Suppression by citrus auraptene of phorbol ester- and endotoxin-induced inflammatory responses: role of attenuation of leukocyte activation

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Auraptene (AUR), a citrus coumarin derivative, is one of the promising chemopreventive agents against skin, tongue, esophagus and colon carcinogenesis in rodents. We reported previously that AUR suppresses superoxide anion (O2−) generation from inflammatory leukocytes in in vitro experiments. In the present study, we investigated the anti-inflammatory activities of AUR using a 12-O-tetradecanoyl-phorbol-13-acetate-treated mouse skin model, and compared them with those of umbelliferone (UMB), a structural analog of AUR that is virtually inactive toward O2− generation inhibition. Double pre-treatments of mouse skin with AUR, but not UMB, markedly suppressed edema formation, hydrogen peroxide production, leukocyte infiltration, and the rate of proliferating cell nuclear antigen-stained cells. These inhibitory effects by AUR are attributable to its selective blockade of the activation stage, as revealed by single pre-treatment experiments. In a murine macrophage line, RAW 264.7, AUR significantly attenuated the lipopolysaccharide-induced protein expression of inducible isoforms of both nitric oxide synthase and cyclooxygenase, with decreased production of nitrite anion and prostaglandin E2, and yet suppressed the release of tumor necrosis factor-α. Conversely, UMB did not show any inhibitory effect. This contrasting activity profile between AUR and UMB was rationalized to be a result of their distinct differences in cellular uptake efficiencies, i.e. the geranyloxyl group in AUR was found to play an essential role in incorporation. Thus, our findings indicate that AUR is an effective agent to attenuate the biochemical responsiveness of inflammatory leukocytes, which may be essential for a greater understanding of the action mechanism that underlies its inhibition of inflammation-associated carcinogenesis.

Introduction

The ingestion of citrus fruit has been reported to be beneficial for the reduction of certain types of human cancer (1). Several classes of citrus phytochemicals, including monoterpenes (2), limonoids (3) and flavonoids (4,5), have been recognized as effective chemopreventive agents in rodent carcinogenesis models. In addition, our research group has recently isolated auraptene (7-geranyloxycoumarin, AUR), a coumarine derivative, from citrus fruit (e.g. grapefruit) and demonstrated its anti-tumor promoting effect in mouse skin (6) and anti-carcinogenesis activities in rat tongue (7), esophagus (8) and colon (9,10).

Oral administration of AUR to rats significantly elevated the activity of such xenobiotic phase 2 enzymes as glutathione S-transferase and quinone reductase in tongues, livers and colons (7,9,10). Furthermore, AUR was found to increase liver glutathione S-transferase activity in a dose-dependent manner, but did not affect hepatic cytochrome P450 activity (11). Therefore, selective induction of phase 2, but not phase 1, enzyme activity by AUR may play an essential role in the action mechanism during the initiation stage of carcinogenesis. However, little is known regarding the action mechanisms of AUR in the post-initiation stage.

Inflammation is a universal and physiological response in the processes of carcinogenesis. Treatment with UV light, endotoxins or chemical tumor promoters is known to lead to chemotaxis, differentiation and infiltration of inflammatory leukocytes, including neutrophils and macrophages which produce reactive oxygen species (ROS), prostaglandins (PGs) and cytokines. In fact, there is ample evidence showing that treatment(s) of mouse skin with single or multiple doses of TPA induce(s) superoxide anion (O2−) generation (12), hydrogen peroxide (H2O2) formation (13), PGE2 and PGF2α production (14), cyclooxygenase (COX)-2 expression (15), as well as the release of tumor necrosis factor α (TNF-α) (16) and interleukin-1α (IL-1α) (17). Thus, activated inflammatory leukocytes are considered to play an important role in carcinogenesis. We reported previously that AUR is a potent suppressor of 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced O2− and H2O2 production in dimethyl sulfoxide (DMSO)-differentiated human promyelocytes (HL-60) which mimic neutrophils (6). However, the suppressive efficacy of AUR on activated leukocyte-induced inflammatory responses in vivo remains to be demonstrated. Wei et al. (13) reported that double applications of TPA to mouse skin led to excessive ROS production. Ji and Marnett (18) designated these two application stages as ‘priming’ (the first stage, as illustrated by leukocyte recruitment, maturation and infiltration of inflammatory leukocytes into inflamed lesions) and ‘activation’ (the second stage, as illustrated by ROS production from accumulated leukocytes), respectively.

In the present study, we attempted to determine whether AUR inhibits the priming and/or activation stages when conducting this double TPA application model with the use of umbelliferone (UMB), a structural AUR analog virtually inactive toward suppressing in vitro O2− generation (6). In addition, the suppressive effects of AUR on lipopolysaccharide
Double TP A treatments of mouse skin was shaved with an electrical clipper 2 days before treatment. Separated on 10% polyacrylamide gels and electrophoretically transferred onto

TNF-α (DMEM) and fetal bovine serum were purchased from Gibco BRL, NY. LPS (Escherichia coli serotype 0127, B8) was purchased from Difco Labs (Detroit, MI) and N-inoiminoethyl-l-ornithine (L-NIO) from Cayman (Ann Arbor, MI). All other chemicals were purchased from Wako Pure Chemical Industries Co. Ltd (Osaka, Japan), unless specified otherwise.

Cells and animals

RAW 264.7 cells were kindly donated by Ohtsuka Pharmaceutical Co. Ltd (Ohtsu, Japan), and cultured in DMEM with 10% fetal bovine serum in a humidified 5% CO2 incubator. Female ICR mice aged 7 weeks (Japan SLC, Shizuoka, Japan) were used in the animal experiments. The mice were kept under an artificial day–night rhythm and were fed CE-2 rodent pellets (Japan SLC), while fresh tap water was available ad libitum. The mice’s back skin was shaved with an electrical clipper 2 days before treatment.

Double TPA treatments of mouse skin

A double TPA treatment experiment was performed as reported previously (19). Each experimental group consisted of five mice. AUR or UMB (810 nmol in 100 µl acetic or acetone (100 µl) was topically applied to the shaved area of the dorsal skin 30 min before application of a TPA solution (8.1 nmol in 100 µl acetone). After 24 h, the same dose of the test compounds, or acetone and TPA, was applied to the same region. Mice were killed by cervical dislocation 1 h after the second TPA treatment, and then biochemical parameters were measured as described below. We divided the mice into six groups as follows: group 1 (acetone ×2acetone ×2); group 2 (acetone–TPA/acetone–TPA); group 3 (AUR–TPA/AUR–TPA); group 4 (AUR (priming)–TPA/acetone–TPA); group 5 (acetone–TPA/AUR (activation)–TPA); group 6 (UMB–TPA/UMB–TPA).

H2O2 and edema formation

Measurements of the levels of H2O2 and edema formation were performed as reported previously (19). In brief, skin punches (epidermis and dermis) were obtained from excised dorsal skins with an 8 mm diameter cork borer and reported as means ± SD. The supernatants were subjected to a Griess assay to measure the concentrations of NO2–

Materials and methods

Materials

AUR was isolated from satsuma mandarins, as reported previously (6). UMB was obtained from Extrasyntese (Genay, France) and TPA from Research Biochemicals International, Natural, MA. Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum were purchased from Gibco BRL, NY. LPS (Escherichia coli serotype 0127, B8) was purchased from Difco Labs (Detroit, MI) and N-inoiminoethyl-l-ornithine (L-NIO) from Cayman (Ann Arbor, MI). All other chemicals were purchased from Wako Pure Chemical Industries Co. Ltd (Osaka, Japan), unless specified otherwise.

Protein determination

Protein concentrations were determined using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). BSA was employed as the standard. Each experiment was done in triplicate, and the data are reported as means ± SD.

Statistical analysis and inhibitory rate (IR)

The statistical significance of differences between groups in each assay was assessed by a Student’s t-test (two-sided) that assumed unequal variance. The IR in each assay was calculated by the following equation. IR (%) = (1 – [test sample data] – [vehicle control data]) × (positive control data) × 100.
and H2O2 generation (12.5-fold), in the dermis and epidermis. Suppressive effects of AUR on iNOS and COX-2 protein in each) led to a dramatic elevation of edema weight (2.7-fold).

Suppression of double-dose TPA-induced edema and H2O2 formation

In groups 2–6 (Table I), TPA (8.1 nmol) was topically applied to the dorsal skin of mice twice within a time interval of 24 h. Treatment with double-dose TPA (group 2, 8.1 nmol each) led to a dramatic elevation of edema weight (2.7-fold) and H2O2 generation (12.5-fold), in the dermis and epidermis 1 h after the second TPA application, as compared with the vehicle control (group 1). AUR or its analog UMB (Figure 1) was also topically applied 30 min prior to each TPA treatment. UMB is a negative control that has no inhibitory potential for O2− generation in vitro (6). As shown in Table I, double pre-treatment with AUR substantially suppressed both edema and H2O2 formation, by 43 (P < 0.01) and 85% (P < 0.001), respectively, whereas that with UMB (810 nmol, group 6) failed to decrease these inflammatory indicators (IR = 21 and 1%, respectively, neither statistically significant). Subsequently, we addressed a question regarding which stage, either priming (leukocyte infiltration) or activation (oxidative burst), can be blocked by a single AUR pre-treatment. Although a single pre-treatment with AUR (810 nmol) during the activation stage remarkably suppressed edema (IR = 29%, P < 0.02) and H2O2 (IR = 84%, P < 0.001) formation, the same during the priming stage was much less effective (IR = −15 and 35%, P < 0.001, respectively).

Histological examination of mouse cutis

Leukocyte infiltration and expression of a cell proliferation marker were investigated histologically. No detectable acute toxicity was observed in any group. A great number of leukocytes were observed infiltrated into the cutis (dermis and dermis) after double-dose TPA treatments, when compared with group 1 (27-fold) (Table I, Figure 2A and B), as revealed by hematoxylin and eosin staining. Double pre-treatments with AUR (group 3) caused a nearly complete blockade of leukocyte infiltration (IR = 92%, P < 0.001) (Table I and Figure 2C). Furthermore, this suppressive efficacy was notably reproduced by a single application during the activation stage (group 5) (IR = 90%, P < 0.001) (Table I; Figure 2E), but not the priming stage (IR = 8%) (Table I; Figure 2D). A quite similar tendency was observed when measuring the PCNA-labeling index, a cell proliferation marker. In the epidermis, the PCNA-labeling index increased by double-dose TPA (1.7-fold as compared with group 1; P < 0.001) (Table I; Figure 3A and B). The inhibitory effect shown by a single pre-treatment of AUR during the activation stage (IR = 84%, P < 0.001) was comparable with that of double pre-treatment (IR = 90%, P < 0.001) (Table I; Figure 3C and E). Conversely, pre-treatment during the priming stage had no effect at all (IR = 6%) (Table I; Figure 3D).

Suppression of iNOS and COX-2 expression in RAW 264.7 cells

Suppressive effects of AUR on iNOS and COX-2 protein expression were examined by western blotting using LPS (100 ng/ml)-stimulated RAW 264.7 cells, a murine macrophage cell line. Concentrations of NO2− and PGE2 (the major products synthesized by iNOS and COX-2, respectively) in the media, were also measured. L-NIO, a substrate analog iNOS inhibitor, and UMB were used as positive and negative controls, respectively. Cell viability, as measured by an MTT assay, in each experiment was consistently ≥90% when compared with the cells treated only with LPS (data not shown). Though barely detectable in non-stimulated cells, iNOS protein was highly (25-fold) expressed after LPS-treatment for 12 h (Figure 4A), and the NO2− concentration in the media reached 20.0 ± 3.3 nmol/ml/mg protein. AUR (at 10 and 20 µM) significantly suppressed iNOS protein expression (IR = 16%, P < 0.05; and 59%, P < 0.001, respectively) as well as NO2− formation (IR = 25%, P < 0.05; and 60%, P < 0.01, respectively). However, and unexpectedly, L-NIO at a concentration of 20 µM also attenuated iNOS expression (IR = 57%, P < 0.01),

Table I. Inhibitory effects of AUR and UMB on TPA-induced oxidative and inflammatory parameters in mouse skin

<table>
<thead>
<tr>
<th>Group</th>
<th>Edema (mg/punch, IR)</th>
<th>H2O2 (mmol/punch, IR)</th>
<th>No. leukocytes in the cutis (no./mm², IR)</th>
<th>PCNA-stained cell nuclei index (%) (IR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Ac/Ac→Ac/Ac</td>
<td>16.6 ± 5.2</td>
<td>0.6 ± 0.2</td>
<td>11 ± 2</td>
<td>53.7 ± 6.9</td>
</tr>
<tr>
<td>2 Ac/TPA→Ac/TPA</td>
<td>44.7 ± 3.6</td>
<td>7.5 ± 2.1</td>
<td>298 ± 57</td>
<td>89.1 ± 7.9</td>
</tr>
<tr>
<td>3 AUR/TPA→AUR/TPA</td>
<td>32.5 ± 6.9, 43%</td>
<td>1.6 ± 0.1, 85%</td>
<td>35 ± 4.1, 92%</td>
<td>56.8 ± 7.1, 90%</td>
</tr>
<tr>
<td>4 AUR/TPA→Ac/TPA</td>
<td>48.8 ± 5.4, −15%</td>
<td>5.5 ± 0.4, 35%</td>
<td>275 ± 98, 8%</td>
<td>87.0 ± 8.2, 6%</td>
</tr>
<tr>
<td>5 Ac/TPA→AUR/TPA</td>
<td>36.5 ± 5.1, 29%</td>
<td>1.7 ± 0.3, 84%</td>
<td>39 ± 17, 90%</td>
<td>59.4 ± 7.0, 84%</td>
</tr>
<tr>
<td>6 UMB/TPA→UMB/TPA</td>
<td>38.8 ± 8.9, 21%</td>
<td>7.3 ± 1.6, 1%</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

Mice were treated as described in Materials and methods, and killed 1 h after the second TPA treatment (8.1 nmol). Skin punchs were then obtained for biological and histological analyses.

aP < 0.001 versus G1.
bP < 0.01 versus G2.
cP < 0.001 versus G2.
dP < 0.02 versus G2.

Fig. 1. Structures of AUR and UMB.
Fig. 2. Suppression by AUR of TPA-induced skin morphological changes, observed by hematoxylin and eosin staining. AUR (810 nmol in 100 µl acetone) was topically applied to the shaved area of dorsal skin 30 min before application of a TPA solution (8.1 nmol in 100 µl acetone). After 24 h, the same dose of AUR or acetone was applied 30 min prior to a second TPA application. Methods for histological staining are described in Materials and methods. Ac, acetone. Original magnification: (A–E) ×80.

Fig. 3. Suppression by AUR of the TPA-induced increase in PCNA-positive cells in mouse epidermis. AUR (810 nmol in 100 µl acetone) was topically applied to the shaved area of dorsal skin 30 min before application of a TPA solution (8.1 nmol in 100 µl acetone). After 24 h, the same dose of AUR or acetone was applied 30 min prior to a second TPA application. Methods for PCNA staining are described in Materials and methods. Ac, acetone. Original magnification: (A–E) ×80.

whereas it reasonably blocked NO₂⁻ formation (IR = 20–85% at 5–20 µM). As shown in Figure 4B, COX-2 protein expression was highly increased in the positive control (33-fold), and the media PGE₂ concentration was 4.5 ± 1.3 ng/ml/mg protein. AUR showed a concentration-dependent suppression of COX-2 expression (IR = 44–86%...
Auraptene attenuates skin inflammation

**Fig. 4.** Suppression by AUR and L-NIO of LPS-induced NOS/COX-2 protein expression and NO$_2^–$/PGE$_2$ production in RAW 264.7 cells. RAW 264.7 cells were incubated with LPS (100 ng/ml) and DMSO [0.5% (v/v) as a final concentration] or test compounds (5, 10, 20 or 100 µM) for 12 h. NO$_2^–$ and PGE$_2$ concentrations were determined using a commercial experimental kit and the Griess method, respectively. Western blotting was performed as described in Materials and methods. The iNOS/COX-2 band levels were corrected using β-actin as the internal standard. (A) iNOS and NO$_2^–$; (B) COX-2 and PGE$_2$. *P < 0.05, **P < 0.01, ***P < 0.001 versus positive control using Student’s t-test. One representative picture is shown. Each experiment was done independently in duplicate twice, and data are reported as means ± SD.

at 5–20 µM) and PGE$_2$ production (IR = 20–73% at 5–20 µM). L-NIO at a concentration of 20 µM also inhibited protein expression and prostanoid synthesis (IR = 60%, P < 0.01; and 43%, P < 0.05, respectively).

**Suppression of TNF-α release**

The suppressive effect of AUR (5–20 µM) was also evaluated using 100 ng/ml LPS-stimulated RAW 264.7 cells. After stimulation of the cells for 3 h, no marked cytotoxicity was observed in any of the experiments (data not shown). Whereas no detectable TNF-α release was observed in non-stimulated cells, the media concentration of TNF-α was 7.2 ± 1.0 ng/ml/mg protein (Figure 5). AUR at concentrations of 10 and 20 µM suppressed TNF-α release in a concentration-dependent manner by 24.9 (% P < 0.02) and 83.9% (P < 0.001), respectively, whereas UMB, even at a concentration of 100 µM, did not suppress TNF-α release.

**Cellular uptake**

To understand the distinct differences between AUR and UMB in their suppressive activities toward iNOS and COX-2 protein expression, as well as pro-inflammatory cytokine release, we examined their incorporating efficiencies in RAW 264.7 cells. The intra- and extracellular distribution of AUR or UMB at a concentration of 50 µM was quantified using HPLC at time points of 0, 0.5, 1 and 7 h after sample addition. As shown in Figure 6A, while a marked cellular uptake of AUR (12.5–22.4% of the total incubated) was observed in the time range of 0.5–7 h, UMB was found to be barely (0.5–2.2%) incorporated into the cells at all times tested. Accordingly, the extracellular amounts of AUR (72.3–85.6%) were significantly (P < 0.05 or P < 0.01) lower than those of UMB (95.3–97.2%) (Figure 6B).
organisms as a virus or bacteria, one of the notable immune bergamottin (8-geranyloxypsoralen) to suppress NO generation.

In this study, AUR at the priming stage did not show any metalloproteinase and plasminogen activator (43), expression as for the induction of IL-6 that enhances cancer cell invasion through attenuation of the responsiveness of activated leukocytes. In the double-dose TPA model, AUR during the priming stage did not show any suppressive effect on TPA-induced oxidative stress and inflammation through attenuation of the responsiveness of activated leukocytes. In the double-dose TPA model, AUR during the priming stage did not show any suppressive effect on TPA-induced oxidative stress and inflammation through attenuation of the responsiveness of activated leukocytes. 

Interestingly, UMB was found to be consistently inactive towards suppression of TPA-induced EB virus infection (6), O$_2^-$ generation (6) and H$_2$O$_2$ and edema formation, as well as towards attenuation of LPS-induced NOS/COX-2 expression and TNF-α release. To our knowledge, there is no report showing the chemopreventive effect of UMB in contrast to AUR. The distinct differences in their cellular uptake efficiencies may reasonably explain their in vitro and in vivo activity differences. The geranyloxyl group, presumably acting as the carrier group of AUR for incorporation into cells, has also been reported to be an important structural moiety of bergamottin (8-geranylxyxypsoralen) to suppress NO generation (20). It is of interest to note that both geraniol and geranyl acetate showed weak suppressive activity towards LPS-induced NO$_2^-$ production in RAW 264.7 cells since their IRs at a concentration of 100 μM were 40 and 4%, respectively, and they were completely inactive at 20 μM (unpublished data). These results suggest the importance of both the coumarin and geranyl moieties in AUR for suppressing LPS-induced signaling pathways in RAW264.7 cells. On the other hand, we have reported that 7-alkylxylcoumarins are notable mono-functional inducers for phase 2 enzyme activity, since 7-ethoxyxymarin and AUR (7-geranlyoxycoumarin) selectively induce glutathione S-transferase activity, while UMB and other types of substituted coumarins are bi-functional or mono-functional inducers of cytochrome P450 activity (11). Thus, AUR may be one of the most promising coumarin-related chemopreventive agents, since it bears two key structural characteristics, the geranyloxyl (efficient cellular uptake) and 7-alkylxyl (phase 2 induction) groups.

There are an increasing number of reports showing that the expression of COX-2 and iNOS is closely associated with the development of cancers (34,35). Suh et al. (36) synthesized novel synthetic triterpenoids that suppressed iNOS and COX-2 protein expression, and demonstrated their potent differentiating, anti-proliferating and anti-inflammatory activities (37). Elevation of COX-2 activity is responsible for the enhanced synthesis of prostanoids, including PGE$_2$, that stimulate bcl-2 activity and thereby inhibit apoptosis, as well as for the induction of IL-6 that enhances cancer cell invasion (38). On the other hand, NO is rapidly and non-enzymatically reacted with O$_2^-$ to form peroxynitrite anion, a highly toxic molecule causing a wide range of DNA and protein modifications (39). Pro-inflammatory cytokines, TNF-α and IL-1 (40–42), play some critical roles in the processes of inflammation and tumor development. TNF-α, exhibiting versatile physiological functions, induces the biosynthesis of matrix metalloproteinase and plasminogen activator (43), expression of COX-2 (44) and iNOS (45), and skin tumor promotion (41,42). While IL-1α is an initial pro-inflammatory mediator released from mouse keratinocytes in the process of tumor...

**Discussion**

Chronic inflammation disorders are estimated to cause 21% of the new cancer cases in developing countries and 9% of those in developed countries (21). Upon infection with such organisms as a virus or bacteria, one of the notable immune responses is leukocyte infiltration and activation. The present study was undertaken to provide experimental evidence that citrus AUR can attenuate TPA-induced inflammatory responses in activated leukocytes, and to understand its action mechanisms that underlie a series of anti-carcinogenesis effects in the initiation and post-initiation stages (6–10). ROS generated from stimulated leukocytes are thought to play some substantial roles in the initiation stage through the activation of certain procarcinogens (22). In the post-initiation stage as well, excess amounts of leukocyte-produced ROS kill neighboring normal cells, giving rise to the clonal expansion of initiated or premalignant cells that have already acquired phenotypic resistance to oxidative stress and apoptosis. The TPA-induced mouse skin inflammation model is a convenient system to investigate leukocyte infiltration and its oxidative burst (13,19). We have reported previously that 1'-acetoxy-chavicol acetate, showing multiple suppressive effects on rodent chemical carcinogenesis (23–28), is a selective inhibitor of the activation stage (19). In addition, AL-1 from a subtropical herb has been found to specifically inhibit the activation stage (29). On the other hand, curcumin (30), sarpaphyrol (31) and geneestein (13) are priming or dual inhibitors.

The results in the present study provide biological and histological evidence that AUR has a prominent ability to suppress TPA-induced oxidative stress and inflammation through attenuation of the responsiveness of activated leukocytes. In the double-dose TPA model, AUR during the priming stage did not show any suppressive effect on TPA-induced edema or H$_2$O$_2$ formation, which is in accordance with our recent study where AUR did not suppress single TPA application-induced edema formation in mouse ears (32). UMB, showing no suppressive effect on O$_2^-$ generation in cell cultures (6), exhibited no in vivo antioxidative potential. Thus, leukocyte activation is involved in TPA-induced oxidative stress mechanisms and can be counteracted efficiently by AUR. In this study, AUR at the priming stage did not show any suppression of leukocyte infiltration, while double pre-treatments or treatment only at the activation stage were remarkably effective to attenuate infiltration. Our previous observation...
promotion (42), we did not see any suppressive effect of AUR on LPS-induced IL-1β release (data not shown). To our knowledge, this is the first report demonstrating the inhibitory effect of coumarin-related compounds on COX-2 expression and TNF-α release, whereas suppressive effects of this class of compounds on iNOS expression have recently been reported by us (46) and others (47,48). As shown in Figure 4, l-NIO, a synthetic iNOS inhibitor, unexpectedly suppressed iNOS and COX-2 protein expression. These results may reflect previous findings where the reduction of NO levels led to diminished expression of these genes, since NO itself was reported to enhance the expression of iNOS (49) and COX-2 (50). Though the molecular mechanisms of AUR for attenuation of inflammation-associated protein formation remain to be addressed, some coumarins, including dicoumarol, are potential agents to block stress-activated protein kinase and nuclear factor-κB pathways to potentiate apoptosis in HeLa cells (51). These pathways are responsible for iNOS (52)/COX-2 (53) expression and TNF-α (54) release. Thus, AUR may suppress inflammation-associated protein formation through disruption of the above pathways. Since O$_2^-$ attenuates apoptosis of macrophages through the same nuclear factor-κB and activator protein-1 activation that induces COX-2 expression (55), it is likely that AUR is a useful molecular probe to investigate the biological cross-talks between these pathways.

In conclusion, we demonstrated that citrus AUR attenuated the in vitro inflammatory leukocyte activation that led to decreased levels of edema formation, H$_2$O$_2$ production, leukocyte infiltration and PCNA-labeling index. In in vitro tests as well, AUR markedly suppressed the expression of iNOS/COX-2 and the release of TNF-α. Our previous and present results thus imply the use of AUR for the prevention and medication of inflammation-related disorders, including cancer, through attenuation of leukocyte activation.

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