K1115 A, a New Anthraquinone Derivative that Inhibits the Binding of Activator Protein-1 (AP-1) to its Recognition Sites

I. Biological Activities

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K1115 A, a new anthraquinone derivative, was isolated from the culture broth of *Streptomyces griseorubiginosus* (Mer-K1115). K1115A inhibited the direct binding of activator protein-1 (AP-1) to AP-1 oligonucleotide ($IC_{50}=100\,\mu M$), and the production of collagenase in IL-1α-stimulated rat synovial cells ($IC_{50}=60\,\mu M$). *In vivo*, the application of K1115 A decreased phorbol myristate acetate (PMA)-induced mouse ornithine decarboxylase (ODC) activity. These results indicated that K1115 A is able to attenuate the inflammatory response mediated by AP-1.

Activator protein-1 (AP-1) was first identified as a nuclear factor that activates the expression of the human metallothionein IIa promoter[1], and is now known to be a critical transcriptional factor for various genes involved in immune and inflammatory responses, such as collagenase and ornithine decarboxylase[2,3]. It consists of a family of dimers of Fos and Jun proteins, including Jun homodimer and Fos/Jun heterodimers[4]. AP-1 binds to palindomic recognition sequence motifs of the general form 5'-TGA(C/G)TCA-3'. Various inflammatory and mitogenic stimulations lead to AP-1 activation, and AP-1 is considered to play an important role in diseases such as rheumatoid arthritis, transplant rejection, and tumor growth[5]. Therefore, inhibitors of AP-1 are considered to be candidate drugs for the treatment of such diseases.

In the course of screening for compounds that inhibit direct binding of AP-1 to AP-1 oligonucleotide by using gel retardation assay, we found K1115 A, a new anthraquinone derivative, in the fermentation broth of *Streptomyces griseorubiginosus* (Mer-K1115). The producing organism, fermentation procedure, isolation, physicochemical properties, and structure determination of K1115 A will be reported in the succeeding paper[6]. Here we describe the biological activity of K1115 A *in vitro* and *in vivo*.

Materials and Methods

Animals

Male 4-week-old Lewis rats were obtained from Charles River Japan. Female 9-week-old hairless mice, Hos:HR-1, were obtained from Hoshino Laboratory Animals (Yashio-shi, Saitama).

Materials

Sources of materials were as follows: phorbol 12-myristate 13-acetate (PMA), MTT, DTT, pyridoxal 5-phosphate and L-ornithine, Sigma Chemical Co. (St. Louis, MO.); 40% acrylamide/bis solution, 37.5:1 (2.6% C), 10x Tris/boric acid/EDTA buffer (1x=89 mM Tris, 89 mM boric acid, and 2 mM EDTA) and ammonium persulfate, Bio-Rad Laboratories (Richmond, CA); Klenow fragment, Takara Shuzo Co. (Kyoto, Japan); [α-^{32}P]dCTP (220 TBq/mmol), DL-[1-^{14}C]ornithine hydrochloride (2.04 GBq/mmol), NCS-II and ACS-II, Amersham (Arlington Heights, IL); NAP-5 columns, Pharmacia (Uppsala, Sweden); Jurkat phorbol (a nuclear extract from phorbol ester treated-Jurkat cells), Santa Cruz Biotechnology (Santa Cruz, CA); DULBECCO's modified EAGLE's medium (D-MEM), GIBCO-BRL (Grand Island, NY); endotoxin-free fetal calf serum, Nippon B. M. A. Co. (Osaka, Japan); Collageno-kit, Collagen Gijyutu Kensyukai (Kiyose, Japan). GF/C

filter, Whatman International Ltd. (Maidstone, England): BCA-protein assay kit, Pierce Chemical Co. (Rockford, IL) . DNA probes of the AP-1 site (5'-GGAGCT*TGAGTCA*GCCGGATC-3' and 3'-TCGA*ACTCAGT*CGGCCTAGGG-3') and NF-κB site (5'-GGGATCGAG*GGGACTTTCC*CTAGC-3' and 3'-CTAGCTC*CCCTGAAAGG*GATCGGG-3') were synthesized according to the solid-phase phosphate triester method with a model 394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA). Italics show consensus sequences of AP-1 and NF-κB, respectively[7]. Recombinant rat interleukin-1α (IL-1α) was prepared previously in our laboratory[8].

Gel Shift Assay

Gel shift assay was performed as previously described[9]. Double-stranded oligonucleotides were labeled with [α-^{32}P]dCTP by use of the Klenow fragment. The labeled DNA probes were purified on NAP-5 columns. One μg of nuclear extracts prepared from Jurkat phorbol was incubated with the AP-1 or NF-κB probe (50,000 cpm/0.1 ng) in the presence or absence of K1115 A for 15 minutes at room temperature. DNA/protein complexes were separated from free DNA probes on native 5% polyacrylamide gel. The gels were vacuum-dried and exposed to an X-ray film or an imaging plate of the BAS 2000 system (Fuji Photo Film Co., Tokyo, Japan).

Collagenase Production

Rat synovial cells were obtained from knee joints of normal male inbred Lewis rats as reported previously[10]. The cells were cultured in D-MEM supplemented with 10% fetal calf serum for 2 passages. The cells were seeded into 96-well culture plates (Falcon 3072) at 4×10^3 cells/100 μl/well. After 3 days of culture, the supernatants were replaced with fresh D-MEM. Then the cells were stimulated with 10 ng/ml of recombinant rat IL-1α in the presence or absence of K1115 A for 48 hours. After the stimulation, the supernatant was drawn from each well for collagenase activity assay and the remaining cells were tested for viability by the MTT colorimetric method[11]. In order to generate the active form of collagenase from the latent form, the supernatants were treated with 167 μg/ml trypsin at 25°C for 10 minutes. Then the collagenase activity was determined with reagents of the Collageno-kit according to the product manual. One unit of enzyme activity is the amount of protein necessary to degrade 1 μg collagen/minute under the conditions employed.

AP-1 Activation

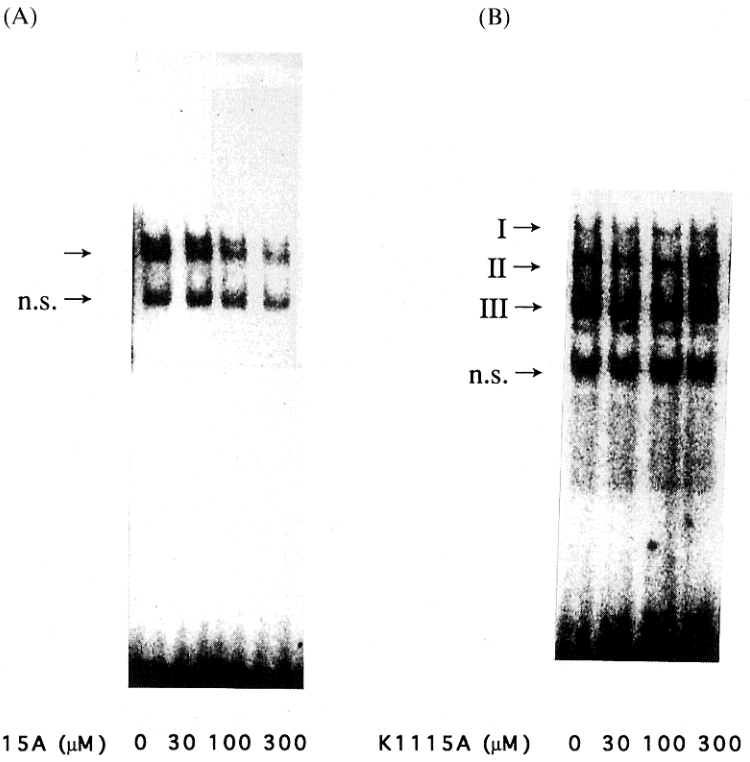
Rat synovial cells were seeded onto 100-mm dishes (Costar 3100). After sub-confluent culture, the supernatants were replaced with fresh D-MEM. The cells were cultured in the presence or absence of K1115 A for 50 minutes, followed by stimulation with 10 ng/ml of recombinant rat IL-1α for 2 hours. Then, the nuclear extracts were isolated according to the method previously reported[8]. Gel shift assay was performed by the method described above.

Ornithine Decarboxylase (ODC) Assay

ODC assay was performed as previously described with a slight modification[13]. K1115 A was dissolved in 200 μl of acetone, and the solution was topically applied to the dorsal skin of Hos:HR-1 mice over an area of about 6 cm^2. One hour later, 17 nmol of TPA in 200 μl of acetone were painted on the same area. After 4.5 hours, the mice were killed by cervical dislocation, and the treated area of their dorsal skin was excised. The epidermis from individual mice was placed in water at 55°C for 30 seconds, then reimmersed in ice-cold water. The epidermal preparation was homogenized in 1 ml of 50 mM sodium phosphate buffer (pH 7.2) containing 0.4 mM pyridoxal-5-phosphate and 5 mM DTT. Then the homogenate was centrifuged at 30,000 × g for 30 minutes and the supernatant was recovered and stocked at −70°C until estimation of the enzyme activity.

ODC activity in an epidermal sample was determined by measuring $^{14}CO_2$ release from DL-[1-^{14}C]ornithine. A 100 μl aliquot of each supernatant was mixed with 50 μl of 50 mM sodium phosphate buffer (pH 7.2) containing 0.4 mM pyridoxal-5-phosphate, 5 mM DTT, 1 mM L-ornithine and 18.5 kBq of DL-[1-^{14}C]ornithine in a polystyrene tube. This tube was placed in a sealed scintillation vial, and incubated at 37°C for 1 hour. The reaction was stopped by injecting 300 μl of 2 M citric acid. The $^{14}CO_2$ released during the incubation was trapped directly on a GF/C filter soaked with NCS II tissue solubilizer. After removal of the tube, 10 ml of ACS II scintillator was added to the scintillation vial and the radioactivity of the sample was counted with liquid scintillation system (LSC-3500, ALOKA Co., Ltd., Tokyo, Japan). The protein concentration of each sample was determined by using a BCA-protein assay kit. The results were expressed as nmol of CO_2 production per mg of protein and statistically analyzed by applying Dunnet's multiple comparison test.

Fig. 1. Effect of K1115 A on direct binding between AP-1 (A) or NF-κB (B) and oligonucleotide containing the respective recognition sequence.



n.s.: non-specific.

Results

Inhibitory Effect of K1115 A on AP-1 Binding to the AP-1 Binding Sequence

Fig. 1A shows the inhibitory effect of K1115 A on the direct binding of AP-1 and AP-1 oligonucleotide. The arrow indicates a specific band of AP-1/AP-1 oligonucleotide complex; this band was shifted by anti-c-*fos* or by anti-c-*jun* antibody (data not shown). K1115 A inhibited the formation of this complex in a dose-dependent manner. Image analysis indicated that the inhibition of AP-1 binding by K1115 A at 100 and 300 μM was 58% and 68%, respectively. On the other hand, the inhibition of non-specific binding by K1115A at 100 and 300 μM amounted to 6, 17%, respectively. NF-κB, another transcriptional factor in Jurkat-phorbol nuclear extract was detected with oligonucleotide containing the binding site of NF-κB (Fig. 1B). The result of supershift assay with specific antibodies reactive to NF-κB protein suggested that complex I was p50/c-*rel*, complex II was p50/p65 heterodimer and complex III was p50 homodimer (data not shown). K1115 A did not affect the formation of these complexes even at 300 μM.

Inhibitory Effect of K1115 A on Collagenase Production and AP-1 Activation

Collagenase gene expression is regulated by AP-1[2]. We therefore investigated the effect of K1115 A on collagenase production in IL-1α-stimulated rat synovial cells. Unstimulated cells did not generate collagenase, while the cells produced 2.08 units/ml of collagenase in the culture medium after IL-1α stimulation for 48 hours (Fig. 2A). K1115 A inhibited the production of collagenase in a dose-dependent manner. The inhibitory effect amounted to 50% and 95%, at 60 and 90 μM K1115 A, respectively. At 90 μM, K1115 A showed distinct cytotoxicity, causing a 26% reduction in the MTT reducing activity (data not shown). Furthermore, K1115A at 90 μM did not inhibit collagenase activity directly (data not shown).

IL-1 stimulation leads to AP-1 activation in many types of cells[12]. So we examined the effect of K1115 A on AP-1 activation in IL-1α-stimulated rat synovial cells. As shown in Fig. 2B, although AP-1 was activated and translocated to nuclei from cytosol after IL-1α stimulation, K1115 A did not affect these processes even at 100 μM.

Fig. 2. Effect of K1115 A on IL-1α stimulated-collagenase production (A), and AP-1 activation (B).

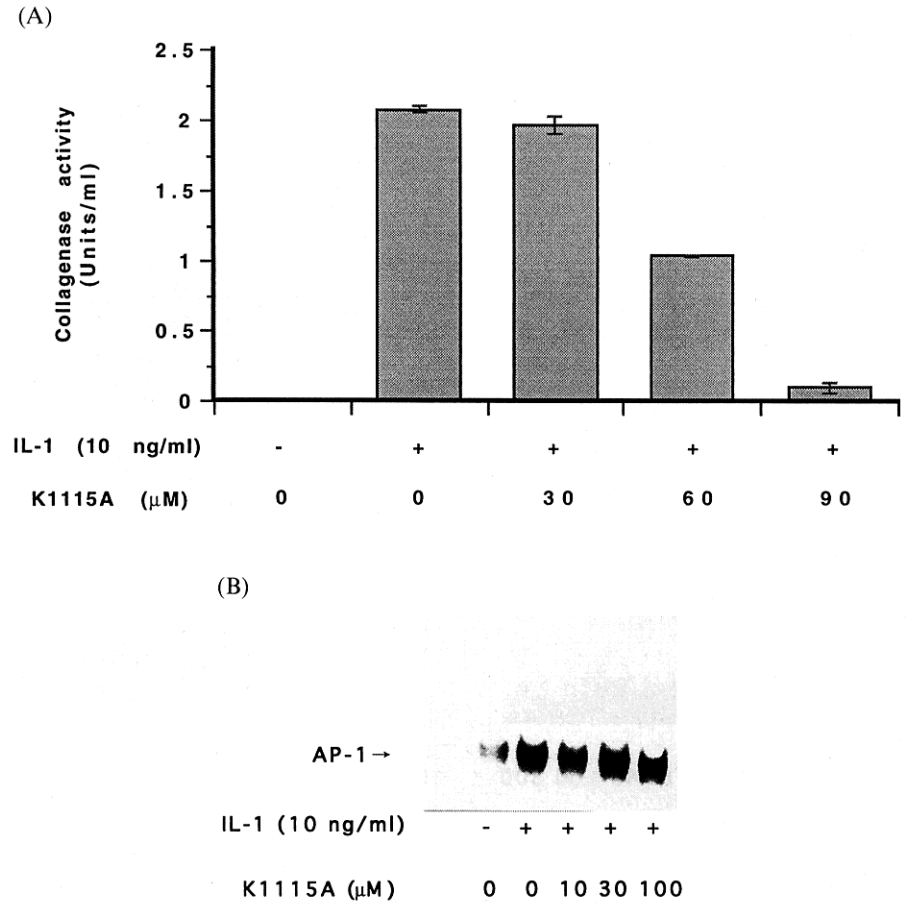
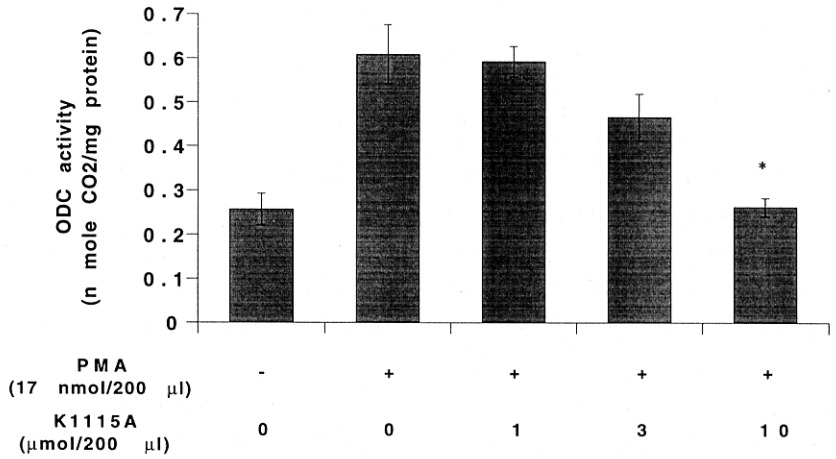


Fig. 3. Effect of K1115 A on PMA-induced ODC activity.



Each value is the mean ± standard error of eight rats.

*: $p < 0.05$, one way ANOVA, Dunnett multiple comparison *vs.* PMA stimulation group in the absence of K1115 A.

Inhibitory Effects of K1115 A in a Mouse PMA-induced ODC Activation Model

In order to examine the effect of K1115 A *in vivo*, we tested its activity in a mouse PMA-induced ODC activation model[13], because it has been reported that a pivotal AP-1 binding site exists in the ODC gene[3]. At 4.5 hours after a single application of PMA, epidermal ODC activity was enhanced 2.37-fold over the basal level (Fig. 3). Pretreatment with K1115 A before PMA application decreased the enhancement of ODC activity by PMA in a dose-dependent manner. The inhibitory effects of K1115 A at 3 and 10 μmol/200 μl were 40% and 99%, respectively. On the other hand, 1 mM of K1115A did not inhibit ODC activity directly. In the course of experiments in PMA-induced ODC activation model, apparent toxicity was not observed.

Discussion

In the course of screening of microbial metabolites to find novel AP-1 binding inhibitors, we have discovered a new anthraquinone derivative, K1115 A. K1115 A inhibited AP-1 binding to an oligonucleotide containing its recognition sequence. The inhibition amounted 58% and 68% at 100 and 300 μM, respectively. On the other hand, K1115 A did not affect binding between NF-κB and an oligonucleotide containing its recognition site, even at 300 μM. These data suggest that the inhibitory effect of K1115 A is selective for AP-1 though the reason for this is unknown.

In IL-1α-stimulated rat synovial cells, 90 μM K1115 A completely abolished the production of collagenase, whose gene is regulated by AP-1[2] (Fig. 2A). The effect was not due to cytotoxicity, since K1115 A did not affect cell viability as measured in terms of MTT reducing activity after the cultivation. In IL-1α-stimulated rat synovial cells, AP-1 protein was activated and translocated to nuclei from cytosol. K1115 A did not affect these processes even at 100 μM (Fig. 2B). Thus activated AP-1 is thought to be located in the nuclei of the cells. However, the activated AP-1 protein did not bind to AP-1 binding site, because K1115 A directly inhibited this binding (Fig. 1A). This inhibition prevented the transcription of collagenase gene and consequently inhibited the production of collagenase. We consider that this is one of the mechanisms how K1115 A can inhibit the production of collagenase (Fig. 2A).

Although there is a difference in IC_{50} values of K1115 A for AP-1/oligonucleotide direct binding and collagenase production (Fig. 1A and Fig. 2A), it might be resulting from the synergistic effect of transcriptional factors on gene activation. Transcriptional activation is regulated by several transcriptional factors through their binding to DNA and interaction among the transcriptional factors. If binding between DNA and a transcriptional factor which plays a key role in a certain gene transcription is inhibited by a drug, the gene transcription might be reduced markedly even if the inhibitory effect of the drug on the transcription is relatively small. For example, the IC_{50} value of MG341, an NF-κB inhibitor, for the inhibition of activation of NF-κB is 0.5 μM, but at this concentration MG341 inhibited IL-2 expression almost completely[14]. The IC_{50} value for the inhibition of IL-2 expression was an order of magnitude lower, 0.05 μM. We anticipate that the same phenomena occurred in the experiment of K1115 A concerning the inhibition of AP-1 binding and collagenase production.

The results obtained in the mouse PMA-induced ODC activation model showed that topical application of K1115 A could decrease the enhancement of ODC activity. The inhibitory effect of K1115 A on ODC activity might be due to the same mechanism as in the case of collagenase, since a pivotal AP-1 binding site also exists in the ODC gene[3].

Which does K1115 A bind AP-1 or the oligonucleotide? We do not have direct evidence showing the interaction between K1115 A and DNA. However, we concluded that the inhibitory effect of K1115 A is not due to the intercalation of the compound into DNA because of the following reasons. First, K1115 A inhibited the direct binding of AP-1 to AP-1 oligonucleotide, but not nonspecific binding (Fig. 1A). Second, K1115 A at 300 μM did not affect the direct binding of NF-κB to NF-κB oligonucleotide or the binding of NF-AT to NF-AT oligonucleotide (Fig. 1B and the latter data not shown in this paper). Third, the inhibitory effect of K1115 A on the direct binding of AP-1 to AP-1 oligonucleotide did not persist after adding 5-fold amount of the nuclear extract from phorbol ester treated-Jurkat cells in gel shift assay (data not shown). Finally, 1 mM of K1115 A was negative in Ames test for strain TA2637 regardless of the presence or absence of S9 mix (data not shown). It is, therefore, unlikely that K1115 A interacts intensively to DNA. The precise mechanisms for the inhibition by K1115 A on the binding of AP-1 to AP-1 oligonucleotide is yet to be elucidated.

It has been reported that some of anthraquinone derivatives are intercalated into DNA[15]. Does all members of anthraquinone inhibiting protein-DNA

interactions? 1-Hydroxy-anthraquinone and 1-amino-anthraquinone at 0.1~1 mM and 1,8-dihydroxy-anthraquinone, 1,5-dihydroxy-anthraquinone, chrysophanic acid and emodine at 1 mM inhibited both AP-1 and NF-κB binding (data not shown). On the other hand, neither anthraquinone-β-carboxylic acid nor 1-chloro-anthraquinone even at 1 mM inhibited AP-1 and NF-κB binding (data not shown). These data indicate that most of the anthraquinone derivatives inhibit DNA-protein binding but not all derivatives do.

AP-1 is a critical transcriptional factor for various genes involved in immune and inflammatory responses[4]. It has been reported that glucocorticoid and retinoic acid inhibit AP-1 activity through the interaction between their nuclear receptor and AP-1 protein[16,17] and show anti-inflammatory activity. We have found that K1115 A, a new anthraquinone derivative, is able to suppress AP-1 action through a different mechanism from that of the above drugs.

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