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Evaluation and Mechanistic Analysis of The Cytotoxicity of The Acyl Glucuronide of Nonsteroidal Anti-inflammatory Drugs

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Running title: Cytotoxicity of acyl glucuronide

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ABBREVIATIONS: AG, acyl glucuronide; Dic, diclofenac; ERK, extracellular signal-regulated kinase; GSH, glutathione; HEK, human embryonic kidney; Ibu, ibuprofen; IL, interleukin; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MCP, monocyte chemoattractant protein; Nap, naproxen; NSAID, nonsteroidal anti-inflammatory drugs; PBMCs, peripheral blood mononuclear cells; Pro, probenecid; TNF, tumor necrosis factor; Tol, tolmetin; UGT, UDP-glucuronosyltransferase.

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Abstract

The chemical reactivity of acyl glucuronide (AG) has been thought to be associated with the toxic properties of drugs containing carboxylic acid moieties, but there has been no direct evidence showing that AG formation is related to the observed toxicity. In the present study, the cytotoxicity of AGs, especially that associated with the inflammatory response, was investigated. The changes in the mRNA and protein expression levels of interleukin 8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) induced by the treatment of human peripheral blood mononuclear cells (PBMCs) with diclofenac (Dic), probenecid (Pro), tolmetin (Tol), ibuprofen (Ibu), naproxen (Nap), and their AGs were investigated by real-time RT-PCR, and the viabilities of $CD3^+$, $CD14^+$, and $CD19^+$ cells were measured by flow cytometry. Treatment with Dic-AG, Pro-AG, and Tol-AG significantly increased the expression levels of IL-8 and MCP-1. In addition, Dic-AG, Pro-AG, and Tol-AG significantly decreased the viability of $CD14^+$ cells. Of these three AGs, Dic-AG showed the most potent changes, followed by Tol-AG and Pro-AG. Treatment with Ibu-AG and Nap-AG affected neither the expression levels of IL-8 and MCP-1 nor the viability of $CD14^+$ cells. None of the drugs affected the $CD3^+$ and $CD19^+$ cell populations. Dic-AG increased the phosphorylation of p38 MAP kinase and JNK1/2. The pretreatment of PBMCs with SB203580 (p38 inhibitor) significantly suppressed the Dic-AG-induced expression of inflammatory factors and cytotoxicity of $CD14^+$ cells. In conclusion, AGs induce inflammatory responses and cytotoxicity against $CD14^+$ cells via the p38 MAPK pathway. These factors may be useful biomarkers for evaluating the toxicity of AGs.

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Introduction

Acyl glucuronidation is one of the major metabolic routes of drugs that contain carboxylic acid moieties. Glucuronidation is one of the most important phase II metabolic pathways for endogenous and exogenous substrates and is generally considered a detoxification pathway. However, it is well known that acyl glucuronides (AGs) are unstable under physiological conditions and consequently undergo hydrolysis or intramolecular rearrangement through the migration of the drug moiety from the 1-*O*-position to the 2-, 3-, and 4-positions on the glucuronic acid ring (Smith et al., 1990; Benet et al., 1993; Bailey and Mickinson, 2003). AGs covalently modify endogenous proteins due to their electrophilic capacity to cause substitution reactions with the nucleophilic groups located on proteins or other macromolecules, and this effect can ultimately lead to adverse drug toxicities associated with carboxylic acid-containing drugs (Fead, 1984; Boelsterli, 2002). To date, both direct toxic effects and immune-mediated toxicity have been suggested as possible mechanisms of idiosyncratic liver injury. With direct toxicity, covalent protein binding via AG may disrupt the normal physiological function of a “critical” protein or some critical regulatory pathway to lead to cellular necrosis (Pirmohamed et al., 1996). In addition, it has been reported that electrophilic AGs can covalently interact with nucleic acids. Clofibrate AG and gemfibrozil AG can form DNA adducts that result in genotoxicity, and these adducts can be measured through a single-cell gel electrophoresis (comet) assay (Sallustio et al., 2006). Furthermore, probenecid and clofibric acid have been found to induce DNA damage in isolated hepatocytes and UDP-glucuronosyltransferase (UGT)-transfected HEK293 (HEK/UGT) cells via a glucuronidation-dependent pathway (Sallustio et al., 2006; Southwood et al., 2007). Thus, there is increasing evidence that the formation of drug-protein adducts is involved in idiosyncratic reactions. However, we previously

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reported that the AGs of naproxen, diclofenac, ketoprofen, and ibuprofen do not lead to cytotoxicity or genotoxicity in HEK/UGT cells and human hepatocytes (Koga et al., 2011). Therefore, it is necessary to elucidate the possibility of immune- and/or inflammation-mediated toxicity to clarify the toxicity of AG. The potentially fatal adverse drug reactions most often appear to be immunologically based. These include anaphylactic reactions and severe dermatological reactions, such as Stevens-Johnson syndrome and fatal epidermal necrolysis (Bailey et al., 2003). In fact, it has been reported that mycophenolic acid, which is the active metabolite of the immunosuppressant mycophenolate mofetil, is primarily metabolized by glucuronidation to form an AG, which was found to result in the induction of cytokine (TNFα and IL-6) formation in leukocytes in a cell-based study (Wieland et al., 2000). It could be envisaged that the induction of immune modulators can lead to immune- and/or inflammation-related adverse drug reactions.

Of the drugs containing carboxylic acid moieties, diclofenac (Dic), probenecid (Pro), tolmetin (Tol), ibuprofen (Ibu), and naproxen (Nap), and their AGs were selected for the present study (Fig. 1). These drugs containing carboxylic acid are associated with some degree of hepatotoxicity, immune cytopenias, and hypersensitivity reactions in patients (Bailey and Dickinson, 2003) and have been categorized as potentially IDT drugs in RxList (the internet drug index system) or in Japanese drug labeling. Therefore, it is suggested that the AGs of these drugs may be related to their toxicity. The purpose of this study was to investigate whether AGs induce the observed cytotoxicity, particularly through inflammatory responses, and to clarify the involvement of cell signaling in the cytotoxicity.

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Materials and Methods

Materials

Diclofenac sodium salt (Dic) and (S)-(+)-6-methoxy-α-methyl-2-naphthaleneaceatic acid (Nap) were purchased from Sigma-Aldrich (St. Louis, MO). Ibuprofen (Ibu) and probenecid (Pro) were purchased from Wako Pure Chemicals (Osaka, Japan). Tolmetin (Tol) was purchased from LKT Laboratories (St. Paul, MN). 4'-Hydoroxy diclofenac (4'-OH Dic), 5-hydroxy diclofenac (5-OH Dic), diclofenac acyl-β-D-glucuronide (Dic-AG), and other AGs were obtained from Toronto Research Chemicals (North York, ON, Canada). Propidium iodide (PI) and 7-amino-actinomycin D (7-AAD) were purchased from BD Pharmingen (San Diego, CA). Monoclonal antibodies against ERK1/2 and JNK1/2 and the polyclonal antibody against p38 MAPK were purchased from Cell Signaling Technology (Beverly, MA). Monoclonal antibodies against anti-Thr202/Tyr204 phosphorylated extracellular signal-regulated kinase (ERK) 1/2, anti-Thr180/Tyr182 phosphorylated p38 mitogen-activated protein kinase (MAPK), and anti-Thr183/Tyr185 phosphorylated c-Jun N-terminal kinase (JNK) 1/2 were also obtained from Cell Signaling Technology. IRDye680-labeled goat anti-rabbit or anti-mouse secondary antibody and Odyssey Blocking Buffer were purchased from Li-COR Biosciences (Lincoln, NE). All of the primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). All other reagents were of the highest commercially available grade.

Cell culture

The human monocytic leukemia cell line THP-1 was obtained from Riken Gene Bank (Tsukuba, Japan). Human peripheral blood mononuclear cells (PBMCs; lot# 48) and CTL-Test medium for the culture of PBMCs were obtained from Cellular Technology

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(Shaker Heights, OH). Human “total liver cells” (primary culture of the mixed population of all native human liver cells) were obtained from SciKon Innovation (Chapel Hill, NC). The THP-1 cells were cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA). The total liver cells were cultured in HBM^{TM} supplemented with HCM^{TM} Single Quots (Lonza, Basel, Switzerland), and these cells were maintained at 37°C under an atmosphere of 5% CO_2.

The THP-1 cells, PBMCs, and total liver cells were seeded at densities of 1×10^6, 3×10^6, and 7.5×10^5 cells/well, respectively, in a 24-well plate with medium containing the indicated concentration of the selected carboxylic acid-containing drugs and their AG and then incubated at 37°C. The final concentration of methanol in the culture medium was 0.1% in all of the experiments. The supernatants were separated from the cell cultures by centrifugation and stored at -80°C until assayed.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA was extracted from each cell using RNAiso (Takara Bio, Shiga, Japan) according to the protocol supplied by manufacturer. The reverse transcription was performed with ReverTra Ace (Toyobo, Tokyo, Japan) according to the manufacturer's protocol. For quantitative analysis of the mRNA levels of inflammatory cytokines, real-time RT-PCR was performed using an MX3000P real-time PCR system (Stratagene, La Jolla, CA). The primers used in this study were human IL-8 (forward: 5’-CAGCCTTCCTG ATTTCTCTGCAG-3’, reverse: 5’-AGACAGAGCTCTCTTCCATCAG-3’) and human MCP-1 (forward 5'-ACCGAGAGGCTGAGACTAAC-3', reverse: 5'-CAGGTGACTGGGGCATTGAT-3'). A 1-μl volume of the reverse-transcribed

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mixture was added to a PCR mixture containing 10 pmol of each primer and 10 μl of SYBR Premix ExTaq solution; the final volume of the reaction mixture was 20 μl. After an initial denaturation at 95°C for 30 s, the amplification was performed through 45 cycles of either denaturation at 94°C for 20 s and annealing and extension at 64°C for 20 s or denaturation at 94°C for 5 s, annealing at 64°C for 10 s, and extension 74°C for 20 s. To normalize the RNA loading and PCR variations, the signals of the targets were normalized to the signals of human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA (forward: 5'-CCATGAGAAGTATGACAACAGCC-3', reverse: 5'-TGGGTGGCAGTGATGGCATGGA-3').

Enzyme-linked immunosorbent assay (ELISA)

The levels of the inflammatory chemokines IL-8 and MCP-1 in the cell supernatants were measured using the Human IL-8 ELISA Ready-SET-GO! ^{TM} and Human CCL2 (MCP-1) ELISA Ready-SET-GO!^{TM} kits (eBioscience, San Diego, CA), respectively, according to the manufacturer's instructions.

Immunoblot analysis

SDS-polyacrylamide gel electrophoresis and immunoblot analysis were performed. The cell homogenates (30 μg) were separated on 10% polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore Corporation, Billerica, MA). The membranes were probed with the monoclonal antibodies against anti-Thr202/Tyr204 phosphorylated ERK1/2, anti-Thr180/Tyr182 phosphorylated p38 MAPK, and anti-Thr183/Tyr185 phosphorylated JNK1/2 and incubated with IRDye680-labeled goat anti-rabbit or anti-mouse IgG secondary antibody diluted with PBST. An Odyssey Infrared Imaging system (Li-COR Biosciences, Lincoln,

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NE) was used for the detection. The relative expression levels were quantified using the ImageQuant TL Image Analysis software (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Flow cytometry analysis

The PBMCs were washed with PBS containing 0.1% BSA. The cells were transferred to a 96-well plate and maintained on ice throughout the procedure until their analysis by flow cytometry. The PBMCs were stained using the following monoclonal antibodies: anti-human CD3-Pacific Blue™ (clone UCHT1, Invitrogen, Carlsbad, CA), anti-human CD14-PE (clone TüK4, Invitrogen) anti-human CD19-PE-Cy®7 (clone SJ25-C1, Invitrogen). The monoclonal antibodies were diluted 1:10 in PBS/0.1% BSA, and the PBMC were incubated with these antibodies for 30 min in the dark. The PBMCs were washed and subsequently incubated with PI (0.625 µg/mL) or 7-AAD (0.25 µg/mL), and the cell viability was measured using an Attune Acoustic Focusing Cytometer (Applied Biosystems, Foster, CA).

Statistical analyses

The data are presented as the means ± SD. The comparison of the multiple groups was performed using ANOVA followed by Dunnett's or Turkey's test. Differences with a *p* value of less than 0.05 were considered statistically significant.

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Results

Effects of Dic-AG on mRNA expression levels of IL-8 and MCP-1 in THP-1 cells, PBMCs, and total liver cells

To investigate whether Dic-AG affects the expression levels of IL-8 and MCP-1 in THP-1 cells, PBMC, and total liver cells, the cells were treated with 100 μM Dic or Dic-AG for 24 h, and the increase in the IL-8 and MCP-1 mRNA levels was measured. In THP-1 cells and PBMCs, the expression levels of IL-8 and MCP-1 were significantly increased by treatment with Dic-AG but not with vehicle (Ctl) or Dic (Figs. 2A and 2B). The changes in the expression levels of these cytokines were higher in PBMCs compared with THP-1 cells: IL-8 (8.3-fold vs. 1.7-fold) and MCP-1 (8.5-fold vs. 4.7-fold). The total liver cells showed no response to Dic-AG (Fig. 2C). It was suggested that Dic-AG induced immune responses, although the changes in the expression levels of IL-8 and MCP-1 by Dic-AG were different in different cell types. PBMCs were used for the subsequent analyses because these cells demonstrated the highest sensitivity to AGs.

Dose- and time-dependent effects of Dic-AG on IL-8 and MCP-1 mRNA expression in and protein release from human PBMCs

To investigate whether a low concentration of Dic-AG can affect the expression levels of inflammatory factors in PBMCs, these cells were treated with Dic, Dic-AG (0, 50 or 100 μM), or vehicle (1% methanol, Ctl) for 24 h. As shown in Fig. 3A, Dic-AG increased the mRNA expression levels of IL-8 and MCP-1 in a dose-dependent manner. The time-dependent changes in the levels of IL-8 and MCP-1 mRNA in PBMCs after treatment with Dic-AG were investigated. Treatment with Dic-AG significantly increased the mRNA expression levels and the release of IL-8 and MCP-1 12 and 24 h after treatment compared with those observed after treatment with Dic and vehicle (Fig.

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3B). The time-dependent changes in the IL-8 and MCP-1 mRNA levels were reflected at the protein level (Fig. 3C); thus, the subsequent experiments mainly analyzed the changes in mRNA expression.

Effects of Dic-AG on cell populations of human PBMCs

The effect of Dic-AG on the major cell populations of PBMCs (monocytes, T-lymphocytes, and B-lymphocytes) was investigated by flow cytometric analysis. The PBMCs were stained with fluorescent monoclonal antibodies against CD3 (T-lymphocyte), CD14 (monocyte), and CD19 (B-lymphocyte) and labeled with 7AAD or PI to detect the dead cells. Treatment with Dic-AG markedly decreased the subset of $CD14^+$ cells of the total cells in a dose- and time-dependent manner (Fig. 4). This effect was not observed with Dic treatment. In addition, Dic-AG showed no inhibitory effect on either the $CD3^+$ cell population or the $CD19^+$ cell population (Fig. 4). These results suggest that Dic-AG specifically affects the viability of $CD14^+$ cells.

Effects of various AGs on the mRNA expression of IL-8 and MCP-1 in PBMCs and their cytotoxicity on $CD14^+$ cells

To investigate whether various NSAID AGs increase the expression levels of IL-8 and MCP-1 and decrease the subset of the $CD14^+$ cell population, PBMCs were treated with 100 μM NSAID or its AG for 24 h; the mRNA levels of IL-8 and MCP-1 were then measured by real-time RT-PCR, and the cell viabilities of $CD3^+$, $CD14^+$, and $CD19^+$ cells were measured by flow cytometry. As shown in Fig. 5, treatment with Pro-AG, Tol-AG, and Dic-AG significantly increased the expression levels of MCP-1 and IL-8. Furthermore, Pro-AG and Tol-AG significantly decreased the viability of $CD14^+$ cells. Treatment with Ibu-AG and Nap-AG affected neither the expression levels of IL-8 and

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MCP-1 nor the viability of $CD14^+$ cells. None of the AGs showed an inhibitory effect on either the $CD3^+$ cell population of the $CD19^+$ cells population. These results suggest that the AGs induced inflammatory factors and cytotoxicity against $CD14^+$ cells. Because Dic-AG had a pronounced effect on the expression of inflammatory factors and the viability of $CD14^+$ cells, further studies were performed to analyze the effect of Dic-AG.

Effects of Dic and its metabolites on mRNA expression of IL-8 and MCP-1 and cell populations in human PBMCs

Dic is metabolized to Dic-AG by UGT2B7 and to 4'-hydroxy Dic (4'-OH Dic) and 5-hydroxy Dic (5-OH Dic) by CYP2C9 and CYP3A4, respectively, in humans. It has been reported that these hydroxides are potential protoxicants because they can be further oxidized to quinone imine (van Leenwen et al., 2011). To investigate whether Dic-AG and other metabolites might increase the expression levels of IL-8 and MCP-1 and decrease the $CD14^+$ cell population in PBMCs, PBMCs were treated with 100 μM Dic-AG, 4'-OH Dic, or 5-OH Dic for 24 h, and the IL-8 and MCP-1 mRNA levels and the cell viability of $CD3^+$, $CD14^+$, and $CD19^+$ cells were then measured. As shown in Fig. 6, treatment with 4'-OH Dic and Dic-AG significantly increased the expression levels of IL-8 and MCP-1. Furthermore, Dic-AG and 4'-OH Dic significantly decreased the viability of $CD14^+$ cells. None of the compounds decreased the viability of $CD3^+$ and $CD19^+$ cells (data not shown). These results suggest that Dic-AG exhibits the highest cytotoxicity among these metabolites.

Effects of Dic-AG on the activation of MAPK signaling pathways in human PBMCs

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The phosphorylation of MAPKs is a major component of many intracellular signaling pathways. To clarify the MAP kinase activation, the phosphorylation of ERK1/2 (44/42 kDa), p38 MAP kinase (43 kDa), and JNK1/2 (46/54 kDa) in cell lysates was assessed by immunoblot analysis. As shown in Fig. 7, Dic-AG treatment for 0.5 h significantly increased the phosphorylation of p38 MAP kinase and JNK1/2 but not ERK1/2 in human PBMCs, which suggests that Dic-AG activates the p38 MAP kinase and JNK1/2 pathways in PBMCs. The phosphorylation of ERK1/2 was increased 12 h after Dic-AG treatment (data not shown). To confirm the effects of MAP kinase inhibitors on the phosphorylation of ERK1/2, p38 MAP kinase, and JNK1/2, PBMC were pretreated for 1 h with various concentrations of the MEK1/2 inhibitor U0126, the p38 MAP kinase inhibitor SB203580, or the JNK1/2 inhibitor SP600125 before treatment with Dic-AG. The results show that the phosphorylations of p38 MAP kinase and JNK1/2 were significantly suppressed by pretreatment with their specific inhibitors (Fig. 5).

Effects of MAPK inhibitors on Dic-AG-induced inflammatory factors and viability of $CD14^+$ cells in human PBMCs

To clarify which MAP kinase signaling pathway is mainly involved in the increase in the expression levels of IL-8 and MCP-1 and in the decrease of the $CD14^+$ cell population in PBMCs, the effects of MAP kinase inhibitors on the expression levels of IL-8 and MCP-1 and the viability of $CD14^+$ cells in PBMCs treated with Dic-AG were investigated. As shown in Fig. 8, the Dic-AG-induced increase in the level of IL-8 mRNA in PBMCs was significantly suppressed by pretreatment with SB203580 and SP600125, and the expression of MCP-1 was significantly suppressed by pretreatment with SB203580 and U0126. These findings suggest that the MAP kinase pathway plays an important role in the expression of IL-8 and MCP-1 in response to Dic-AG treatment. The

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decreased cell viability of $CD14^+$ cells by Dic-AG treatment was significantly restored by pretreatment with SB203580, which suggests that the p38 MAP kinase pathways are involved in the cytotoxicity of this drug in $CD14^+$ cells (Fig. 8B). These results indicate that the increase in the expression of inflammatory factors by Dic-AG treatment is partly related to the cytotoxic effects observed in $CD14^+$ cells.

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Discussion

There is increasing evidence that the formation of drug-protein adducts is involved in idiosyncratic drug toxicities (IDT). However, little direct evidence demonstrates a link between drug-protein adduct formation and adverse biological consequences. It is well known that AGs are characterized by their electrophilic reactivity, and this reactivity is implicated in a wide range of adverse drug effects, including drug hypersensitivity reactions and cellular toxicity (Ritter, 2000). We previously revealed that the AGs of various NSAIDs, such as naproxen, diclofenac, ketoprofen, and ibuprofen, showed no direct cytotoxicity and genotoxicity in human hepatocytes and cells stably expressing human UGTs (Koga et al., 2011). Therefore, in this study, we investigated whether the AGs exert toxicity through inflammation-related responses. Our previous research revealed that some drugs, such as albendazole, terbinafine, and amiodarone, stimulate THP-1 cells to release IL-8, which suggests the involvement of an inflammation-mediated pathway in drug-induced adverse reactions (Mizuno et al., 2010 and 2011; Endo et al., 2012). Furthermore, it has been reported that cytokines and chemokines, such as MCP-1, are released by THP-1 cells and PBMCs after treatment with flavonoids or amyloid proteins (Song et al., 2009). Thus, the expression levels of IL-8 and MCP-1 as markers for predicting the activation of the inflammatory response induced by AGs were investigated in the present study.

First, the effects of Dic-AG treatment on three different types of cells, namely THP-1 cells, PBMCs, and total liver cells, were compared. We previously revealed that the NSAIDs AGs that were produced in the cells are efficiently released from the cells into the culture medium (Koga et al., 2011). Thus, effect of AGs was evaluated by exposing the AGs to the outside of the cells, i.e., the culture medium. In a preliminary study, we found that Dic-AG treatment increases IL-8 production in human PBMCs (data

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not shown). Therefore, three cell types, namely THP-1 cells, PBMCs, and total liver cells, were exposed to Dic-AG treatment, and the treatment effects were compared. The expression levels of IL-8 and MCP-1 were significantly increased in THP-1 cells and PBMCs but not in total liver cells (Fig. 2). It has been reported that some inflammatory factors, such as IL-8, IL-10, and MCP-1, are released from mixed cell culture at a much higher level than that obtained from pure monocytes treated with proteins or chemicals (Feng et al., 2008). In addition, it has been reported that primary PBMCs are more sensitive to drugs and/or chemicals than tumor cells (Hougee et al., 2005). In this study, as suggested by previous reports, the inducibility of IL-8 and MCP-1 in PBMCs by Dic-AG treatment was much higher than that observed in THP-1 cells. We intended to evaluate the inflammatory responses under the condition extrapolating the in vivo in the liver by using total liver cells. However, the total liver cells are less sensitive to Dic-AG compared to THP-1 cells and PBMC. The total liver cells are primary culture of the mixed population of all native human liver cells, and consisted mainly of hepatocytes and contained low percentage of immune-related cells. From the present result, it was suggested that the total liver cells were not suitable for the sensitive in vitro cell-based assay.

Of the commercially available NSAIDs AGs, Dic-AG, Pro-AG, and Tol-AG induced the expression of IL-8 and MCP-1 and decreased the viability of $CD14^+$ cells. However, Ibu-AG and Nap-AG had no effect (Figs. 4 and 5). It was reported that the half-lives of Dic-AG, Pro-AG, and Tol-AG in potassium phosphate solution or human serum albumin are shorter than those of Ibu-AG and Nap-AG (Sawamura et al., 2010). Furthermore, a covalent binding study using a small peptide demonstrated that the degree of AG reactivity is affected by its chemical structure (Wang et al, 2004) (in descending order): acetic acid derivative > isopropionic acid derivative > benzoic acid derivative. It

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was hypothesized that the benzoic acid derivative exhibits the lowest reactivity due to the resonance stabilization provided by the aromatic moiety and that the isopropionic acid derivative displays a lower reactivity than that of the acetic acid derivative likely due to the higher steric hindrance capacity of the isopropyl group compared with the acetyl group (Wang et al., 2004). These reports suggest that the stability of AGs serve as a useful key predictor for their IDT risk. Thus, it was surmised that the observed increase in the levels of inflammatory factors and the cytotoxicity of $CD14^+$ cells by Dic-AG, Pro-AG, and Tol-AG may be related to the structural properties of the AGs.

Dic-AG is one of the most studied AGs due to its related toxicity. Dic-AG is excreted into bile and transported to the small intestine, where it can produce erosions and ulcers in a dose-dependent manner in rats (Seitz and Boelsterli, 1998). Dic is metabolized to Dic-AG by UGT2B7 and to 4'-hydroxy Dic (4'-OH Dic) and 5-hydroxy Dic (5-OH Dic) by CYP2C9 and CYP3A4, respectively. These hydroxides are further metabolized to form benzoquinone imine, which leads to the production of oxidative stress and covalent binding with endogenous proteins (Tang et al., 1999). Of these metabolites, 4'-OH Dic and Dic-AG increased the expression levels of IL-8 and MCP-1 (Fig. 6). The inducibility of these genes by Dic-AG was much higher than that obtained with 4'-OH Dic, which suggests that Dic-AG shows the highest cytotoxicity among these metabolites. It appeared important to evaluate the toxicity of not only the AG but also the quinone imines of Dic.

The activation of MAP kinases, such as ERK1/2, p38 MAP kinase, and JNK1/2, is important for the mediation of the monocyte and macrophage functions, including the activation of various transcription factors, the production of pro-inflammatory cytokines, and cell death (Payne et al., 1991; Defranco et al., 1998). In this study, Dic-AG activated the p38 MAP kinase and JNK1/2 pathways in PBMCs (Fig. 7A). The blocking of these

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MAP kinases by several MAP kinase inhibitors prevented the transcription and/or translation of IL-8 and MCP-1 from LPS-stimulated monocytes and PBMCs (Guha and Mackman, 2001; Isalam et al., 2006). To determine the involvement of MAP kinases in the Dic-AG-induced increase in the expression levels of IL-8 and MCP-1 and the cytotoxicity to $CD14^+$ cells, blocking studies were performed using specific inhibitors of these MAP kinases, including U0126, SB203580, and SP600125 (English and Cobb, 2002). In this study, the p38 MAP kinase pathway was shown to be involved in the stimulation of the increase in the expression levels of IL-8 and MCP-1 in PBMCs (Fig. 8A) and the cytotoxicity to $CD14^+$ cells (Fig. 8B). In the future, it should be clarified whether $CD14^+$ monocytes are the main source of cell death-related inflammatory cytokines and chemokines (IL-8 and MCP-1) after the treatment of PBMCs with a drug.

Notably, the circulating cell population of monocytes ($CD14^+$ cells) in the blood is continuously supplied *in vivo* from the bone marrow, and these cells migrate into the tissues (Hougee et al., 2005), which is an effect that cannot be reproduced in the *in vitro* experimental conditions. It is reported that $CD14^+$ cells were specifically eliminated in PBMC by apigenin and its structural analogues chrysin and luteolin. Thus, it is considered that the structure of $CD14^+$ cell surface and that of drugs are important for the onset of toxicity. Therefore, the *in vivo* evaluation of the mechanism through which AGs exert their selective cytotoxicity to $CD14^+$ cells is required.

In conclusion, we demonstrated that AGs increase the inflammatory responses and cytotoxicity of CD14+ cells via the p38 MAP kinase pathway in human PBMCs. These factors could be useful biomarkers for evaluating the toxicity of AGs. This study provides new insights into the evaluation of the toxicity of AGs in drug development.

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Authorship Contributions

Participated in research design: Miyashita, Fukami, Nakajima and Yokoi

Conducted experiments: Miyashita and Kimura

Contributed new reagents or analytic tools: Miyashita and Yokoi

Performed data analysis: Miyashita

Wrote or contributed to the writing of the manuscript: Miyashita and Yokoi

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Footnote

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Figure Legends

Fig. 1. Chemical structures of the NSAIDs investigated in this study: diclofenac, probenecid, tolmetin, ibuprofen, and naproxen.

Fig. 2. Effects of Dic-AG on the expression levels of IL-8 and MCP-1 mRNA in THP-1 cells (A), human PBMCs (B), and total liver cells (C). The cells was treated with Dic, Dic-AG (100 μM), or vehicle (1% methanol, Ctl) for 24 h. The IL-8 and MCP-1 mRNA levels were measured by real-time RT PCR and normalized with GAPDH. The data represent the means ± SD (n = 4) or the mean (n = 2). $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with Ctl. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with Dic.

Fig. 3. Dose- and time-dependent effects of Dic-AG on the IL-8 and MCP-1 mRNA expression levels and protein release in human PBMCs. PBMCs were treated with Dic, Dic-AG (0, 50, or 100 μM), or vehicle (1% methanol, Ctl) for 24 h (A) or with Dic or Dic-AG (100 μM) for 0, 3, 6, 12, and 24 h (B). The IL-8 and MCP-1 mRNA levels were measured by real-time RT PCR and normalized with GAPDH. The release of IL-8 and MCP-1 protein into the supernatant was measured by ELISA (C). The data represent the means ± SD (n = 4). $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with Ctl. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ compared with Dic.

Fig. 4. Flow cytometry analysis of human PBMCs treated with Dic or Dic-AG. Human PBMCs were treated with Dic, Dic-AG (0, 50, or 100 μM), or vehicle (1% methanol, Ctl) for 24 h (A) or with Dic or Dic-AG (100 μM) for 0, 3, 6, 12, and 24 h (B). The viability of $CD3^+$, $CD14^+$, and $CD19^+$ cells in PBMCs treated with Dic or Dic-AG was measured by

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PI or 7AAD assay, as described in Material and Methods. The data represent the means ± SD (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with Dic.

Fig. 5. Effects of NAIDs and their AGs on the mRNA expression levels of IL-8 and MCP-1 and their cytotoxicity to $CD3^{+}$, $CD14^{+}$, and $CD19^{+}$ cells in human PBMCs. Human PBMC were treated with Dic, Dic-AG, Pro, Pro-AG, Tol, Tol-AG, Ibu, Ibu-AG, Nap, Nap-AG (100 μM), or vehicle (1% methanol, Ctl) for 24 h. The IL-8 and MCP-1 mRNA levels were measured by real-time RT PCR and normalized with GAPDH (A). The cell viability of $CD14^{+}$ cells treated with Dic-AG was measured by 7AAD assay as described in Material and Methods (B). The data represent the means ± SD (n = 4). $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with the parent drugs.

Fig. 6. Effects of Dic and its metabolites on the mRNA expression levels of IL-8 and MCP-1 in human PBMCs. Human PBMCs were treated with Dic, Dic-AG, 4'-OH Dic, 5-OH Dic (100 μM), or vehicle (1% methanol, Ctl) for 24 h. The expression levels of IL-8 and MCP-1 mRNA were measured by real-time RT PCR and normalized with GAPDH. The data represent the means ± SD (n = 4). $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with Ctl. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with Dic.

Fig. 7. Activation of MAP kinase signaling pathways in PBMCs treated with Dic-AG. Immunoblot analyses of the MAP kinase proteins in PBMCs were performed and quantified (A). Before treatment with 100 μM Dic-AG, the PBMCs were pretreated with the indicated concentrations of the MAP kinase inhibitors for 1 h (B). U0126, SB203580, and SP600125 were used as specific inhibitors of MEK1/2, p38 MAP kinase, and JNK1/2, respectively. After 0.5, 1, and 3 h of incubation with Dic-AG, the cell lysates were

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subjected to immunoblot analyses using anti-Thr202/Tyr204 phosphorylated ERK1/2, anti-Thr180/Tyr182 phosphorylated p38 MAP kinase, and anti-Thr183/Tyr185 phosphorylated JNK1/2 antibodies. The data represent the means ± SD (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with Ctl. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with Dic. $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ compared with Dic-AG.

Fig. 8. Effects of MAP kinase inhibitors on Dic-AG-induced IL-8 and MCP-1 expression and cytotoxicity to $CD14^{+}$ cells in human PBMCs. Before treatment with 100 μM Dic-AG, the PBMCs were pretreated with the indicated concentrations of the MAP kinase inhibitors for 1 h. After 24-h incubation with Dic-AG, the expression levels of IL-8 and MCP-1 mRNA were measured by real-time RT-PCR, and the viability of $CD14^{+}$ cells treated with Dic-AG was measured by 7AAD assay as described in Material and Methods. The data represent the means ± SD (n = 3). $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with Ctl. $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ compared with Dic-AG.

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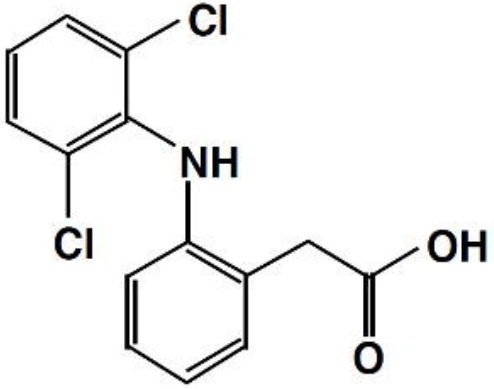
Figure 1



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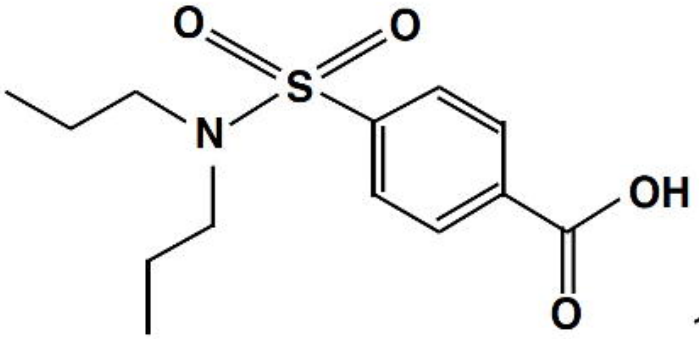

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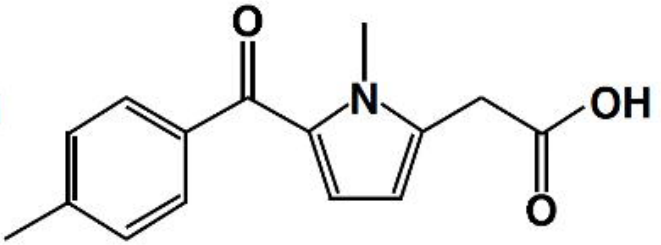

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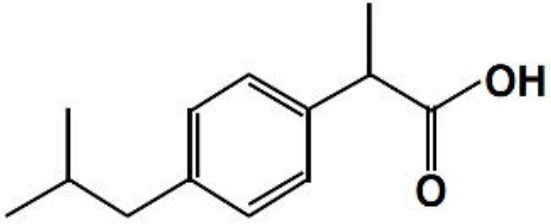
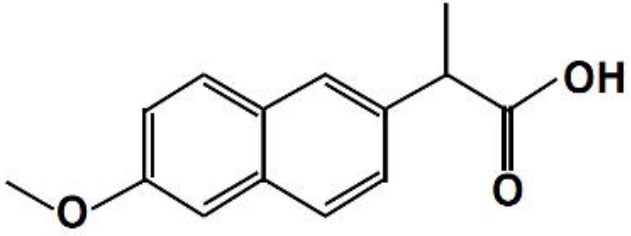
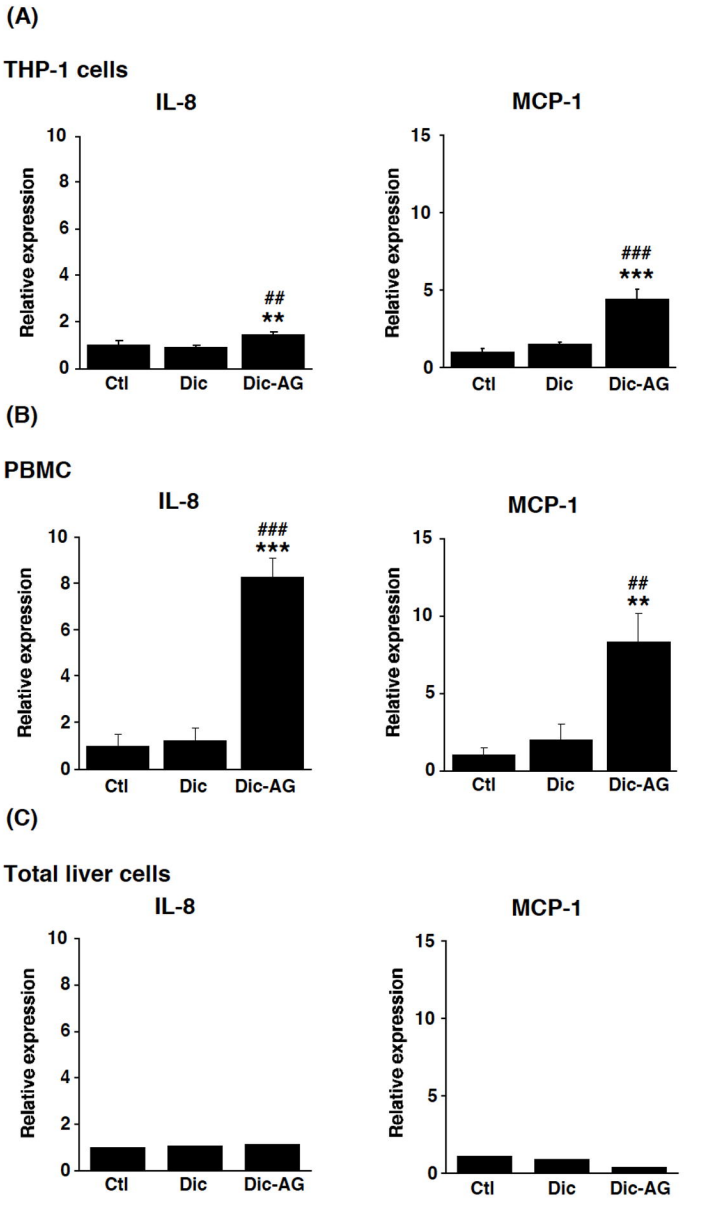
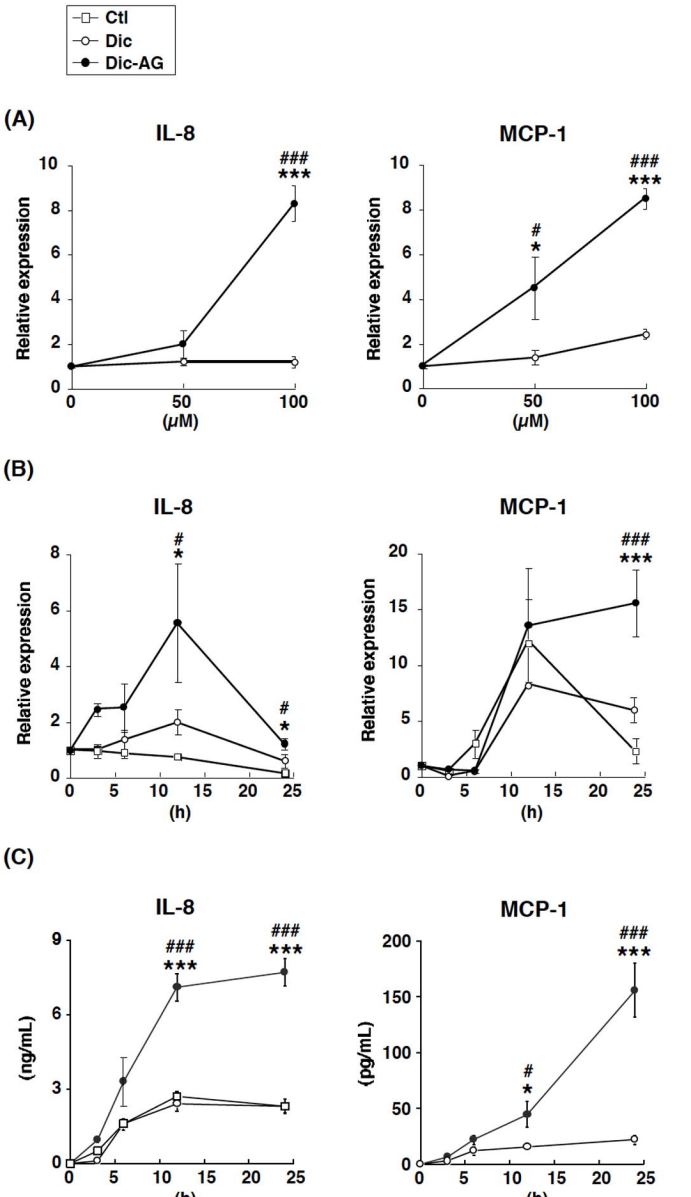


Ibuprofen



Naproxen


(A)
THP-1 cells
IL-8
MCP-1
Relative expression
10
8
6
4
2
0
15
10
5
##
**
###
***
Ctl
Dic
Dic-AG
(B)
PBMC
IL-8
MCP-1
Relative expression
###
***
##
**
Ctl
Dic
Dic-AG
(C)
Total liver cells
IL-8
MCP-1
Relative expression
Ctl
Dic
Dic-AG


Ctl
Dic
Dic-AG
(A)
IL-8
MCP-1
Relative expression
(μM)
(B)
(h)
(C)
(ng/mL)
(pg/mL)
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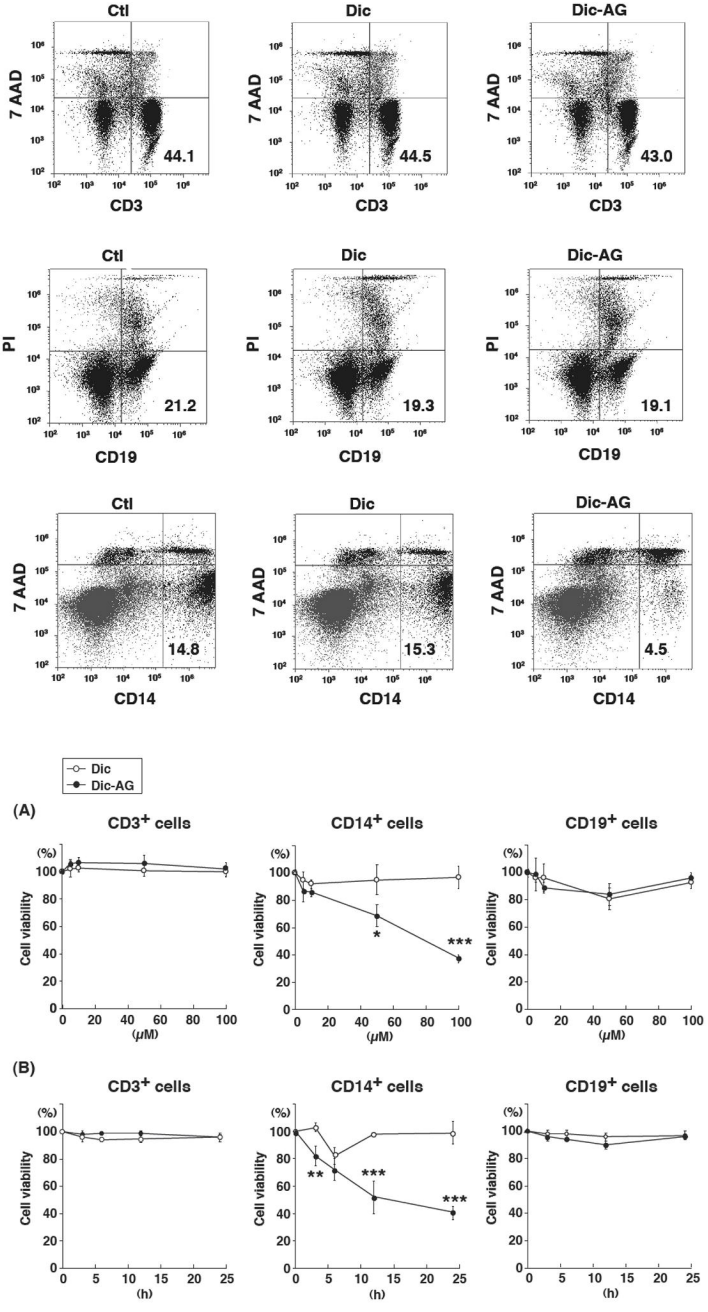

Ctl
Dic
Dic-AG
7 AAD
CD3
44.1
44.5
43.0
PI
CD19
21.2
19.3
19.1
CD14
14.8
15.3
4.5
Dic
Dic-AG
(A)
CD3+ cells
CD14+ cells
CD19+ cells
Cell viability
(%)
(μM)
*
***
(B)
(h)
**

Figure 5

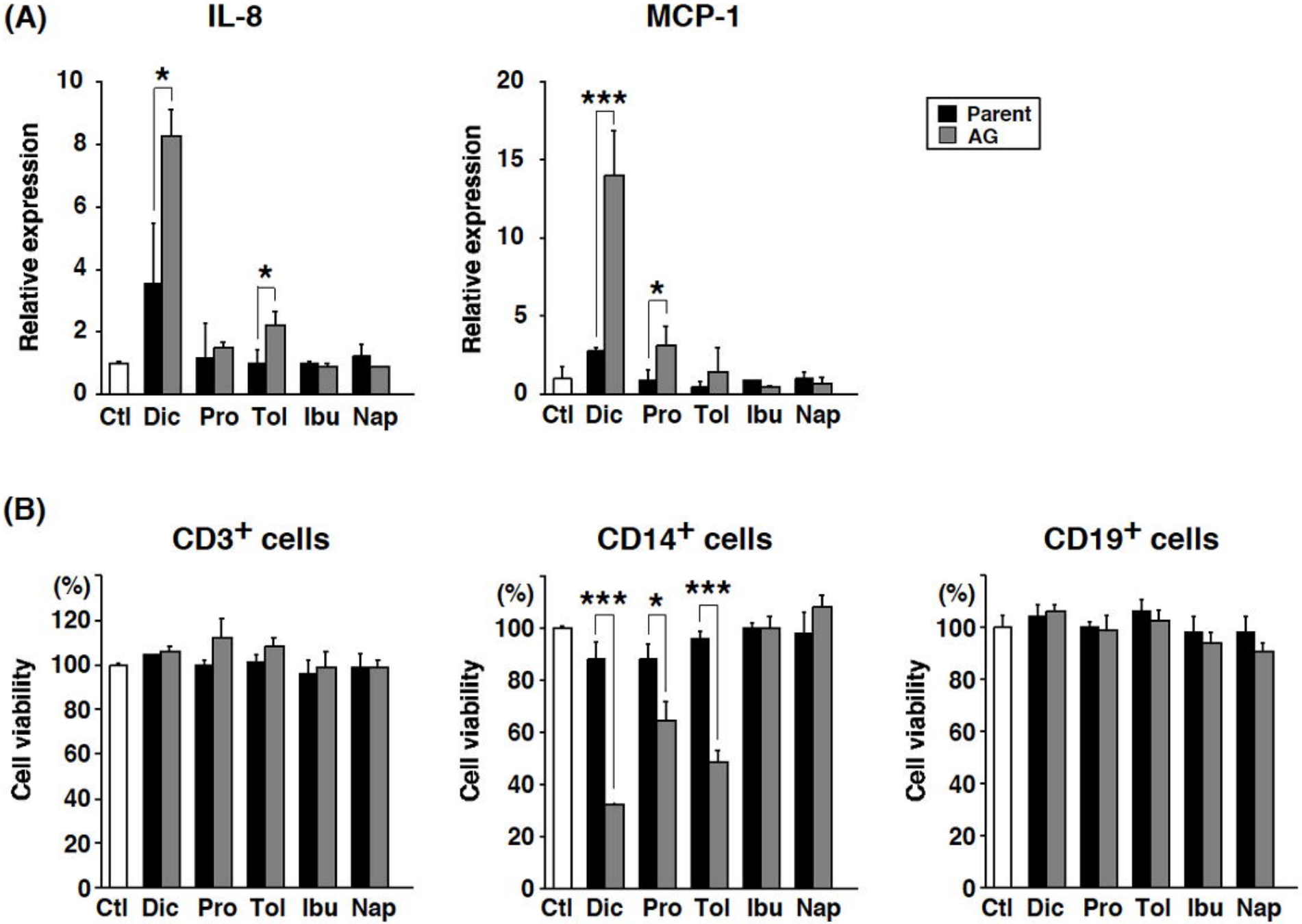
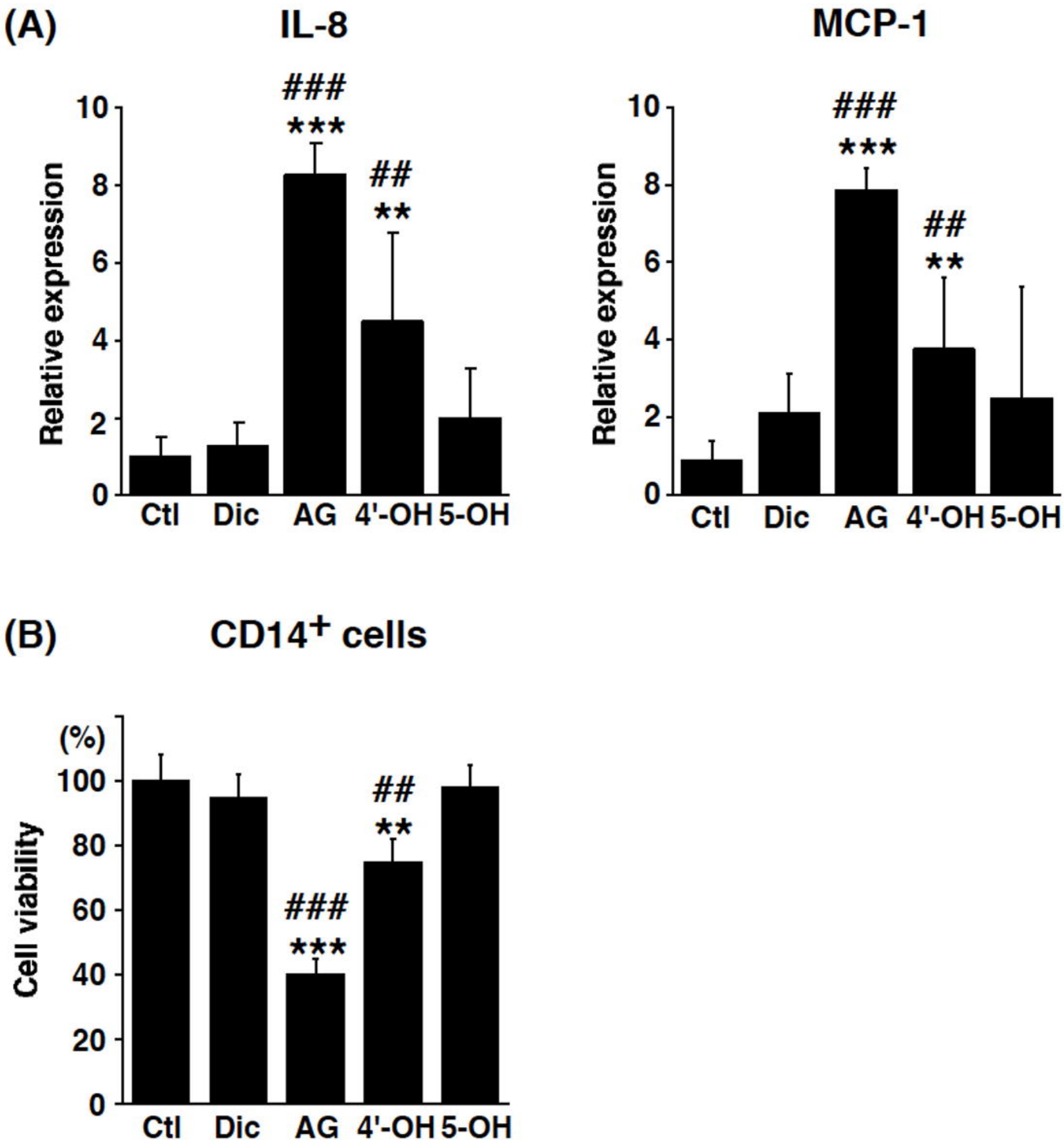

(A)
IL-8
MCP-1
Relative expression
Parent
AG
Ctl Dic Pro Tol Ibu Nap
(B)
CD3+ cells
CD14+ cells
CD19+ cells
Cell viability
(%)

Figure 6


(A)
IL-8
MCP-1
Relative expression
10
8
6
4
2
0
###
***
##
**
Ctl
Dic
AG
4'-OH
5-OH
(B)
CD14+ cells
Cell viability
(%)
100
80
60
40
20
0

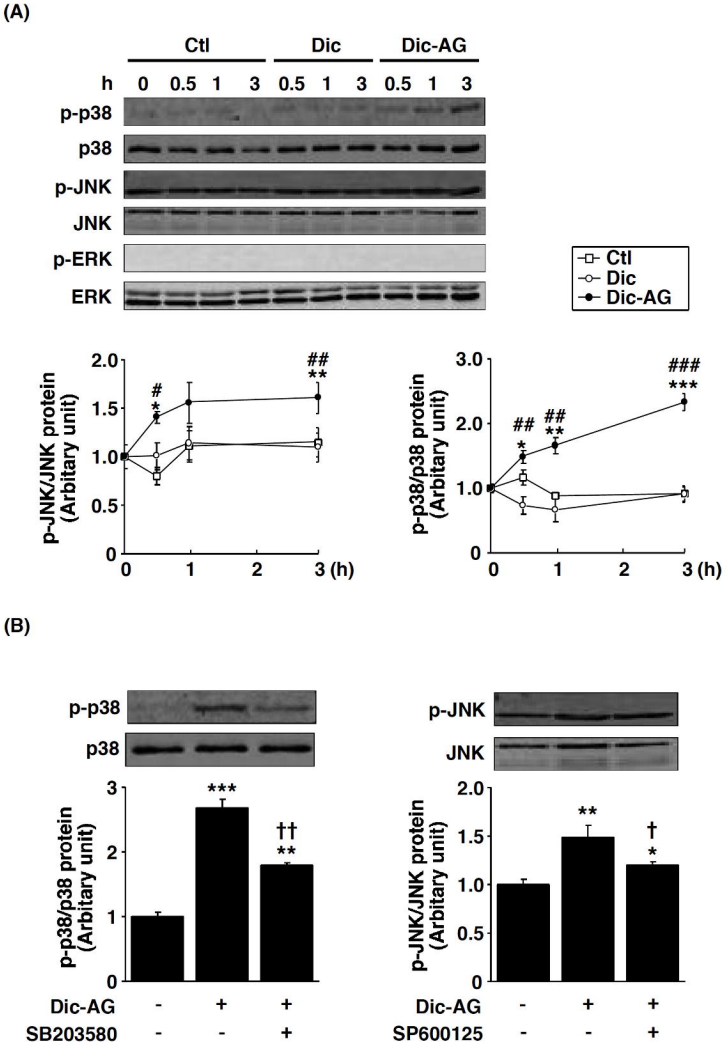
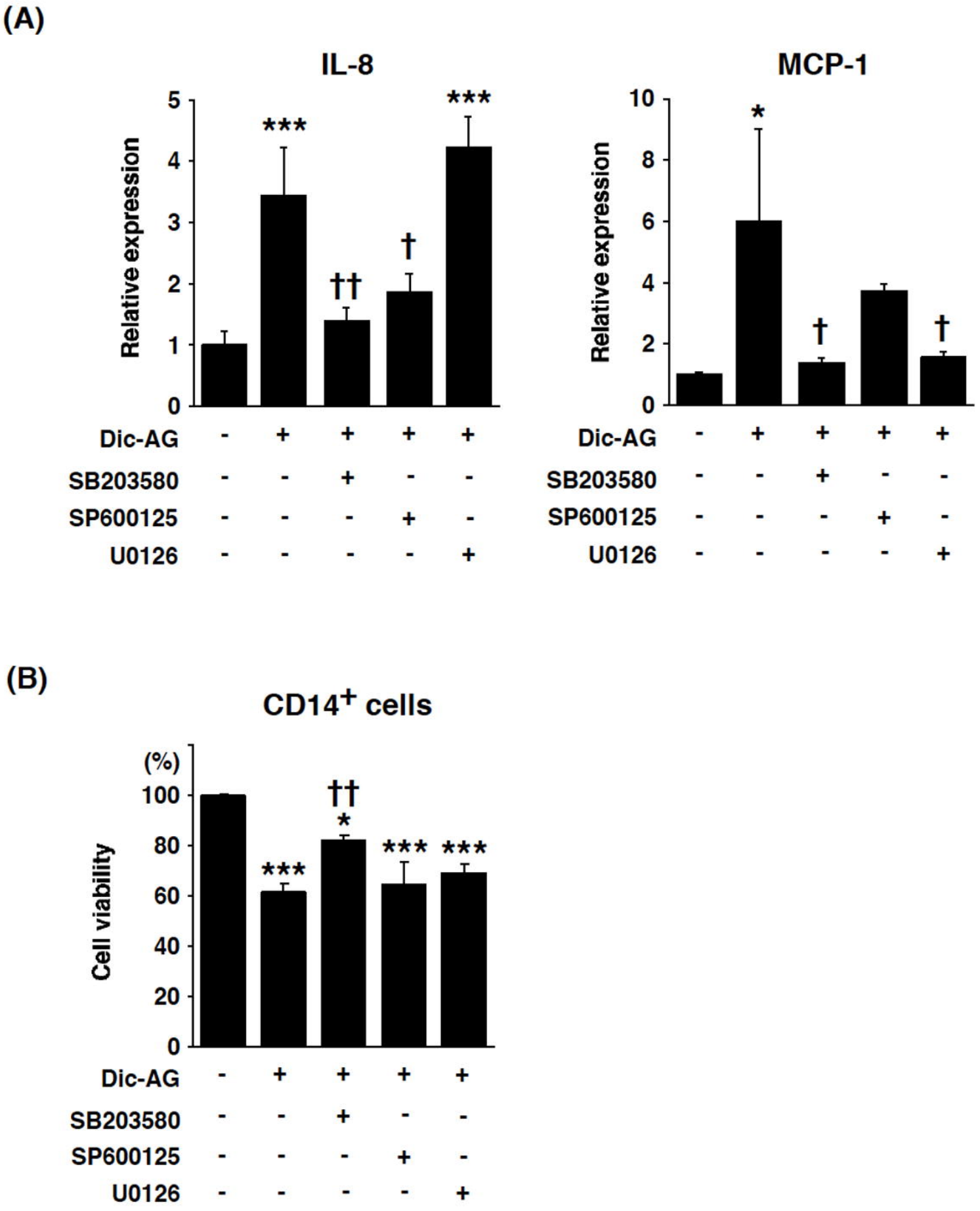

(A)
Ctl
Dic
Dic-AG
h
0
0.5
1
3
0.5
1
3
0.5
1
3
p-p38
p38
p-JNK
JNK
p-ERK
ERK
Ctl
Dic
Dic-AG
p-JNK/JNK protein (Arbitary unit)
p-p38/p38 protein (Arbitary unit)
3 (h)
(B)
p-p38
p38
p-JNK
JNK
p-p38/p38 protein (Arbitary unit)
p-JNK/JNK protein (Arbitary unit)
Dic-AG
SB203580
SP600125

Figure 8


(A)
IL-8
Relative expression
0
1
2
3
4
5
***
***
††
†
Dic-AG - + + + +
SB203580 - - + - -
SP600125 - - - + -
U0126 - - - - +
MCP-1
Relative expression
0
2
4
6
8
10
*
†
†
Dic-AG - + + + +
SB203580 - - + - -
SP600125 - - - + -
U0126 - - - - +
(B)
CD14+ cells
Cell viability
(%)
0
20
40
60
80
100
***
††
*
***
***
Dic-AG - + + + +
SB203580 - - + - -
SP600125 - - - + -
U0126 - - - - +