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Original Article

Aristolochic acid C displays cytotoxic effect and remarkable enhancing effect on the all-*trans* retinoic acid-induced superoxide-generating ability in U937 cells

Hidehiko Kikuchi[1], Kaori Harata[1], Harishkumar Madhyastha[2], Hitomi Mimuro[3] and Futoshi Kuribayashi[4]

[1]Department of Food and Nutrition, Shokei University Junior College, 2-6-78 Kuhonji, Chuo-ku, Kumamoto 862-8678, Japan
[2]Department of Cardiovascular Physiology, Faculty of Medicine, University of Miyazaki, 5200, Kihara, Kiyotake, Miyazaki 889-1692, Japan
[3]Division of Genome-wide Infectious Microbiology, Research Center for GLOBAL and LOCAL Infectious Diseases, Oita University, 1-1 Idaigaoka, Hasama, Yufu, Oita, 879-5593, Japan
[4]Department of Biochemistry, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama, 701-0192, Japan

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ABSTRACT — Aristolochic acids (AAs) with strong bio-toxicity are the natural compounds that consist in Aristolochiaceae plants. There are the governing health issues regarding toxicities of AAs although Aristolochiaceae plants have been used as herbal medicines. For example, AAs are known as significant risk factors for nephropathy, urological cancer, liver cancer and so on. However, the understandings about the molecular mechanisms of toxicity of each AA derivative have been still poor and insufficiently studied. In this study, we investigated the effects of four AA derivatives (AA-A, AA-B, AA-C and AA-D) on the viability and the all-*trans* retinoic acid (ATRA)-induced superoxide anion (O_2^-)-generating ability of human leukemia U937 cells. AA-A and AA-C remarkably reduced cell viability when co-treated with ATRA while AA-B and AA-D had little effect on viability of U937 cells. On the other hand, only AA-C among the four AAs dramatically up-regulated the ATRA-induced O_2^--generating ability. Quantitative RT-PCR and immunoblotting analyses showed that AA-C significantly enhances the ATRA-induced O_2^--generating ability via up-regulating gene expression levels of gp91-phox, which is an essential factor for the O_2^--generating ability of phagocytes. These findings revealed that AA-C has not only the ATRA-enhanced cytotoxic effect but also the remarkable enhancing effect on the ATRA-induced O_2^--generating ability via up-regulating transcription of gp91-phox gene.

Key words: Aristolochic acid, Superoxide, Cytotoxicity, All-*trans* retinoic acid, Gp91-phox, Leukocyte

INTRODUCTION

Aristolochiaceae plants show strong toxicity against human resulting in development of various renal diseases such as nephropathy and kidney cancer although Aristolochiaceae plants have been used as herbal medicines. At the present time, aristolochic acids (AAs) that consist in Aristolochiaceae plants have been identified as the causative substances of these renal diseases caused by ingested Aristolochiaceae plants (Anger *et al.*, 2020; Ji *et al.*, 2021; Wang *et al.*, 2023). Thus, kidney complications caused by AAs is especially called AA nephrop-

Correspondence: Hidehiko Kikuchi (E-mail: masakari@shokei-gakuen.ac.jp)

athy (AAN). In addition, AAs are also known as significant risk factors for urologic and hepatobiliary cancers (Das *et al*., 2022). On the other hand, very interestingly, AAs play an important role as not only the larval feeding stimulants of an Aristolochiaceae plants-feeding swallowtail butterfly, but also the defensive substances against vertebrate predators such as tree sparrows (Nishida and Fukami, 1989).

While AAs are thought to bring about their toxicity via generating DNA adducts in the TP53 tumor suppressor gene, findings about the molecular mechanisms of AAs-induced diseases have been still poor (Yang *et al*., 2019; Anger *et al*., 2020). Recently, it was reported that AA should activate transcription of gp91-phox in kidney, resulting in AAN via enhancing superoxide anion (O_2^-) production (Declèves *et al*., 2016; Kim *et al*., 2019). As is well known, gp91-phox was originally discovered as a component of the O_2^--generating system in phagocytes. Therefore, in this paper, we examined the effect of AAs on the O_2^--generating system in phagocytes.

The O_2^--generating ability of phagocytes (macrophages, neutrophils) is essential for innate immune system. This system is formed from a membrane cytochrome b_{558} which is a heterodimer protein composed of the small [p22-phox] and the large [gp91-phox] subunits, three specific cytosolic proteins (p40-phox, p47-phox and p67-phox), and ubiquitous small G-protein Rac (Dagher and Pick, 2007; Panday *et al*., 2015). The gp91-phox (large subunit of the cytochrome b_{558}) is also called as NADPH oxidase 2 (Nox2) because this system carries an electron from NADPH to molecular oxygen via heme in the gp91-phox protein, resulting in generation of O_2^-. On the other hand, various differentiation inducers including all-*trans* retinoic acid (ATRA) can promote differentiation of human monoblastic leukemia U937 cells to macrophage-like cells (Harris and Ralph, 1985). This *in vitro* differentiation system using U937 cells has been used as a powerful model for *in vitro* studies on induction of the O_2^--generating system in phagocytes. While undifferentiated U937 cells generate an undetectable level of O_2^-, differentiated U937 cells able to generate O_2^- in response to phorbol 12-myristate 13-acetate (PMA) (Kikuchi *et al*., 1994; Kikuchi *et al*., 2011). We have investigated the effects of various phytochemicals on the ATRA-induced O_2^--generating ability of U937 cells, such as curcumin (Kikuchi *et al*., 2010), resveratrol (Kikuchi *et al*., 2018), chalcones (Kikuchi *et al*., 2019), sulforaphane (Akiyoshi *et al.*, 2019), ellagic acid and urolithin A (Kikuchi *et al*., 2021a), hydroxyflavones (Kikuchi *et al*., 2021b), L-theanine (Kikuchi *et al.*, 2022a), polymethoxyflavones (Kikuchi *et al*., 2022b) and cafestol and kahweol (Kikuchi *et al*., 2023).

In this communication, we studied the effects of various AA derivatives on viability and the ATRA-induced O_2^--generating ability of U937 cells, and revealed that the particular AA derivatives exhibit remarkable cytotoxicity in collaboration with ATRA and significantly enhance the ATRA-induced O_2^--generating ability via up-regulating the gene expression of gp91-phox.

MATERIALS AND METHODS

Materials

AA-A, PMA, ATRA, luminol (Sigma, St Louis, MO, USA), AA-B, AA-C (LKT Laboratories Inc, St. Paul, MN, USA), AA-D (ChemFaces, Hubei, China), ReverTra Ace qPCR RT Master Mix with gDNA remover (Toyobo, Osaka, Japan), RPMI-1640 culture medium (Thermo Fisher Scientific, Waltham, MA, USA), GeneAce SYBR qPCR mix α (Nippon Gene, Tokyo, Japan), Diogenes-luminol chemiluminescence probes (National Diagnostics, Atlanta, GA, USA), fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA), plasmocin (InvivoGen, San Diego, CA, USA), Trizol reagent (Invitrogen, Carlsbad, CA, USA), monoclonal anti-gp91-phox antibody (BD Biosciences, San Jose, CA, USA), monoclonal anti-p22-phox antibody (GeneTex, Irvine, CA, USA), monoclonal anti-Na^+/K^+-ATPase antibody (Abcam, Cambridge, UK), and horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin (Promega, Madison, WI, USA) were obtained from the companies indicated.

Cell culture, treatment with AAs in the presence of ATRA and measurement of cell viability

A human monoblastic leukemia cell line U937 (RCB0435) was provided by the RIKEN BRC (Saitama, Japan) through the National Bio-Resource Project of the MEXT, Japan. U937 cells were grown in RPMI-1640 culture medium containing 10% FBS and 5 μg/mL plasmocin as described (Kikuchi *et al*., 2019). Cells (1.0×10^6) in 5 mL of the culture medium were incubated with (20, 50 and 100 μM) or without AAs in the presence or absence of 1 μM ATRA at 37°C for 48 hr. Viable cells were counted by the trypan blue dye exclusion method using a hemocytometer under a microscope. (Kikuchi *et al*., 2020).

Measurement of O_2^- generation

Cells (1.0×10^6) in 5 mL of the culture medium were incubated with (10 or 20 μM) or without AAs in the presence or absence of 1 μM ATRA at 37°C for 48 hr. Meas-

urement of O_2^- generation was performed using Lumat^3 LB9508 luminometer (Berthold Technologies, Bad Wildbad, Germany). To enhance chemiluminescence, luminol and Diogenes-luminol chemiluminescence probes were used as described (Kikuchi *et al*., 2018).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Cells (1 x 10^6) in 5 mL of the culture medium were incubated with or without 20 μM AA-C in the presence of 1 μM ATRA at 37°C for 48 hr. Total RNAs were isolated from the cells using Trizol reagent. The cDNAs were synthesized using a first strand cDNA synthesis kit, and RT-qPCR using GeneAce SYBR qPCR mix α was performed by the MyGo Mini S real-time PCR instrument (IT-IS Life Science, Cork, Ireland). Resulting PCR data were analyzed according to the attached manufactures' protocols. Sequence data of PCR primers were also listed in our previous report (Kikuchi *et al*., 2022b). RT-qPCR data of all genes tested were normalized to those of GAPDH gene.

Immunoblot analysis

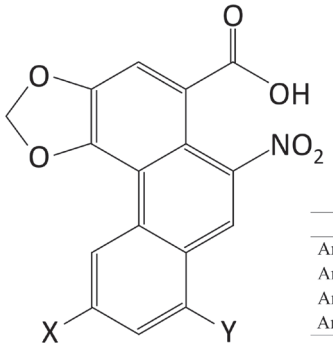
Cells (1 x 10^6) in 5 mL of the culture medium were incubated with or without 20 μM AA-C in the presence of 1 μM ATRA at 37°C for 48 hr under standard cell culture protocol. Cells were disrupted in 100 μL of 50 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose, 2 mM EDTA and 1 mM PMSF. The disrupted cells were centrifuged at 15,000 rpm for 10 min in a TMP-21 rotor (TOMY SEIKO, Tokyo, Japan). The precipitations (membrane fractions) were treated with 10% trichloroacetic acid, and centrifuged at 15,000 rpm for 5 min in a TMP-21 rotor. The precipitates were dissolved in a solubilizing solution containing 9 M urea, 2% Triton X-100 and 5% 2-mercaptoethanol, and added a loading buffer [0.5 M Tris-HCl (pH 6.8) containing 5% SDS and 20% glycerol]. These protein samples were subjected to SDS-PAGE followed by immunoblot analysis using anti-p22phox or anti-gp91phox antibodies as primary antibodies. Data analyses were carried out using a luminescent image analyzer STAGE-5100 (AMZ System Science, Osaka, Japan) and the Quant-AMZ software (TotalLab., Newcastle upon Tyne, UK). Human Na^+/K^+-ATPase was used as a control (Kikuchi *et al*., 2019).

Statistical analysis

Quantitative data are presented as averages of three separate experiments. Error bars indicate standard deviation. Statistical differences were calculated with Student's *t* test.

RESULTS AND DISCUSSION

Chemically, AAs are nitrophenanthrene carboxylic acid derivatives including various analogs (Nishida and Fukami, 1989; Tanaka *et al*., 2000; Wei *et al*., 2005; Wang *et al*., 2017). In this study, we investigated the influence of AAs on the O_2^--generating system using four commercially available AA derivatives: AA-A, AA-B, AA-C and AA-D. As shown in Fig. 1, these four AA derivatives are structurally different by the presence or absence of the methoxy group at the 8-position and hydroxyl group at the 10-position. To know the influences of the four AAs on viability of U937 cells in the presence or absence of ATRA, the cells were treated with up to 100 μM of each compound with or without 1 μM ATRA for 48 hr. ATRA showed no cytotoxicity at 1 μM (data not shown). As shown in Fig. 2, all AAs tested displayed no effect on the viability of U937 cells up to 20 μM with or without ATRA for 48 hr. While AA-D had no effect on viability of U937 cells up to 100 μM with or without ATRA for 48 hr, 100 μM AA-B presented a weak cytotoxicity at 48 hr in the presence of 1 μM ATRA (viability: to ~81.5%). In contrast, other two AAs (AA-A and AA-C) significantly reduced the viability of U937 cells at a concentration of 50 or 100 μM (50 μM AA-A: to ~84.3%, 100 μM AA-A: to ~4.6%, 50 μM AA-C: to ~89.1%, 100 μM AA-C: to ~54.3%) respectively at 48 hr in the absence of ATRA. In addition, 1 μM ATRA reduced the viability of U937 cells in synergy with 50 or 100 μM AA-A and AA-C at 48 hr (50 μM AA-A: to ~36.8%, 100 μM AA-A: to ~4.4%, 50 μM AA-C: to ~38.9%, 100 μM AA-C: to ~5.0%). These data revealed that the four AAs tested have no effect on viability of U937 cells up to 20 μM with or without ATRA and 50 or 100 μM AA-A and AA-C show significant cytotoxicity effects that are enhanced by 1 μM ATRA in U937 cells.



	X	Y
Aristolochic acid A	H	OCH_3
Aristolochic acid B	H	H
Aristolochic acid C	OH	H
Aristolochic acid D	OH	OCH_3

Fig. 1. Chemical structures of AA derivatives.

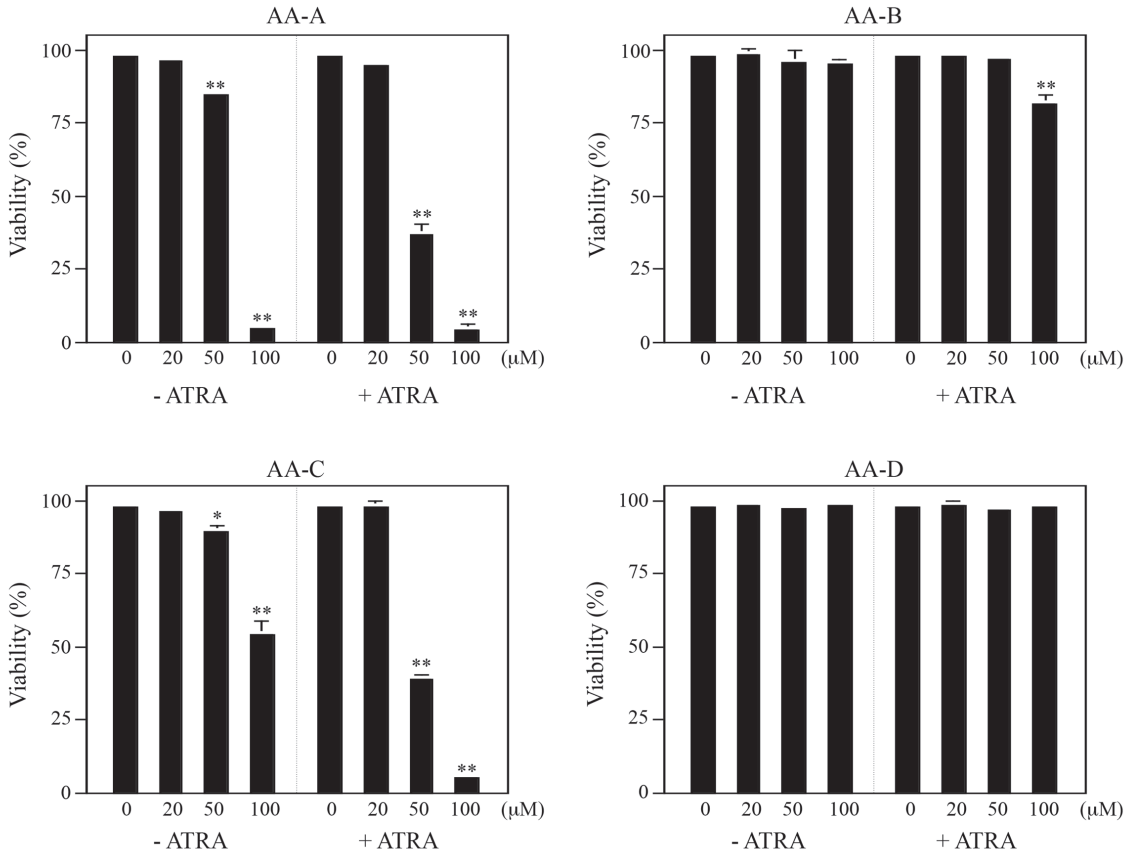


Fig. 2. Effects of four AA derivatives on cell viability of U937 cells. Cells (1.0 x 10^6) in 5 mL of culture medium were incubated without or with 20 μM, 50 μM or 100 μM AA derivatives in the absence or presence of 1 μM ATRA for 48 hr. Viable cells were counted by the trypan blue dye exclusion method. Data represent the averages of three separate experiments. Statistical differences were calculated by Student's *t* test. *, $p < 0.05$, **, $p < 0.01$ compared with the data without AA derivatives.

To examine the effects of these four AAs on the ATRA-induced O_2^--generating ability of U937 cells, the cells were treated with 10 μM or 20 μM of each AA in the presence of 1 μM ATRA for 48 hr. As mentioned above, these AAs showed no effect on viability of U937 cells up to 20 μM in the presence of 1 μM ATRA (Fig. 2). As shown in Fig. 3, AA-C dramatically enhanced the ATRA-induced O_2^--generating ability of U937 cells (10 μM AA-C: to ~221% of the ATRA-treated cells, 20 μM AA-C: to ~301% of the ATRA-treated cells). In addition, only 20 μM AA-C could slightly enhance the O_2^--generating ability of U937 cells even in the absence of ATRA (data not shown). On the other hand, 10 μM and 20 μM of AA-A showed the opposite effect on the ATRA-induced O_2^--generating ability of U937 cells (10 μM AA-A: to ~131% of the ATRA-treated cells, 20 μM AA-A: to ~83% of the ATRA-treated cells). Unfortunately, this reason is not clear. On the other hand, AA-B and AA-D displayed no influence on the ATRA-induced O_2^--generating ability of U937 cells. These results demonstrated that only AA-C has a strong enhancing effect on the ATRA-induced O_2^--generating ability of U937 cells. However, it is difficult to guess the structure-activity relationships from the chemical structures of these four AAs tested as shown in Fig. 1.

To reveal the mechanism of the enhancement of the ATRA-induced O_2^--generating ability by AA-C, we examined the effects of AA-C on the transcription levels of p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox genes that are essential for the leukocyte O_2^--generating system by RT-qPCR technique. After 48 hr of treatment, total RNAs were extracted from the 1 μM ATRA-treated, the 1 μM ATRA plus 10 μM AA-C-treated, and the 1 μM ATRA plus 20 μM AA-C-treated U937 cells. Quantitative

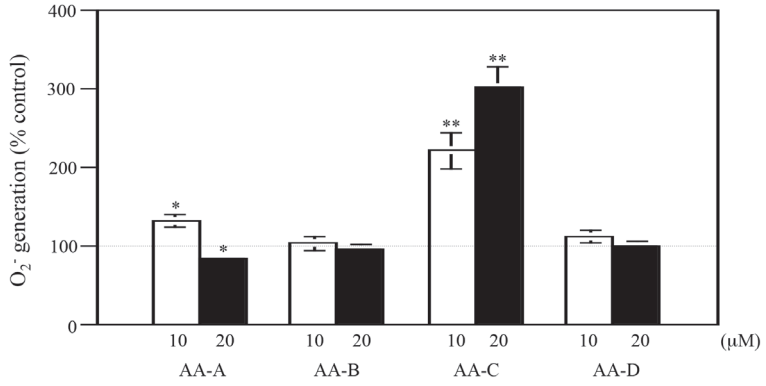


Fig. 3. Effects of AA derivatives on the ATRA-induced O_2^--generating activity of U937 cells. After cultivation without or with 10 or 20 μM AA derivatives in the presence of 1 μM ATRA for 48 hr, cells (1 x 10^5 cells/mL) were stimulated with 200 ng/mL PMA at 37°C. PMA-induced chemiluminescence was measured at 10 min after stimulation using a Lumat^3 LB9508 luminometer as described previously (Kikuchi *et al*., 2018). Quantitative data are indicated as percentages of control value obtained from ATRA-treated U937 cells, and presented as the average of three separate experiments. Error bars indicate standard deviation. Statistical differences were calculated by Student's *t* test. *, $p < 0.05$, **, $p < 0.01$ compared with the data of ATRA-treated U937 cells.

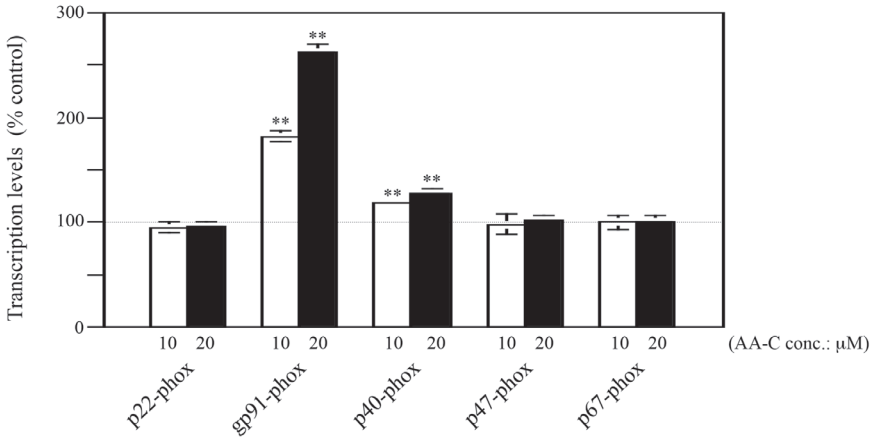


Fig. 4. Effects of AA-C on the transcription levels of the O_2^--generating system-related factors. The transcription levels of p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox were determined by RT-qPCR using total RNA extracted from ATRA-treated, ATRA plus 10 μM AA-C and ATRA plus 20 μM AA-C-treated U937 cells as described in "MATERIALS AND METHODS". All qPCR data of p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox genes were normalized to qPCR data of GAPDH gene. Quantitative data are indicated as percentages of control value obtained from ATRA-treated U937 cells, and presented as the average of three separate experiments. Error bars indicate standard deviation. Statistical differences were calculated using Student's *t* test. **, $p < 0.01$ compared with the data of ATRA-treated U937 cells.

data of RT-qPCR were indicated as percentages of control values obtained from the ATRA-treated U937 cells (Fig. 4). AA-C at a concentration of 10 μM and 20 μM showed no effect on the transcription levels of p22-phox, p47-phox and p67-phox genes during co-treatment with 1 μM ATRA. On the other hand, the transcription levels of p40-phox gene were slightly enhanced in the 1 μM ATRA plus 10 μM or 20 μM AA-C-treated U937 cells (10 μM AA-C: to ~119% of the ATRA-treated cells, 20 μM AA-C: to ~127% of the ATRA-treated cells). Moreover, interestingly, the transcription levels of gp91-phox gene were remarkably up-regulated in the 1 μM

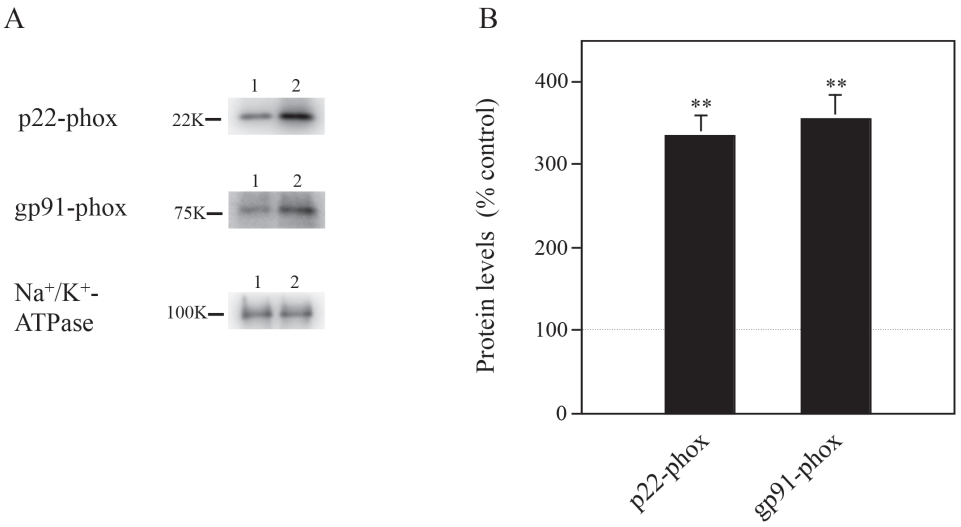


Fig. 5. Effects of AA-C on the protein levels of cytochrome b_{558} composed of p22-phox and gp91-phox proteins. (A) Typical immunoblot profiles. Protein levels of p22-phox and gp91-phox were determined by immunoblot analysis using membrane fractions prepared from ATRA-treated (lane 1) and ATRA plus 20 μM AA-C-treated (lane 2) U937 cells. Human Na^+/K^+-ATPase was used as a control. (B) Quantitative data of immunoblot analysis. Data of ATRA plus 20 μM AA-C-treated U937 cells are indicated as percentages of control value obtained from ATRA-treated U937 cells, and presented as the average of three separate experiments. Error bars indicate standard deviation. Statistical differences were calculated using Student's *t* test. **, $p < 0.01$ compared with the data of ATRA-treated U937 cells.

ATRA plus 10 μM or 20 μM AA-C-treated U937 cells (10 μM AA-C: to ~182% of the ATRA-treated cells, 20 μM AA-C: to ~263% of the ATRA-treated cells). These results demonstrated that AA-C enhances the ATRA-induced O_2^--generating ability of U937 cells mainly through activating the transcription of gp91-phox gene. Subsequently, to study the effects of 10 μM or 20 μM AA-C on the amounts of cytochrome b_{558} composed of p22-phox and gp91-phox proteins during co-treatment with 1 μM ATRA, immunoblot analyses were performed using antibodies specific for p22-phox or gp91-phox proteins. Typical immunoblot profiles for p22-phox and gp91-phox proteins in the 1 μM ATRA-treated and the 1 μM ATRA plus 20 μM AA-C-treated U937 cells are shown in Fig. 5A. Quantitative immunoblot data were indicated as percentages of control values obtained from the 1 μM ATRA-treated U937 cells (Fig. 5B). Expectedly, the protein levels of both p22-phox (to ~334% of the 1 μM ATRA-treated cells) and gp91-phox (to ~354% of the 1 μM ATRA-treated cell) were significantly enhanced in the 1 μM ATRA plus 20 μM AA-C-treated U937 cells. As described in some previous reports, p22-phox and gp91-phox proteins are cooperatively assembled resulting in formation of cytochrome b_{558} heterodimer in the plasma membrane (Dagher and Pick, 2007; Panday *et al*., 2015). Furthermore, our previous studies clarified not only that the expression level of gp91-phox protein is the most essential limiting factor of the O_2^--generating ability in U937 cells among the five essential protein factors (p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox) but also that the amounts of p22-phox protein mostly depend on those of gp91-phox in U937 cells (Kikuchi *et al*., 1994; Kikuchi *et al*., 2021a; Kikuchi *et al*., 2021b; Kikuchi *et al*., 2022a; Kikuchi *et al*., 2022b; Kikuchi *et al*., 2023). As expected, the degree of enhancement of the ATRA-induced O_2^--generating ability by 20 μM AA-C (to ~301% of the ATRA-treated cells) was roughly consistent with the degree of increases of p22-phox (to ~334% of the ATRA-treated cells) and gp91-phox (to ~358% of the ATRA-treated cells) proteins. Taken together, our data in this study showed that AA-C strongly enhanced the transcription levels of gp91-phox gene, resulting in significant increase of cytochrome b_{558} protein followed by remarkable up-regulation of the ATRA-induced O_2^--generating ability in U937 cells.

In this paper, as a first report, we showed the effects of AA-C, a derivative of AA, on the ATRA-induced O_2^--generating ability via enhancing transcription level of gp91-

phox. Although the relationship between the O_2^--generation enhancing activity against leukocytes of AA-C and AAN should be considered as unknown, we think that the O_2^--generation-enhancing activity of AA-C cannot be ignored in elucidating the mechanism of AAN.

As mentioned before, injection of AA to mice causes the enhancement of gp91-phox transcription in kidney (Declèves *et al*., 2016; Kim *et al*., 2019), suggesting that gp91-phox is main source of reactive oxygen species in the pathogenesis of AAN. Our data in this paper clarified the definite differences among the four AA derivatives (AA-A, AA-B, AA-C and AA-D) in the enhancing activity against the ATRA-induced O_2^--generating ability of U937 cells. Therefore, our results suggested that each purified AA derivative should be used for investigation of the effect of AA on the gene expression of gp91-phox even if AA-A and AA-B are thought to be the most abundant AA derivatives (Dickman *et al*., 2011). Indeed, a possibility that different components of AAs could cause different clinical lesions was indicated previously (Tanaka *et al*., 2000).

On the other hand, our data also revealed not only that AA-A and AA-C showed strong cytotoxicity against U937 cells in collaboration with ATRA but also that AA-C has the remarkable promoting effects on the ATRA-induced differentiation of U937 cells in a dose-dependent manner. That is to say, AAs may be expected to be effective modifiers in therapy for certain types of leukemia in combination with ATRA.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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