All trans retinoic acid impairs platelet function and thrombus formation and inhibits protein kinase CβI/δ phosphorylation

Running title: ATRA inhibits platelet function and thrombosis

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Abstract

All-trans retinoic acid (ATRA) is widely used for induction of complete remission in patients with acute promyelocytic leukaemia. ATRA also regulates protein kinase C (PKC) activity. Therapeutic use of ATRA reportedly interferes with hemostatic function in APL patients, including effects on coagulation or other vascular cells, although effects of ATRA on platelets remain unclear. This study aims to investigate the effect of therapeutic-relevant doses of ATRA on platelet function. Human platelets were pre-incubated with ATRA (0-20 µM) for 1 hour at 37°C, followed by analysis of aggregation, granule secretion, receptor expression by flow cytometry, platelet spreading or clot retraction. Additionally, ATRA (10 mg/kg) was injected intraperitoneally into mice and tail bleeding time and arterial thrombus formation were evaluated. ATRA inhibited platelet aggregation and ATP release induced by collagen (5µg/ml) or thrombin (0.05U/ml) in a dose-dependent manner without affecting P-selectin expression or surface levels of glycoprotein (GP)Ibα, GPVI or αIIbβ3. ATRA-treated platelets demonstrated reduced spreading on immobilized fibrinogen or collagen and reduced thrombin-induced clot retraction together with reduced phosphorylation of Syk and PLCγ2. In addition, ATRA-treated mice displayed significantly impaired hemostasis and arterial thrombus formation in vivo. Further, in platelets stimulated with either collagen-related peptide or thrombin, ATRA selectively inhibited phosphorylation of PKCβI (Ser661) and PKCδ (Thr505), but not PKCα or PKCβII phosphorylation (Thr638/641). In conclusion, ATRA inhibits platelet function and thrombus formation, possibly involving direct or indirect inhibition of PKCβI/δ, indicating that ATRA might be beneficial for the treatment of thrombotic or cardiovascular diseases.

Key words: All trans retinoic acid; Platelet; Hemostasis; Thrombus formation; Protein kinase CβI/δ.
Introduction

Platelets play critical roles in thrombosis and haemostasis. At site of vascular injury, platelets attach to the subendothelial matrix through binding to exposed von Willebrand factor (vWF) or collagen via surface receptors glycoprotein (GP) Ibα or GPVI (1, 2). Engagement of platelet receptors triggers transduction of intra-platelet signaling, resulting in activation of integrin αIIBβ3 which binds fibrinogen and mediates platelet aggregation. This process is called αIIBβ3 “inside-out” signaling (3). Ligand binding to αIIIb3 also initiates αIIBβ3 “outside-in” signaling and subsequent activation of c-Src, Syk or PLCγ2, which mediates platelet spreading, clot retraction and thrombus formation to prevent blood loss (4). Therefore, abnormal platelet function is closely associated with bleeding or thrombosis under pathological conditions.

As the synthetic and natural forms of vitamin A, retinoids have been shown to regulate cell differentiation, growth or apoptosis through binding to their cognate nuclear receptors (5, 6), which include retinoic-acid-receptor (RAR) and retinoid-x-receptor (RXR) family members. RARs can bind to all trans retinoic acid (ATRA) and 9-cis RA, whereas, RXRs bind specifically to 9-cis RA (7). ATRA is an active metabolite of vitamin A under the family retinoid and has been widely used for induction of complete remission in patients with acute promyelocytic leukemia (APL) as the only well-established differentiation therapy (8, 9). In APL, RARα on chromosome 17 is fused with promyelocytic leukemia protein (PML) on chromosome 15, leading to formation of a fusion protein PML-RARα, which exerts negative effect on RA signaling and blocks cell differentiation (10). Pharmacological concentrations of ATRA are capable to bind PML-RARα and overcome its inhibitory effects, resulting in promotion of terminal differentiation of leukemic promyelocytes (11). ATRA has been reported to have effects on the hemostatic system of APL patients as demonstrated by decrease or normalization of clotting and fibrinolytic variables (12-14) as well as reduced proteolysis of the vWF (15). In addition, ATRA has also been reported to interfere with the hemostatic properties of different cells, such as
promyelocytic blast cells, normal human endothelial cells and monocytes (16). Furthermore, a previous study has demonstrated that rapid cytoskeletal events (platelet spreading) and actin-dependent morphological changes (extended cell body formation) were all significantly inhibited in ATRA-treated human platelets (17). However, whether ATRA affects platelet function remains unclear.

In the present study, through incubation of human platelets with different concentrations of ATRA, we aims to investigate the role of ATRA in platelet function.

**Materials and methods**

**Reagents**

ATRA was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in 0.5% v/v (final concentration) DMSO. Collagen and thrombin (≥ 10 NIH units/vial) were from Chrono-log Corporation (Havertown, PA, USA). Collagen-related peptide (CRP) was prepared as previously described (18). FITC-conjugated mouse anti-human CD41a was from BD Biosciences (San Jose, CA, USA). PE-conjugated anti-human/mouse CD62p (P-Selectin) and anti-human Glycoprotein VI purified antibody were purchased from eBioscience (San Diego, CA, USA). FITC-conjugated anti-CD42b antibody was from Abcam (Cambridge, MA, USA). FITC-conjugated goat anti-mouse IgG was purchased from ZSGB-BIO (Beijing, China). β-actin antibody and anti-rabbit IgG (HRP-linked) antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Tirofiban was purchased from Grand Pharma (China) CO. LTD.

**Animals**

All experimental procedures involving animals were complied with ARRIVE guidelines and approved by the Ethic Committee of Xuzhou Medical University. C57BL/6 mice, aged 8-10 weeks and weighted 24-28g were purchased from SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All mice were housed in specific
pathogen free (SPF) grade environment with 12 h light/dark cycle and free access to food and water.

**Platelet preparation**

All experimental procedures involving collection of human and mouse blood were approved by the Ethic Committee of Xuzhou Medical University. Informed consent has been obtained from all participants. Platelets were prepared from human and mouse blood as described previously (19). For human platelets, venous blood was collected into a tube anti-coagulated with trisodium citrate, glucose and citric acid (ACD) and centrifuged for 20 min at 120 x g at room temperature to obtain platelet-rich plasma (PRP). Platelet pellets were then collected by centrifugation of PRP at 1,350 x g for 15 min, followed by washing three times in CGS buffer and resuspended in Tyrode’s buffer. Mouse platelets were isolated from ACD anti-coagulated blood, washed using CGS buffer and resuspended in Tyrode’s buffer.

**Treatment of platelets with ATRA**

Isolated human platelets were incubated with different concentrations of ATRA (0, 1, 10 and 20 μM) at 37°C for 1 h followed by relevant analysis.

**Platelet aggregation and ATP release**

Platelet aggregation was performed in the presence of fibrinogen (0.5 mg/ml). After ATRA treatment, platelet aggregation in response to collagen (5 μg/ml) and thrombin (0.05 U/ml) was evaluated in a Lumi-Aggregometer Model 700 (Chrono-log Corporation, Havertown, PA, USA) at 37°C with stirring (1000 rpm). Platelet aggregation was quantified as the percentage of maximum platelet aggregation in the absence of drug. The release of adenosine triphosphate (ATP) was monitored in parallel with platelet aggregation after addition of luciferin/luciferase reagent (Chrono-log Corporation) to the platelet suspension according to the manufacturer's instructions. ATP release was quantified relative to the vehicle (0 μM ATRA) treatment.
Platelet alpha-granule release

Platelet alpha-granule release was assessed through measuring the surface expression of the α-granule glycoprotein, P-selectin by flow cytometry as described previously (20). Briefly, after ATRA treatment, human platelets were stimulated with collagen (5 and 10 μg/ml), thrombin (0.05 and 0.1 U/ml) or CRP (2 μg/ml) in the presence of PE-conjugated anti-P-selectin antibody followed by analyzing platelet P-selectin expression by flow cytometry. P-selectin expression was defined as the percentage of platelets in platelet-specific gate with positive staining of anti-P-selectin antibody.

Expression of platelet receptors

After treatment with ATRA, FITC-conjugated anti-CD42b antibody (GPIbα), FITC-conjugated mouse anti-human CD41a antibody (αIIb) or anti-human GPVI antibody (detected by FITC-conjugated goat anti-mouse IgG) were added and incubated for 30 min at room temperature followed by flow cytometry analysis of the expression of platelet receptors as described previously (21).

Platelet spreading

Human platelets were placed on glass coverslips which were pre-coated with fibrinogen (10 μg/ml) or collagen (10 μg/ml) (4°C overnight) at 37°C for 90 min followed by washing with PBS. Then, platelets were fixed, permeabilized, stained with Alexa Fluor-546-labelled phalloidin and observed under a fluorescence microscopy (Nikon-80i) using an X100 oil objective. The surface coverage was quantified using Image J software.

Clot retraction

Human platelets-mediated clot retraction was initiated by addition of thrombin (1 U/ml) in the presence of 2 mM Ca²⁺ and 0.5 mg/ml fibrinogen a at 37°C as described previously (19). Images were captured every 30 min.

Tail bleeding assay

Mice received intraperitoneal injection of ATRA (10mg/kg). After 30 min, tail
bleeding time was measured as described previously (19, 21).

**FeCl₃-induced arterial thrombosis**

After treated with ATRA (20 μM) or vehicle at 37°C for 30 min, mouse platelets (1 x 10⁸) were labelled with calcein and infused into ATRA-treated mice or wild-type mice respectively via tail vein injection. After 30 min, 10% w/v (final concentration) FeCl₃ was used to cause damages to mesenteric arterioles and thrombus formation was monitored by a fluorescence microscopy (Olympus BX53) (19, 21).

**Western blotting**

Washed human platelets were treated with CRP (5 μg/ml) or thrombin (under the condition of clot retraction) (1 U/ml) in the presence different concentrations of ATRA or vehicle for 15 min. Levels of total and phosphorylated Syk (anti-Tyr-525 and pan-Syk, Bioworld Technology), PLCγ2 (anti-Tyr-1217 and pan-PLCγ2; Bioworld Technology), PKCα/βII (anti-Thr638/641, Cell Signaling Technology, and pan-PKCα, Affinity Biosciences), PKCβI (anti-Ser661 and pan-PKCβI, Affinity Biosciences) or PKCδ (anti-Thr505, Cell Signaling Technology, and pan-PKCδ, Affinity Biosciences) were assessed by SDS-PAGE/western blot. The density of protein band was quantified using Image J software and the phosphorylation level was presented as a ratio to the total level.

**Statistical analysis**

Data are represented as mean ± standard deviation (SD) or mean ± standard error (SE) and analyzed using GraphPad Prism software. One-way ANOVA was conducted for comparison of difference among different groups. Two-way ANOVA with Bonferroni post-tests was performed for comparison among different groups over time. P < 0.05 indicates statistically significance.
Results

**ATRA inhibits platelet aggregation and dense-granule secretion**

To investigate the effect of ATRA on platelet aggregation, we incubated human platelets with different concentrations of ATRA (0, 1, 10 and 20 μM) followed by measuring collagen- or thrombin-induced platelet aggregation. As seen in Figure 1A, compared to vehicle treatment (0 μM ATRA), ATRA significantly impaired platelet aggregation in response to collagen (5 μg/ml) or thrombin (0.05 U/ml) in a dose-dependent manner. To further evaluate whether ATRA affects platelet dense-granule secretion, we also measured ATP release in collagen or thrombin-stimulated platelets and showed ATRA significantly inhibited ATP release from collagen- or thrombin-treated platelets in a dose-dependent manner (Figure 1B). Interestingly, ATRA did not affect platelet alpha-granule secretion after collagen or thrombin stimulation even at a higher dose as demonstrated by no significant changes of P-selectin expression after ATRA treatment (Figure 1C). Taken together, these data show that ATRA inhibits platelet aggregation and dense-granule secretion.

**ATRA does not affect the surface expression of platelet receptors**

Platelet surface receptors GPIbα, GPVI and αIibβ3, were demonstrated to play an important role in the regulation of platelet aggregation through engagement of their specific ligand, such as von Willebrand factor (GPIbα), collagen (GPVI) and fibrinogen αIibβ3 (22, 23). Given impaired platelet aggregation in response to agonist stimulation, surface expression of platelet receptors GPIbα, GPVI and αIibβ3 after ATRA treatment was measured by flow cytometry. As shown in Figure 2, ATRA treatment did not affect the surface expression of GPIbα (Figure 2A), GPVI (Figure 2B) and αIibβ3 (Figure 2C) after treated with different concentrations of ATRA even at a higher dose as indicated by no significant changes of the expression of these platelet receptors compared with vehicle treatment.

**Impaired platelet spreading and clot retraction after ATRA treatment**

To assess whether ATRA affects platelet spreading, after treated with different
concentrations of ATRA, human platelets were allowed to spread on immobilized fibrinogen or collagen and found platelet spreading was significantly impaired in ATRA-treated platelets in a dose-dependent manner as the surface coverage area of spread platelets on fibrinogen (Figure 3A) or collagen (Figure 3B) after ATRA treatment was significantly smaller than platelets after vehicle treatment, consistent with a previous study showing platelet spreading was inhibited in ATRA-treated human platelets (17). In addition, we also evaluate the effect of ATRA on platelet-mediated clot retraction, a process regulated by αIIbβ3 outside-in signaling (24, 25). Consistent with platelet spreading, clot retraction in ATRA-treated platelets was significantly inhibited in a dose-dependent manner as demonstrated by the significantly higher clot volume of ATRA-treated platelets than vehicle-treated platelets (Figure 3C). These data suggest that ATRA inhibits platelet αIIbβ3 outside-in signaling transduction.

**Reduced phosphorylation of Syk and PLCγ2 in ATRA-treated platelets**

Since ATRA inhibits platelet aggregation, spreading and clot retraction, we next assessed the effect of ATRA on platelet intracellular signaling transduction through measuring the phosphorylation level of Syk and PLCγ2, which have been shown to be important in the regulation of platelet function and αIIbβ3 signaling transduction (25). Our data showed that the phosphorylation of Syk and PLCγ2 in collagen-related peptide (CRP) (5 μg/ml)-stimulated platelets was significantly impaired after ATRA treatment in a dose dependent manner (Figure 4A). To investigate whether the impaired Syk phosphorylation is due to reduced GPVI signaling or integrin αIIbβ3 outside-in signaling, platelets were treated with tirofiban to block integrin αIIbβ3 followed by analysis of Syk phosphorylation and found there was no difference of Syk phosphorylation in platelets treated with ATRA compared with that in platelets treated with vehicle in the presence of tirofiban (supplementary Figure S1), indicating that the impaired Syk phosphorylation is due to impaired integrin αIIbβ3 outside-in signaling. Since thrombin-mediated clot retraction was inhibited in ATRA-treated platelets, the phosphorylation level of Syk and PLCγ2 which have been demonstrated
to regulate clot retraction (26) was also measured in thrombin (1 U/ml)-treated platelets under the conditions of clot retraction and found reduced phosphorylation of Syk and PLCγ2 after ATRA treatment (Figure 4B). Taken together, these data show that ATRA inhibits αIIbβ3 outside-in signaling transduction possibly through inhibition of the phosphorylation of Syk and PLCγ2.

**ATRA impairs hemostasis and arterial thrombosis in vivo**

To investigate the effect of ATRA on mouse platelet function in vivo, ATRA (10 mg/kg) was intraperitoneally injected into mice followed by analysis of platelet aggregation and ATP release as well as tail bleeding time. As seen in Figure 5, ATRA-treated mice displayed significantly reduced CRP- or thrombin-induced platelet aggregation (Figure 5A) and ATP release (Figure 5B) compared with vehicle-treated mice. Regarding the effect of ATRA on in vivo hemostasis and thrombosis, mice receiving injection of ATRA showed a significantly prolonged tail bleeding time (265.80 ± 46.93 s) compared with mice receiving vehicle (96.00 ± 23.59 s) (P < 0.05) (Figure 5C). Meanwhile, the arterial thrombus formation which was induced by FeCl3 was also significantly delayed in ATRA-treated mice receiving infusion of ATRA-treated platelets (29.67 ± 3.51 min) compared with that in mice receiving vehicle-treated platelets (16.50 ± 1.36 min) (P < 0.01) (Figure 5D). Taken together, these data show that ATRA impairs hemostasis and arterial thrombosis *in vivo*.

**ATRA inhibits the phosphorylation of PKCβI and PKCδ**

A previous study has demonstrated a direct interaction of ATRA with protein kinase C (PKC) (27) and PKC has been shown to play an important role in the regulation of platelet aggregation, granule secretion and spreading (28). To evaluate whether ATRA affects platelet function through regulation of PKC, we measured the phosphorylation level of PKCα, PKCβI/II and PKCδ in CRP (5 μg/ml) - or thrombin (1 U/ml)-stimulated platelets in the presence of different doses of ATRA, and found the phosphorylation level of PKCβI (Ser661) and PKCδ (Thr505) was significantly reduced in CRP- (Figure 6A) and thrombin-treated (Figure 6B) platelets in the
presence of ATRA in a dose-dependent manner. However, ATRA did not affect the phosphorylation of PKCα or PKCβI phosphorylation (Thr638/641). Consistently, ATRA administration into mice also decreased the phosphorylation of PKCβI and PKCδ in CRP-stimulated platelets (Figure 6C).

Discussion

As an active metabolite of vitamin A under the family retinoid, ATRA has been demonstrated to be widely applied for induction of complete remission in APL patients as the only well-established differentiation therapy (8, 9). However, therapeutic use of ATRA is reported to interfere with the haemostatic function in APL patients (13, 16), including effects on coagulation or other vascular cells, such as promyelocytic blast cells, normal human endothelial cells, and normal human monocytes, although effects of ATRA on platelets remain poorly understood. In the present study, through incubating human platelets with different doses of ATRA, we investigated the effect of ATRA on platelet function and showed ATRA inhibits platelet aggregation, spreading, clot retraction as well as hemostasis and arterial thrombosis in vivo.

ATRA has been previously shown to downregulate tumor necrosis factor-induced the expression of tissue factor in vascular endothelial cells (29), increase tissue plasminogen activator activity in rats as well as thrombomodulin expression in human U937 monoblast-like cells and human MEGO1 megakaryocyte-like cells (30, 31). In addition, ATRA has also been demonstrated to reduce the cancer procoagulant activity in acute promyelocytic leukemia cells (32) and improve the coagulopathy in APL patients (33) as demonstrated by decreased markers of clotting activation and fibrin degradation, increased protein C as well as reduced proteolysis of VWF after ATRA treatment, which was paralleled the improvement of clinical signs of the coagulopathy in APL patients (16). Furthermore, ATRA has been reported to interfere with the hemostatic function of promyelocytic blast cells, normal human endothelial cells, and normal human monocytes to exert beneficial antithrombotic effects (16).
Consistent with the antithrombotic role of ATRA, in the present study, we showed that ATRA inhibits platelet function as demonstrated by reduced platelet aggregation, spreading, clot retraction as well as impaired in vivo hemostasis and arterial thrombus formation after ATRA treatment, suggesting ATRA might also exert antithrombotic effects through inhibition of platelet function.

The platelet surface glycoprotein receptors, such as αIIbβ3, GPIbα and GPVI, play an important role in the regulation of platelet function (34). In response to vascular injury, platelet receptor GPIbα and GPVI adhere to the damaged blood vessel wall through recognition of exposed VWF and collagen respectively, leading to transduction of intra-platelet signaling pathway and subsequent activation of αIIbβ3, which binds to fibrinogen or VWF and mediates platelet aggregation and thrombus formation (2, 34). Considering the inhibition of platelet aggregation and thrombosis by ATRA, as well as importance of platelet receptors αIIbβ3, GPIbα and GPVI in platelet function, we evaluated whether ATRA affects the surface expression of these receptors and found no changes of the surface expression of these receptors in ATRA-treated platelets, suggesting ATRA does not affect the surface expression of platelet glycoprotein receptors.

Protein kinase C belongs to a family of serine/threonine kinases and are classified into three subfamilies, which are conventional isoforms (α, βI/II and γ), novel isoforms (δ, ε, η and θ) and atypical isoforms (ζ and υ/λ). Human platelets express PKCα, PKCβ, PKCδ and PKC0, which are demonstrated to play important role in the regulation of platelet aggregation, granule secretion, integrin activation, spreading as well as procoagulant activity (28). A previous study has demonstrated a direct interaction of ATRA with PKC (27), showing ATRA binding site in three PKC isozymes (α, βI and γ) after amino acid alignments and comparison of the crystal structures of several ATRA-utilizing proteins. Using Photoaffinity Labeling assay, they further showed that other PKC isozymes (α, βI, βIII, γ, δ, ε, and ζ) were also photolabeled with PKCβII having the highest affinity for ATRA and PKCζ having the lowest affinity. Consistent with the interaction between ATRA and PKC, in the present study, we
showed that ATRA treatment selectively inhibited phosphorylation of PKCβI (Ser661) and PKCδ (Thr505), but not PKCα or PKCβII phosphorylation (Thr638/641) in CRP- or thrombin-stimulated platelets. In addition, we also demonstrated that ATRA significantly reduced dense-granule secretion (ATP release) from collagen- or thrombin-treated platelets, which was consistent with a previous study showing that inhibition of PKCα/β or PKCδ significantly reduced dense granule release from human platelets after treatment with convulxin or thrombin receptor (PAR) agonists respectively (35). Interestingly, inhibition of both classical and novel PKC isoforms completely abolished dense granule secretion from convulxin- or PAR agonists-treated platelets (35). In the present study, both classical and novel PKC isoforms (PKCβI and PKCδ) phosphorylation was significantly reduced in CRP- or thrombin-stimulated platelets in the presence of ATRA, which might be the reason why dense granule secretion was both inhibited in collagen- or thrombin-treated platelets. Surprisingly, ATRA treatment did not affect alpha-granule secretion (P-selectin expression) from collagen- or thrombin-treated platelets, which was in accordance with a previous study showing that PKCδ had minimal effect on platelet P-selectin expression (36).

In conclusion, therapeutic dose of ATRA inhibits platelet function, hemostasis and arterial thrombus formation in vivo, which might be through direct or indirect inhibition of the phosphorylation of PKCβI/δ, suggesting it may represent a novel inhibitor of platelet function and thrombus formation.

Acknowledgement

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**Conflict of interest**

All authors have no conflict of interest to declare.

**References**

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integrin alphaIIbbeta3 outside-in signaling, hemostasis and arterial thrombosis. Haematologica 2018;103:1568-76.


Figure legends

Figure 1. Platelet aggregation and ATP release. Washed human platelets were incubated with different concentrations of ATRA (0, 1, 10 and 20 µM) at 37°C for 1 h followed by analysis of platelet aggregation in response to collagen (5 µg/ml) (A) or thrombin (0.05 U/ml) (B) in a Lumi-Aggregometer. Meanwhile, ATP release was measured simultaneously using luciferin/luciferase reagent. ATP release was presented relative to 0 µM ATRA which was defined as 100%. Platelet P-selectin expression was measured in ATRA-treated platelets after collagen or thrombin stimulation by flow cytometry (C). Data were presented as mean ± SE (n=3-4) and analyzed by one-way ANOVA. Compared to 0, *P < 0.05; **P < 0.01.

Figure 2. Expression of platelet surface receptors. After treated with different concentrations of ATRA, the expression of platelet surface receptors αIIβ3 (A), GPIbα (B) and GPVI (C) was measured by flow cytometry. Data were presented as mean ± SE (n=4) and analyzed by one-way ANOVA. IC: Isotype control.

Figure 3. Platelet spreading and clot retraction. After ATRA treatment, washed human platelets were placed on fibrinogen (A) or collagen (B) coated glass coverslips and allowed to spread at 37°C for 90 min followed by staining with Alexa Fluor-546-labelled phalloidin (mean ± SD, n = 3). ATRA-treated platelets were supplemented with 2 mM Ca2+ and 0.5 mg/ml fibrinogen and clot retraction was initiated after addition of thrombin (1 U/ml). Images were captured every 30 min (mean ± SD, n = 3) (C). For panel A and B, data were analyzed by one-way ANOVA. compared with 0, *P < 0.05; **P < 0.01; ***P < 0.001. For panel C, data were analyzed by two-way ANOVA. compared with 1, 10 or 20, ***P < 0.001.

Figure 4. Phosphorylation level of Syk and PLC-2. After ATRA treatment, human platelets were treated with 5 µg/ml CRP (A) or 1 U/ml thrombin (under the conditions of clot retraction) (B) for 15 min followed by analysis of the phosphorylation level of Syk and PLCγ2 by western blot. The protein expression was quantified using Image J software and represented as a ratio of phosphorylation to the total level (mean ± SD, n = 3). Data were analyzed by one-way ANOVA. Compared with 0, *P < 0.05; **P <
0.01; ***P < 0.001.

**Figure 5. Effect of ATRA administration on mouse platelet function.** Mice received intraperitoneal injection of ATRA (10mg/kg) followed by analysis of platelet aggregation (A), ATP release (B) and tail bleeding time (mean, n = 5) (C). After treated with ATRA (20 μM) or vehicle, mouse platelets were labelled with calcein and infused into ATRA-treated or wild-type mice respectively via tail vein injection followed by challenge with 10% FeCl₃ to induce arterial thrombus formation which was monitored by a fluorescence microscopy (Olympus BX53) and the vessel occlusion time was then measured (mean, n = 6) (D). For panel A and B, data were presented as mean ± SE (n=3). Data were analyzed by student t-test. *P < 0.05; **P < 0.01.

**Figure 6. Phosphorylation of PKCα/βII, PKCβI and PKCδ.** ATRA-treated human platelets were stimulated with 5 μg/ml CRP (A) or 1 U/ml thrombin (under the conditions of clot retraction) (B) for 15 min and the phosphorylation level of PKCα/βII, PKCβI and PKCδ was measured by western blot. In addition, platelets were isolated from mice treated with ATRA (10 mg/kg) and treated with 5 μg/ml CRP followed by analysis of the phosphorylation of PKCβI and PKCδ (C). The protein expression was quantified using Image J software and represented as a ratio of phosphorylation to the total level (mean ± SD, n = 3). Data were analyzed by one-way ANOVA Compared with 0, **P < 0.01; ***P < 0.001.
Figure 1

A

ATRA (μM)

Light transmission (%)

Time (5 min)

ATP release (μM)

Time (5 min)

Collagen (5 μg/ml)

Platelet aggregation (MAX%)

ATRA concentration (μM)

ATRA concentration (μM)

Collagen (5 μg/ml)

ATP release (%)

ATRA concentration (μM)

B

ATRA (μM)

Light transmission (%)

Time (5 min)

ATP release (μM)

Time (5 min)

Thrombin (0.06 U/ml)

Platelet aggregation (%)

ATRA concentration (μM)

ATRA concentration (μM)

Thrombin (0.06 U/ml)

C

P-selectin expression(%)

ATRA (μM)

0

1

10

20

0

1

5

10

0.05

0.1

2

Resting

Collagen (μg/ml)

Thrombin (U/ml)

CRP (μg/ml)
Figure 2

A

ATRA concentrations (µM)

B

ATRA concentrations (µM)

C

ATRA concentrations (µM)
Figure 3

A

B

C

![Images of cell culture with ATRA concentration and time points]

- **Panel A**: Graph showing the effect of ATRA concentration on cell area
- **Panel B**: Graph showing the effect of ATRA concentration on cell area
- **Panel C**: Images and graph showing cell volume over time with varying ATRA concentrations
Figure 4

A  CRP (5 μg/ml)

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B  Thrombin (1 U/ml)

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Phosphorylation/Total level

Syk  PLCγ2

At ATRA (μM)
Figure 5

A

B

C

D
Figure 6

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<td></td>
<td>ATRA (μM)</td>
<td>ATRA (μM)</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>20</td>
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</tbody>
</table>

A. Phospho-PKCα/βII
Total PKCα
Phospho-PKCβI
Total PKCβI
Phospho-PKC5
Total PKC5

B. Phospho-PKCα/βII
Total PKCα
Phospho-PKCβI
Total PKCβI
Phospho-PKC5
Total PKC5

C. Phospho-PKCβI
Total PKC-β4
Phospho-PKCα
Total PKCα

![Bar graphs showing phosphorylation levels for different concentrations of CRP and ATRA](image-url)
What is known about this topic?

- All trans retinoic acid (ATRA) has been widely used for induction of complete remission in patients with acute promyelocytic leukemia (APL).
- ATRA has effects on the hemostatic system of APL patients as demonstrated by decrease or normalization of clotting and fibrinolytic variables.
- ATRA inhibits cytoskeletal events (platelet spreading) and actin-dependent morphological changes (extended cell body formation) in human platelets.

What does this paper add?

- ATRA inhibits platelet aggregation, ATP release, spreading on fibrinogen or collagen and clot retraction.
- ATRA impairs in vivo hemostasis and arterial thrombus formation.
- ATRA selectively inhibits phosphorylation of PKCβ1 (Ser661) and PKCδ (Thr505).