In conclusion, the effects of the combined treatment with ATRA and EGCG observed in the present study provide experimental evidence of the potential use of this combination for treatment of patients with AML who harbor *FLT3*-mutations. The novelty of the findings of the present study is that the combination of ATRA and EGCG resulted in an additive but not synergistic effect, as seen in APL and melanoma cells. The underlying mechanism of the combined effect is not understood and requires further study.

### Acknowledgements

We would like to thank Professor Yuko Sato (University of Tokyo, Tokyo, Japan) for providing the cell lines used in the present study. We would also like to thank Dr Yukihiro Hara (Tea Solutions, Hara Office Inc., Tokyo, Japan) for providing the EGCG powder.

### Funding

This study was funded by the Vietnam National Foundation for Science and Technology Development (grant no. 106.02-2019.50).

### Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

### Authors' contributions

BTKL conceived and designed the study, formulated the experimental protocols, and prepared the manuscript. HTC performed the experiments, organized, and analysed the data,

and assisted in the preparation of the manuscript. Both authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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