# Synergistic effect of all-trans retinoic acid in combination with protein kinase C 412 in FMS-like tyrosine kinase 3-mutated acute myeloid leukemia cells

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**Abstract.** Acute myeloid leukemia (AML) is a heterogeneous disease. Numerous molecular abnormalities have been identified in AML and, amongst these, FMS-like tyrosine kinase 3 (*FLT3)* mutations are one of the most common somatic alterations detected. In the present study, an *in vitro* investigation was performed to evaluate the effects of all-trans retinoic acid (ATRA) and PKC412, alone and in combination, in *FLT3*-mutated AML cell lines. Trypan blue exclusion test, as well as morphological, western blot and isobologram analyses were conducted. The results indicated that the combined ATRA and PKC412 treatment exhibited additive or synergistic effects in *FLT3*-mutated AML cell lines. These results provided *in vitro* evidence for the future clinical trials evaluating the effects of a combination treatment using PKC412 and ATRA on AML patients with *FLT3*-mutations.

## Introduction

Acute myeloid leukemia (AML) is a common hematological malignancy, which is characterized by the arrest of myeloid cells at various differentiation stages, resulting in their accumulation as they are unable to differentiate into mature, functional blood cells (1,2). Numerous genetic abnormalities have been identified in AML. Among these, FMS-like tyrosine kinase 3 (*FLT3*) abnormality is common and a previous study has demonstrated that *FLT3* was abnormally activated in 70-90% of patients with AML, including overexpression of wild-type *FLT3* and *FLT3* mutations (3).

AML patients with *FLT3* abnormalities present unfavorable prognoses (4), including a high risk of relapse and lower long-term survival rates compared with patients with wild-type *FLT3* (5,6). The use of cytarabine- and anthracycline-based intensive chemotherapy, in combination with advanced supportive care and the introduction of allogeneic stem cell transplantation has been demonstrated to initially improve the outcome of patient responses (7,8). However, these responses were found to be transient, lasting for weeks to months, followed by progressive disease development and subsequent drug-resistance (9). The median survival of *FLT3*-mutated patients with AML has been reported to be ≤5 months, following the first disease relapse (10,11). To date, multiple small molecule FLT3-tyrosine kinase inhibitors (FLT3-TKIs) have been developed, and their effect in AML patients as single agents or in combination with chemotherapy have been evaluated (12). Protein kinase C (PKC)412 (*N*-benzoyl-staurosporine) is a FLT3-TKI that was originally developed as a PKC and vascular growth factor receptor inhibitor. PKC412 has been used in phase I clinical trials for the treatment of solid tumors, which demonstrated a tolerable dose (13,14). Subsequently, PKC412 was found to specifically and potently inhibit the growth of leukemic cell lines expressing *FLT3*-internal tandem duplication (ITD)-induced-myeloproliferative-like syndrome in the nanomolar range (15). The results of phase II and IIB trials, where patients with *FLT3* mutations were treated with PKC412, indicated that PKC412 was generally well-tolerated. These results suggested a potentially effective strategy comprising

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a combination of PKC412 and other agents, including chemotherapy, in AML patients with *FLT3*-mutations (16,17).

Individuals with a subtype of AML known as acute promyelocytic leukaemia (APL) are frequently treated with all-trans retinoic acid (ATRA). ATRA is a derivative of vitamin A (retinoids), also known as tretinoin (Vesanoid®). ATRA induces leukemia cell maturation and differentiation, and is therefore able to rapidly reduce leukemia symptoms by preventing myeloid cell accumulation (18). In addition, ATRA has been demonstrated to induce cell growth arrest, differentiation and cell death of various types of cancer cells *in vitro* (19,20). However, the clinical applications of ATRA are limited by side-effects, which include acute retinoid resistance, hypertriglyceridemia, mucocutaneous dryness, headaches, disease relapse following a brief remission period and drug resistance (21-23). In addition, its clinical applications are further limited due to its low plasma concentrations. Therefore, in order to circumvent these limitations, combinations of ATRA and other anticancer drugs have been investigated (24,25). Previous studies have indicated that ATRA was able to enhance the cytotoxic effect of chemotherapeutic drugs (26,27). Furthermore, certain preclinical trials have demonstrated the efficacy of using a combination of retinoids and cytotoxic drugs (27-29).

In the present study, an *in vitro* investigation was performed to evaluate the effect of the combination of ATRA and PKC412 in *FLT3*-mutated cell lines. The results of the present study may establish whether a clinical trial on patients with *FLT3* mutations should be conducted.

## Materials and methods

*Cell lines and culture conditions*. Four human leukemia cell lines were used in this study and were obtained as described previously (30). Briefly, the two sister cell lines, MOLM13 and MOLM14, were obtained from a patient with acute monocytic leukemia (M5a) presenting the t(9;11) mutation (31), MV4-11 cell line was acquired from an AML patient carrying the t(4;11) mutation (32) and KOCL-48 cell line was obtained from an infant leukemic patient carrying the t(4;11) mutation (33).

Two mutations within *FLT3* exon 14 were detected in MOLM13 and MOLM14 cells, including an ITD (21 bp) corresponding to codons Phe594-Asp600 and a novel missense nucleotide substitution at codon 599 (Tyr599Phe) (34,35). These mutations were located on the same allele (35). In MV4-11 cells, an ITD (30 bp) within *FLT3* exon 14, corresponding to codons Tyr591-Asp600, and a Tyr591His mutation were detected (34,35). In the KOCL-48 cell line, only the *FLT3*-Asp835Glu mutation was detected (34).

All the cell lines were cultured in RPMI-1640 medium (Sigma-Aldrich Japan K.K., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA), 100 IU/ml penicillin and 0.1 mg/ml streptomycin (Nakalai Tesque, Kyoto, Japan) in a humidified atmosphere containing 5% $CO_2$ at 37˚C.

*Reagents*. PKC412 was provided by Novartis Pharma AG (Basel, Switzerland) and ATRA was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All the drugs were dissolved in dimethylsulfoxide (DMSO; Merck-Millipore, Darmstadt, Germany). Control cells were cultured with the same DMSO concentration as that used for the highest drug dose (1:1000 dilution). To avoid cytotoxicity, the concentration of DMSO was maintained at <0.1%.

*Cell proliferation assays*. Cell proliferation was determined using the trypan blue dye exclusion test as described previously (30). Briefly, the cells were seeded in six-well plates ($1x10^5$ cells/ml) in the presence of various ATRA or PKC412 concentrations for 72 h. Following treatment, 10 $\mu$l cell suspension was mixed with 10 $\mu$l 0.4% trypan blue (Nacalai Tesque, Tokyo, Japan), and live cells were counted manually using a hemacytometer (Erma, Tokyo, Japan). The results were expressed as the percentage of the values obtained when the cells were grown in the absence of reagents.

*Morphological assessment for the detection of apoptotic cells*. In order to detect fragmented nuclei and condensed chromatin, the cells ($1x10^5$ cells/ml) were treated with 1 $\mu$M ATRA. Following incubation for 8 h, the cells were harvested and fixed onto slides using a Cytospin 2 (Shandon Southern Products Ltd., Cheshire, UK). Subsequently, the cells were stained with a Wright-Giemsa solution (Merck Co., Ltd, Tokyo, Japan) and the cell morphology was observed under an inverted microscope (IX70; Olympus Corp. Tokyo, Japan).

*Western blot analysis*. Cells were plated onto 10-cm dishes at a density of $1x10^5$ cells/ml in the presence of 1 $\mu$M ATRA. Following incubation for the indicated time periods, the cells were collected and washed twice with PBS. Next, the cells were dissolved in protein lysis buffer (Wako Pure Chemical Industries, Ltd), consisting of 5 mM EDTA, 50 mM NaF, 10 mM $Na_2H_2P_2O_7$, 0.01% Triton X-100, 5 mM HEPES, 150 mM NaCl, 1 mM $Na_3VO_4$, 1 mM phenylmethylsulfonyl fluoride and 75 $\mu$g/ml aprotinin, on ice for 30 min with a brief vortex of four times every 10 min. The samples were centrifuged at 16,000 x g at 4˚C for 10 min and the total cell lysates were collected for western blot analysis. Subsequently, the protein samples were subjected to 12% polyacrylamide gel electrophoresis and transferred to Hypond-P membranes (GE Healthcare Life Sciences, Little Chalfont, UK) using electroblotting. Following washing with PBS, the membranes were incubated with various antibodies and antibody-binding was detected using enhanced chemiluminescence system (Amersham Pharmacia Biotech, Tokyo, Japan). Rabbit polyclonal anti-actin (A2066; 1:500 in 5% skimmed milk) was obtained from Sigma-Aldrich, rabbit polyclonal anti-caspase-3 (#9662; 1:1,000 in 5% BSA) and mouse anti-caspase-9 (#9508 1:1,000 in 5% BSA) antibodies were purchased from Cell Signaling Technology (Tokyo, Japan) and rabbit-anti-poly-adenosine diphosphate ribose polymerase (PARP) antibody (#9542; 1:1,000 in 5% BSA) was purchased from Wako Pure Chemical Industries, Ltd. Secondary antibodies horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin (Ig)G (sc-2317) and anti-mouse IgG-HRP (sc-2031) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA)

*Isobologram analysis*. The isobologram method described by Steel and Peckham (36) was used to evaluate the dose-response

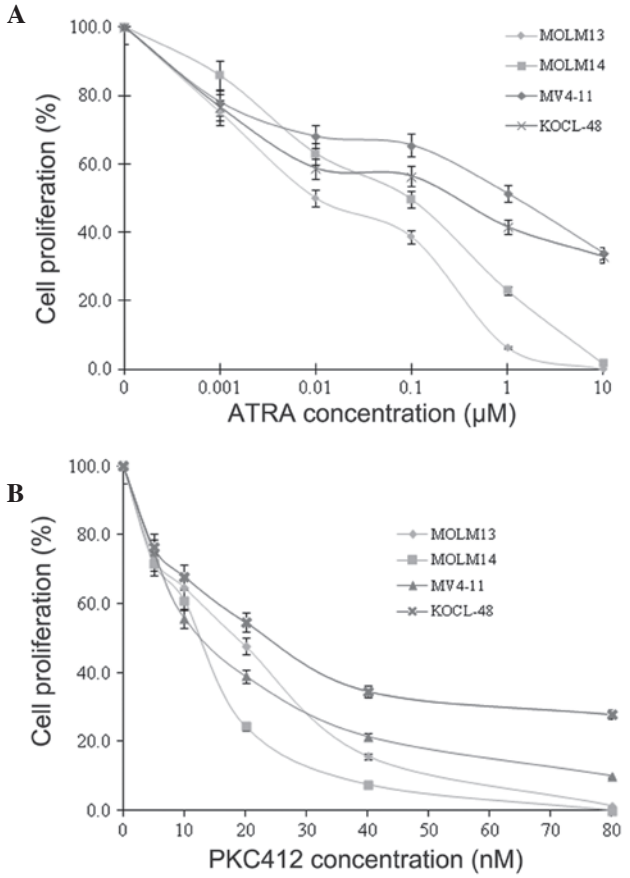


Figure 1. Effect of ATRA and PKC412 on cell proliferation of MOLM13, MOLM14, MV4-11 and KOCL-48 cell lines. MOLM13, MOLM14, MV4-11 and KOCL-48 cells at a density of $1x10^5$ cells/ml were treated with the indicated concentrations of (A) ATRA or (B) PKC412 for 72 h. Control cells were treated with dimethylsulfoxide alone (0 $\mu$M ATRA/PKC412). The number of live cells was counted following trypan blue exclusion test. The results were calculated as a percentage of the control values. Values are expressed as the mean ± standard deviation of three independent experiments. ATRA, all-trans retinoic acid; PKC412, protein kinase C 412.

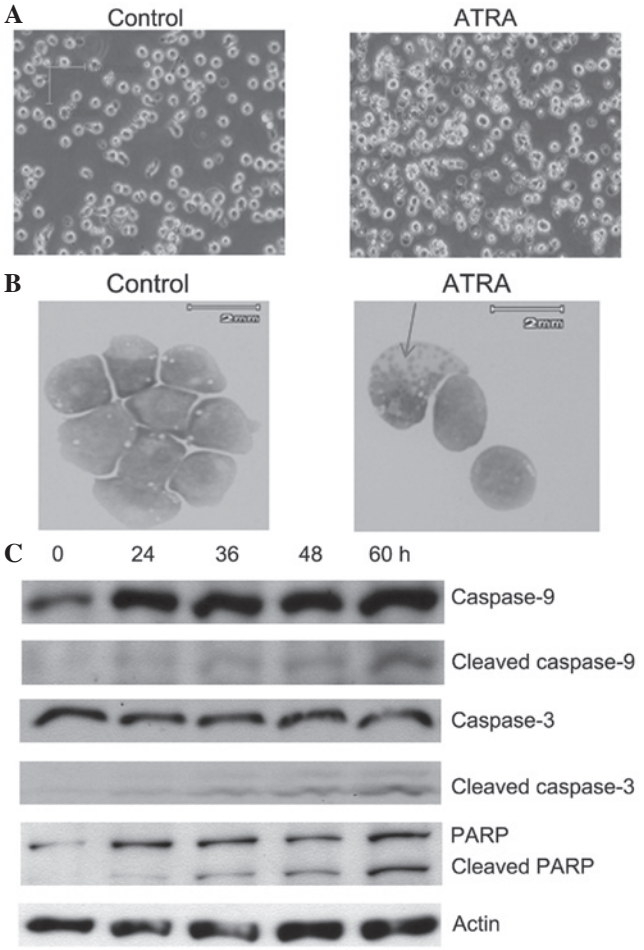


Figure 2. ATRA-induced apoptosis in FLT3-mutated cells. (A) Morphology of MOLM13 cells observed under an inverted microscope 8 h after treatment with 1 $\mu$M ATRA or without ATRA (control). Magnification, x10. (B) Morphology of MOLM13 cells stained with Wright-Giemsa solution, as observed under an inverted microscope. The arrows indicates the nuclei of MOLM13 cells that were fragmented by ATRA treatment. Magnification, x100. (C) Evidence of apoptosis induced by ATRA treatment in MOLM13 cells, observed by western blot analysis. Active forms of caspase-9 and caspase-3 were observed, as well as an inactivated form of PARP following ATRA treatment, demonstrating that ATRA induced apoptosis in these cells. ATRA, all-trans retinoic acid; FLT3, FMS-like tyrosine kinase 3; PARP, poly-adenosine diphosphate ribose polymerase.

interactions between ATRA and PKC412 in the MOLM13, MOLM14, MV4-11 and KOCL-48 cells at the $IC_{50}$ level. $IC_{50}$ was defined as the concentration of the reagent that produced 50% cell-growth inhibition. The isobologram method used in this study was selected since it can be used to investigate anticancer agents with unclear cytotoxic mechanisms and various dose-response curves (36).

*Statistical analysis*. Data obtained from the isobolograms were analyzed as described previously (37). The drug combinations were considered to have a synergistic effect when the majority of the observed data points were found in the area of supra-additivity (mean value of observed data less than predicted minimum values). By contrast, an antagonistic effect was considered between the drug combinations when the majority of data points were located in the areas of subadditivity and protection (mean value of observed data more than predicted maximum value). Statistical analyses were performed to analyze the significance of the detected synergism (or antagonism). The Wilcoxon signed-rank test was used to compare the observed data with the predicted minimum or maximum values for additive effects, which were closest to the observed data. Values are expressed as the mean ± standard deviation of three independent experiments. $P<0.05$ was considered to indicate a statistically significant difference. $P\geq0.05$ was considered to indicate additive to synergistic (or additive to antagonistic) effect. Additional data were analyzed using Student's t-test. Statistical analyses were performed using StatView 4.01 software program (Abacus Concepts, Berkeley, CA, USA).

## Results

*ATRA and PKC412 inhibit proliferation of FLT3-mutated AML cells*. In order to evaluate the effect of ATRA and PKC412 on cell growth, the cell lines (MOLM13, MOLM14, MV4-11 and KOCL-48) were incubated with DMSO alone (0 $\mu$M reagents), with various concentrations of ATRA (0.001-10 $\mu$M) or with various concentrations of PKC412 (5-80 nM) for 72 h. Cell proliferation was evaluated using the trypan blue exclusion

Table I. Mean values of observed data and predicted minimum and maximum values of the combination of all-trans retinoic acid and protein kinase C 412.

| | | | Predicted values for an additive effect | | |
|---|---|---|---|---|---|
| Cell line | n | Observed data | Minimum | Maximum | Effect |
| MOLM13 | 4 | 0.150 | 0.771 | 0.895 | Synergistic (<0.01) |
| MOLM14 | 4 | 0.231 | 0.265 | 0.584 | Synergistic (<0.01) |
| MV4-11 | 5 | 0.442 | 0.245 | 0.653 | Additive |
| KOCL-48 | 6 | 0.432 | 0.335 | 0.632 | Additive |

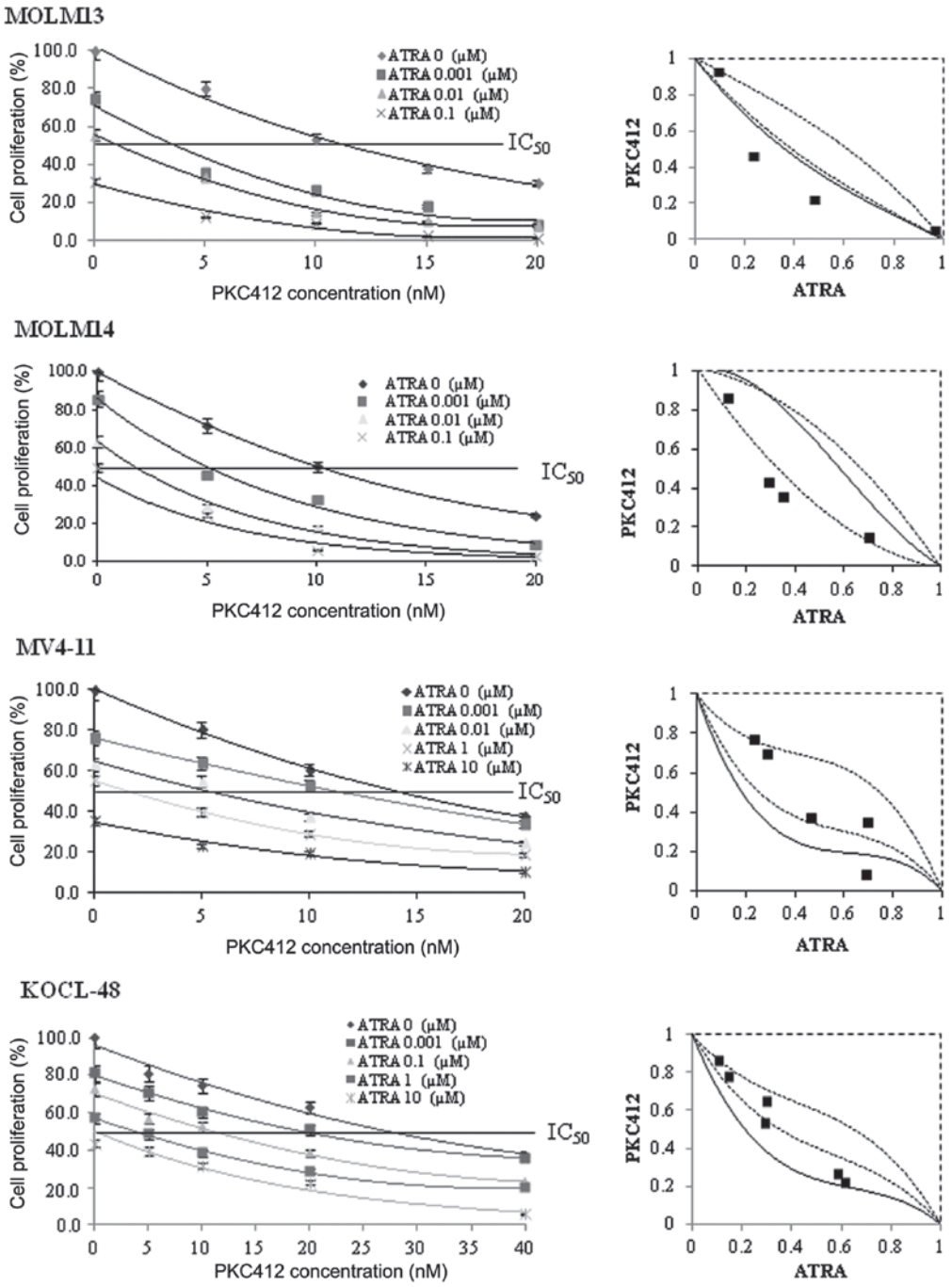


Figure 3. Isobolograms of simultaneous exposure to ATRA and PKC412 in MOLM13, MOLM14, MV4-11 and KOCL-48 cell lines. Each point observed in the isobolograms represents the mean value of at least three independent experiments. Values are expressed as the mean ± standard deviation of three independent experiments. Treatment with a combination of ATRA with PKC412 exhibited a synergistic effect on MOLM13 and MOLM14 cells and an additive effect on MV4-11 and KOCL48 cell lines. ATRA, all-trans retinoic acid; PKC412, protein kinase C 412.

test. The results indicated that ATRA and PKC412 inhibited the cellular proliferation of MOLM13, MOLM14, MV4-11 and KOCL-48 cells in a dose-dependent manner (Fig. 1).

*ATRA induces apoptosis in FLT3-mutated AML cells.* Previous studies demonstrated that ATRA was able to induce the differentiation of immature leukemic blasts into terminally differentiated granulocytic cells (38,39), or the apoptosis of specific tumor cells (340,41). To elucidate the mechanism underlying the ATRA-induced suppression of cellular proliferation in MOLM13, MOLM14, MV4-11 and KOCL-48 cells, the cell morphology and expression levels of apoptotic markers in these cells following treatment with 1 $\mu$M ATRA were evaluated. After 8 h of treatment with 1 $\mu$M ATRA, higher apoptosis was observed in the cell membranes of MOLM13 cells compared with the non-treated cells (0-h treatment; Fig. 2A and B). Furthermore, bands of cleaved caspase-9 were detected after 8 h of incubation with 1 $\mu$M ATRA. Cleaved caspase-9 induced the activation of caspase 3 (indicated by the presence of the cleaved caspase-3 band), which subsequently inactived an enzymes involved in DNA repair, known as PARP (Fig. 2C). The caspase 3-mediated proteolytic cleavage of PARP is a key event in apoptosis. In addition, apoptotic bodies were also observed after 24 h of treatment with 1 $\mu$M ATRA in MOLM13 cells (Fig. 2B), which indicated that ATRA induced apoptosis in cell lines exhibiting *FLT3* mutations. Analogous results were observed following evaluation of apoptosis in the remainder cell lines (data not shown).

*Synergistic effect of combined ATRA and PKC412 treatment.* ATRA was demonstrated to suppress cellular proliferation by inducing apoptosis, whereas PKC412 is known to be a specific *FLT3*-inhibitor that suppresses cell proliferation by inhibiting *FLT3* expression in *FLT3*-mutated cell lines (15). The present study aimed to elucidate whether a combination of ATRA and PKC412 was able to enhance the effect of these drugs on the suppression of cell proliferation in *FLT3*-mutated cell lines. The results revealed that combined treatment with ATRA and PKC412 had a synergistic effect on MOLM13 and MOLM14 cells and an additive effect on KOCL-48 and MV4-11 cells (Fig. 3). In the MOLM13 and MOLM14 cells, the combined data points were detected in the areas of supra-additivity and additivity. The mean values obtained were lower compared with the predicted minimum values (Table I), and the differences were found to be significant ($P<0.01$), indicating synergistic effects. However, in the KOCL-48 and MV4-11 cells, the combined data points were detected within the envelope of additivity, indicating an additive effect. In addition, the mean values detected for the KOCL-48 and MV4-11 cells were lower compared with the predicted maximum valies and higher than the predicted minimum values (Table I), confirming the presence of additive effects.

## Discussion

Despite the positive response to certain therapeutic strategies, the decreased ability of cancer cells to undergo apoptosis by malignant evolution represents a major challenge in the development of effective therapeutic approaches (19). Currently, novel selective strategies aiming to manipulate cancer cells, but not healthy cells, towards apoptosis are under development as potential therapies (42). Therefore, apoptosis-inducing agents that do not induce cytotoxicity in normal cells represent a potential anticancer treatment. The results of the present study demonstrated that ATRA inhibited cell proliferation and induced apoptosis in *FLT3*-mutated cell lines, indicating that ATRA may be a potentially useful drug for the treatment of AML patients with *FLT3* mutations.

ATRA has been demonstrated to inhibit vascular endothelial growth factor, which is crucial for the process of angiogenesis (29), and represents a major development in the treatment of APL with differentiation therapy. However, the duration of remission induced and maintained by ATRA therapy alone has been found to be short-lived, and ATRA alone failed to induce a second remission in the majority of patients following relapse (43,44). To circumvent these limitations, improving the effectiveness of ATRA on first drug application is required. Notably, AML results from by a combination of at least two pathophysiological problems. Therefore, the application of a therapy targeting only one pathophysiological pathway may not be sufficient to induce a major response, unless using a therapeutic combination. Furthermore, anticancer drugs may induce severe cytotoxic side-effects, which limit the doses that can be administered during treatment, thereby limiting the potential effectiveness of these therapeutic approaches (45,46). The use of differential combinations of anticancer drugs may circumvent these limitations by improving the effectiveness of cancer chemotherapy (45-47). The majority of anticancer drugs have distinct mechanisms of action and are associated with specific cytotoxic side-effects. In addition, an upper concentration limit exists for each drug in order to achieve effective inhibition of tumor-cell proliferation, whilst minimizing the extent of damage to healthy cells. Therefore, an ideal anticancer drug combination should maximize the therapeutic efficacy and minimize the associated cytotoxic side-effects (48-50).

Based on the aforementioned principals, the present study aimed to evaluate the effect of a combination of ATRA and PKC412 on *FLT3*-mutated AML cell lines. Preliminary preclinical data obtained in the present study demonstrated that the combination of ATRA and PKC412 had a synergistic/additive cytotoxic effect on *FLT3*-mutated cell lines as compared to each agent alone. For instance, the $IC_{50}$ of ATRA alone in MOLM13 cells was found to be 0.01 $\mu$M; however, upon combined treatment with PKC412 (3.5 nM), the $IC_{50}$ concentration of ATRA was significantly reduced to 0.001 $\mu$M ($P<0.01$). Similarly, the $IC_{50}$ of PKC412 alone in MOLM13 cells was found to be 20 nM; however, upon combined treatment with ATRA (0.001 $\mu$M), the $IC_{50}$ concentration of PKC412 was reduced to 3.5 nM ($P<0.01$).

A previous study indicated that the side-effects associated with PKC412 treatment were associated with the dosage administered (14). Therefore, in the treatment combination, ATRA and PKC412 may maximize the therapeutic efficacy and minimize cytotoxic side-effects. In conclusion, the results of the present study provided experimental evidence for the effect of a combined ATRA and PKC412 therapeutic strategy in the prevention and treatment of AML patients with *FLT3*-mutations.

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