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Author(s)	Okamoto, Keisuke; Saito, Yoshitaka; Narumi, Katsuya et al.
Citation	European journal of pharmacology, 884, 173339 https://doi.org/10.1016/j.ejphar.2020.173339
Issue Date	2020-10-05
Doc URL	https://hdl.handle.net/2115/82868
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Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	journal article
File Information	WoS_95814_Kobayashi.pdf



Title

Comparison of the nephroprotective effects of non-steroidal anti-inflammatory drugs on
cisplatin-induced nephrotoxicity *in vitro* and *in vivo*

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Abstract

Cisplatin (CDDP) is an anticancer drug, often used in the treatment of several types of cancers. CDDP-induced nephrotoxicity (CIN) is one of the most severe adverse events associated with the use of CDDP. It has been suggested that the co-administration of non-steroidal anti-inflammatory drugs (NSAIDs) is a risk factor for CIN. However, the specific NSAIDs that affect CIN and the precise mechanisms underlying this interaction remain unclear. Hence, we aimed to evaluate the effect of NSAIDs on CDDP-induced cytotoxicity *in vitro* and confirmed the results *in vivo*. Using the epithelioid clone of the normal rat kidney cells (NRK-52E cells), we assessed the effects of 17 NSAIDs on CDDP-induced cytotoxicity all at once using the MTT assay. Furthermore, we evaluated two NSAIDs, which significantly attenuated or enhanced CDDP-induced cytotoxicity, *in vivo*. Wistar rats were treated with CDDP (5 mg/kg, i.p., day 1) and NSAIDs (p.o., day 1–4), and the kidneys were excised on day 5. Our results demonstrated that several NSAIDs attenuated, while others enhanced CDDP-induced cytotoxicity. Celecoxib significantly attenuated and flurbiprofen markedly enhanced cell dysfunction by CDDP. These results were reproduced *in vivo* as celecoxib decreased and flurbiprofen increased the expression of kidney injury molecule 1 (Kim-1) mRNA, a sensitive kidney injury marker, compared to the CDDP group. Moreover, celecoxib increased the antioxidant and autophagy markers quantified by qPCR *in vitro* and prevented a decrease in body weight induced by CDDP *in vivo*. In conclusion, we

revealed that celecoxib significantly attenuated CIN *in vitro* and *in vivo*.

Keywords

Cisplatin; Nephrotoxicity; NSAIDs; Celecoxib

1. Introduction

Cisplatin (cis-dichloro-diammine platinum, CDDP) is widely used as an anticancer agent in many types of cancer, including lung, gastric, and head and neck cancer (Rosenberg et al., 1969). CDDP-induced nephrotoxicity (CIN) is one of the most serious adverse effects caused by CDDP (Prestayko et al., 1979), and it is dose-dependent, cumulative, and usually reversible (Miller et al., 2010; Pabla and Dong, 2008). CIN occurs in 30–40% of patients administered CDDP (Yoshida et al., 2014). CIN mainly affects the S3 segment of the proximal tubule located in the outer medulla, and the thick ascending limb of the loop of Henle (Dobyan et al., 1980). The suggested mechanisms of CIN include oxidative stress, mitochondrial dysfunction, DNA damage, increased tumor necrosis factor, and inhibition of protein synthesis (Kawai et al., 2006; Park et al., 2002; Tsuruya et al., 2003). In spite of adopting hydration as an approach to prevent CIN, it fails to sufficiently attenuate CIN (de Jongh et al., 2003).

In a retrospective clinical study, our research group demonstrated that the

co-administration of non-steroidal anti-inflammatory drugs (NSAIDs) enhances CIN (Saito et al., 2017a). NSAIDs demonstrate their anti-inflammatory effect through the inhibition of cyclooxygenase (COX) activity (Vane, 1971). There are two isoforms of COX, COX-1 is constantly expressed and COX-2 is expressed in response to inflammatory signals (Loftin et al., 2002). Some NSAIDs inhibit COX non-selectively, while others inhibit COX-2 selectively (Warner et al., 1999). While the strong inhibition of COX-1 causes gastrointestinal dysfunction, selective COX-2 inhibitors keep the stomach protected by targeting only COX-2. The World Health Organization (WHO) recommends the administration of NSAIDs for cancer pain (World Health Organization, 1996). Hence, NSAIDs and anticancer drugs are often combined in clinical use.

Together with fellow researchers, we have reported that the co-administration of NSAIDs is a risk factor for CIN (Kidera et al., 2014; Sato et al., 2016). However, these results were established based on retrospective clinical studies and secondary assessments. Moreover, the specific NSAIDs that affect CIN and the precise mechanism underlying the possible interaction remain unclear. Although several reports have assessed the relationship between CDDP and some NSAIDs *in vitro* and *in vivo* (Fernández-Martínez et al., 2016; Honma et al., 2013), there is no report comparing multiple NSAIDs under the same condition. In this study, we aimed to assess the effect of NSAIDs on CDDP-induced cytotoxicity all at once *in vitro* and to reproduce these effects *in vivo* by using two NSAIDs which significantly attenuated or

enhanced cell injury induced *in vitro* by CDDP, to determine the appropriate co-administration in patients administered CDDP.

2. Materials and Methods

2.1. Chemicals

CDDP was purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan). Celecoxib was purchased from the Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Flurbiprofen was purchased from Sigma Aldrich (St. Louis, MO, USA). All other chemicals and reagents were commercially available and of the highest purity possible. NSAIDs were chosen for the following reasons: (1) having a dosage form for oral administration; (2) they were not a prodrug; (3) easily available.

2.2. Cell culture

The epithelioid clone of normal rat kidney cells (NRK-52E cells, JCRB Cell Bank, Osaka, Japan) was cultured in DMEM (Sigma Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Sigma Aldrich, St. Louis, MO, USA) in a humidified atmosphere of 5% CO₂ at 37°C. NRK-52E cells with a passage number of 25–40 were used in the experiment.

2.3. Animals and experimental design

Male Wistar rats (7 weeks old) were procured from JLA (Tokyo, Japan). All rats were

housed in an animal maintenance facility, in a room with controlled temperature (23°C) and moisture (60 ± 10%) conditions and a 12 h light–dark cycle. All rats were allowed free access to demineralized diet pellets and water. All animal experiments were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals of Hokkaido University, and all experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the “Guide for the Care and Use of Laboratory Animals” (approval number: 17-0005).

Rats were treated with CDDP (5 mg/kg, 2 mg/ml in saline, i.p., day 1) or saline, and celecoxib (30 mg/kg/day, 30 mg/ml in methyl cellulose, p.o., day 1–4) or flurbiprofen (10 mg/kg/day, 10 mg/ml in methyl cellulose, p.o., day 1–4), or methyl cellulose. Rats were divided into six groups: (1) Control group, saline and methyl cellulose; (2) CDDP group, CDDP and methyl cellulose; (3) CDDP + celecoxib group; (4) CDDP + flurbiprofen group; (5) celecoxib group; (6) flurbiprofen group. On day 5, blood samples (200–300 µl) were collected from the tail vein. After blood collection, the rats were anesthetized with sevoflurane, euthanized, and the kidneys were immediately excised. The kidney tissues were washed in saline and stored at –80°C until further analysis.

2.4. Measurement of mRNA expression

Total RNA was extracted using an ISOGEN II kit (Nippon Gene, Tokyo, Japan), according to the manufacturer’s protocol. The cortex from the kidney was then sliced and

total RNA was extracted. Total RNA was used to prepare complementary DNA by reverse transcription using ReverTra Ace (Toyobo, Osaka, Japan). The level of mRNA was measured by RT-PCR or qPCR. Supplemental Table 1 shows the primer sequences used for the PCR amplification.

RT-PCR was performed using a KAPATaq Extra kit (NIPPON Genetics, Tokyo, Japan) and T100™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The RT-PCR thermocycling protocol was: 30 cycles of denaturation at 95°C for 30 s; annealing at 56.5°C–63.5°C for 30 s; and extension at 72°C for 30 s. Products were size-fractionated on 1.2 % agarose gel and stained using ethidium bromide. The band was detected by using LAS-1000 (GE Healthcare UK Ltd., Buckinghamshire, UK) and band intensities were analyzed by using ImageJ analysis software (NIH, Bethesda, MD, USA). qPCR was performed by using a KAPA SYBR Fast qPCR kit (Kapa Biosystems, Wilmington, MA, USA) and Mx3000p (Agilent Technologies, Santa Clara, CA, USA). The qPCR thermocycling protocol was: 40 cycles of denaturation at 95°C for 5 s; annealing at 58°C–60°C for 20 s; and extension at 72°C for 30 s. Standard curves were constructed for each target and housekeeping gene. The software calculated the relative amount of the target gene and the housekeeping gene based on the threshold cycles.

2.5. Cell viability assay

The cells were separately seeded in 96-well plates at a density of 5,000 cells/well in

the culture medium. After 24 h, the cells were treated with CDDP and/or some reagents (containing 0.1% dimethyl sulfoxide (DMSO)) for 48 h, and cell viability was measured by the MTT assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in accordance with the manufacturer's instructions. The absorbance of the resulting reaction solution was measured at 540 nm for samples and at 690 nm for the reference wavelength. The concentration of 50% inhibition for cell viability (IC_{50}) was calculated using SigmaPlot 14 (HULINKS Inc., Tokyo, Japan).

2.6. Superoxide scavenging assay

The superoxide scavenging ability was measured using 2-methyl-6-p-methoxyphenylethynyl-imidazopyrazinone (MPEC) (ATTO, Tokyo, Japan) according to the manufacturer's protocol. All reagents were dissolved in DMSO. The concentrations of NSAIDs are presented in Table 1. Each reaction solution was placed in a 384-well white plate, and the chemiluminescence was measured.

2.7. Measurement of serum creatinine level

The rat blood samples were centrifuged at $1,200 \times g$ for 20 min at 4°C. The serum creatinine level in the supernatant was measured by using LabAssayTM Creatinine (FUJIFILM Wako Pure Chemical Corp, Osaka, Japan) in accordance with the manufacturer's protocol.

2.8. Histological examination

Kidney samples were fixed in 10% buffered formalin. The fixed tissue was

embedded in paraffin and sectioned. The prepared slides were stained with hematoxylin-eosin and observed by using a BZ-9000 (KEYENCE, Osaka, Japan).

2.9. Statistical analysis

Statistical analyses of data were performed using the unpaired Student's t-test, or one-way ANOVA followed by Tukey's post-hoc test, or one-way ANOVA followed by Dunnett's test or Pearson's correlation coefficient. Data were analyzed using SigmaPlot 14, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. The characteristics of NRK-52E cells treated with CDDP

We compared renal transporter mRNA expression between NRK-52E cells and the rat kidney cortex. Since most of renal transporters, including the organic cation transporter 2 (Oct2), copper transporter (Ctr1) and multidrug and toxin extrusion 1 (Mate1) mainly transporting CDDP (Yokoo et al., 2009), were expressed in the NRK-52E cells, we thought that this cell line reflected the rat kidney function (Fig. 1A). Following exposure of CDDP to NRK-52E cells for 48 h, the IC_{50} was calculated as $7.4 \pm 0.2 \mu M$ (Fig. 1B). Moreover, the mRNA levels of Clusterin, a kidney injury marker, were significantly increased following treatment with $10 \mu M$ CDDP (Fig. 1C).

3.2. The effects of 17 NSAIDs on CDDP-induced cytotoxicity in screening

Cell viability following exposure to NSAIDs for 48 h is shown in Table 1. IC₅₀ values could not be calculated for most NSAIDs, except celecoxib. Next, the ratios of IC₅₀ of co-addition of CDDP and NSAIDs to IC₅₀ of CDDP alone in the MTT assay screening are shown in Fig. 2A, and the concentration of NSAIDs was established as that which did not injure the NRK-52E cells within a range of 95–120% cell viability. Hence, among the 17 NSAIDs screened, it was clearly demonstrated that celecoxib significantly attenuated and flurbiprofen markedly enhanced CDDP-induced cytotoxicity. Moreover, the co-addition of celecoxib significantly decreased and flurbiprofen co-addition increased the Clusterin mRNA level compared to CDDP alone (Fig. 2B and Fig. 2C).

3.3. Celecoxib attenuated CDDP-induced cytotoxicity via antioxidant effects.

Rashtchizadeh et al. reported that CIN is prevented by antioxidant effects (Rashtchizadeh et al., 2019). Hence, we focused on antioxidant markers. Celecoxib co-addition significantly increased the mRNA levels of the antioxidant markers including heme oxygenase 1 (Ho-1, Fig. 3A), superoxide dismutase 1 (Sod1, Fig. 3B) and NF-E2-related factor 2 (Nrf2, Fig. 3C). Moreover, we observed correlations between the IC₅₀ of cell viability and the antioxidant markers (Table 2). To elucidate the mechanism of celecoxib mediated oxidative stress reduction, we examined the superoxide scavenging ability using MPEC (Kobayashi and Kanai, 2013). However, NSAIDs failed to demonstrate superoxide scavenging ability themselves (Supplemental Fig. 1). Therefore, celecoxib

attenuated CDDP-induced cytotoxicity by enhancing the resistance to oxidative stress in cells, and not by directly reducing oxidative stress through superoxide scavenging.

3.4. Autophagy was related to celecoxib attenuation of CDDP-induced cytotoxicity.

It has been suggested that attenuating CIN is related not only to antioxidant effects but also autophagy (Kaushal and Shah, 2016). Thus, we also evaluated autophagy markers. The changes in mRNA levels of autophagy markers, autophagy related gene (Atg) 5, Atg7, Lc 3, and Beclin 1, are shown in Fig. 4A. Celecoxib co-addition increased Atg5, Atg7, and Lc 3 mRNA levels in comparison to CDDP alone. Additionally, on evaluating the changes in Atg5 mRNA level, in each of the 17 NSAIDs co-additions, we observed that celecoxib significantly increased the Atg5 mRNA level (Fig. 4B). Moreover, a correlation was observed between Atg5 mRNA level and cell viability like antioxidant effects (Table 2). Hence, it was suggested that autophagy, as well as antioxidant effects, could be responsible for the attenuation of CDDP-induced cytotoxicity mediated by celecoxib.

3.5. The effects of celecoxib on CIN in vivo

The changes in the body weight of rats administered CDDP, with and without celecoxib and flurbiprofen, are shown in Table 3. In our study, CDDP-treated rats demonstrated a decreased body weight. However, celecoxib co-administration increased the body weight to the same levels as the Control group, with the CDDP + flurbiprofen group weighing less than CDDP group. The changes in the kidney weight indicated similar

tendencies as the body weight changes. However, this was attributed to the decreasing body weight since the kidney weight per body weight was not altered significantly. Kidney injury molecule 1 (Kim-1), kidney injury marker, was increased by CDDP. Notably, celecoxib co-administration decreased and flurbiprofen co-application increased Kim-1 mRNA levels in comparison to CDDP group (Fig. 5A and Fig. 5B). Accordingly, celecoxib attenuated and flurbiprofen enhanced CIN *in vivo*, as well as *in vitro*. Also, the changes in mRNA levels of Ho-1, Sod1, Nrf2, and Atg5 by CDDP are shown in Supplemental Table 2.

4. Discussion

We have examined the effects of 17 NSAIDs on CDDP-induced cytotoxicity *in vitro* and revealed that several NSAIDs demonstrate the potential to attenuate or enhance cell injury by CDDP. Notably, we observed that celecoxib significantly attenuated and flurbiprofen markedly enhanced CIN. Initially, we predicted that the attenuating and enhancing effects of NSAIDs on CIN were due to the differential inhibition selectivity between COX-1 and COX-2, since celecoxib is a COX-2 selective inhibitor and flurbiprofen is highly selective for COX-1. However, on assessing concomitant treatment with rofecoxib, with higher selectivity for COX-2 than celecoxib, and ketorolac tromethamine, highly selective for COX-1 than flurbiprofen, (Warner et al., 1999), they did not affect the results. Moreover, etodolac and meloxicam, which like celecoxib are highly selective for COX-2, did not reduce

CDDP-induced cytotoxicity. Accordingly, the influence of NSAIDs is considered to be independent of selectivity for COX inhibition. This observation cannot be explained without the simultaneous evaluation of this study, as there is no previous report evaluating the influence of NSAIDs on CDDP-induced cytotoxicity under similar conditions. Moreover, previous reports utilized recombinant COX-1 and COX-2 to assess the inhibition of COX activity by NSAIDs (Abdelall et al., 2016), and failed to evaluate the inhibition of COX activity and calculate IC_{50} by NSAIDs in cellular COX-1 and COX-2. To further assess is needed.

Antioxidant effects and autophagy are important approaches for attenuating CIN (Kaushal and Shah, 2016; Rashtchizadeh et al., 2019). Cell dysfunction is induced by CDDP via reactive oxygen species (ROS) production (Kawai et al., 2006). Therefore, eliminating ROS and increasing resistance to oxidative stress by antioxidant substrates are valuable to prevent CIN. In fact, the interaction between CDDP and food substances possessing antioxidant effects has been investigated. For example, many researchers have assessed the effect of curcumin in preventing CIN (Sahin et al., 2014; Trujillo et al., 2016). It has also been reported that CIN is caused by mitochondrial dysfunction (Park et al., 2002), and autophagy is known to remove the injured organelle. Decrease in the autophagy functions could induce CIN (Takahashi et al., 2012). Therefore, it remains important to focus on the antioxidant and autophagy functions. We evaluated whether N-acetylcysteine (NAC), which has a strong

antioxidant effect (Duval et al., 2019), attenuates CDDP-induced cytotoxicity. Co-addition of NAC and CDDP showed higher IC₅₀ than CDDP alone, similar to celecoxib. Hence, we confirmed that the antioxidant effect is important to protect against cell dysfunction induced by CDDP (Supplemental Fig. 2).

Celecoxib upregulated mRNA levels of antioxidant and autophagy markers in comparison to CDDP alone. Flurbiprofen, however, did not downregulate these markers. Therefore, it is speculated that CIN attenuation by celecoxib could be related to these markers. The detailed mechanisms of CIN enhancement by flurbiprofen remain unclear. We have previously reported that decreasing renal platinum accumulation attenuates CIN by regulating the expression of renal transporters (Saito et al., 2017b). CDDP is mainly taken up by OCT2 and CTR1, and effluxed by MATE1 (Yokoo et al., 2009). We evaluated the platinum accumulation in NRK-52E cells, and found that it was not changed by the co-addition of celecoxib or flurbiprofen (data not shown). Therefore, these NSAIDs may not regulate renal transporters, or the contribution of passive diffusion in the transport of CDDP may be significant. In addition, CDDP is conjugated with glutathione followed by cysteine, and CDDP-cysteine conjugation is suggested to be more toxic than CDDP (Townsend et al., 2009). Therefore, NSAIDs may affect the metabolism of CDDP. xCT, a cystine/glutamate transporter, is an important for the production of glutathione in cells (Thomas et al., 2015). Although we evaluated xCT mRNA expression, xCT mRNA was not detected in the rat kidney cortex.

Hence, the involvement of the mechanisms of CDDP metabolism through xCT may be low. However, the combination CDDP and flurbiprofen needs careful consideration. Flurbiprofen axetil, flurbiprofen's prodrug, is used in cancer pain in clinical settings (Wu et al., 2014). Thus, patients administered CDDP may switch from flurbiprofen axetil to celecoxib, or alternative medications such as acetaminophen and tramadol, and a detailed clinical evaluation needs to be undertaken.

We assessed Sod1 and Atg5 mRNA levels to identify the *in vivo* mechanisms of CIN attenuation by celecoxib. However, celecoxib co-administration did not increase these mRNA levels (data not shown). Since the celecoxib mechanism in CIN might differ *in vitro* and *in vivo*, we evaluated apoptosis to confirm this hypothesis. On assessing Bcl-2-associated X protein/B-cell lymphoma-2 (Bax/Bcl-2) mRNA levels, as apoptosis markers, we observed that these variations differed between the *in vitro* and *in vivo* evaluation in the CDDP + celecoxib group (Supplemental Fig. 3). Therefore, the mechanism of actions were likely to be different. We considered that the difference between the *in vitro* and *in vivo* mechanisms might be associated with the NSAID activated gene 1 (NAG-1). NAG-1 is induced by NSAIDs, regardless of COX activity, and is related to apoptosis (Zhang et al., 2014). Furthermore, it has been suggested that celecoxib increases NAG-1 (Vaish et al., 2013). Therefore, regulating Bax/Bcl-2 as an apoptosis marker might not be consistent owing to the differential effect of CDDP and celecoxib on NAG-1 *in vitro* and *in vivo*. Furthermore, NSAIDs affect renal blood

flow (Herboczynska-Cedro and Vane, 1973). This change in renal blood flow may affect the pharmacokinetics and the effect of NAG-1 *in vitro* and *in vivo*. Also, the selected time point may be unsuitable for *in vivo* evaluation. CDDP accumulates in kidney for more than a week (Saito et al., 2017c), whereas we evaluated the mRNA levels of several markers on day 5. Therefore, the selected evaluation time point might not be appropriate, or some markers might be regulated earlier than day 5. We evaluated the effects of celecoxib or flurbiprofen only on Kim-1 mRNA expression. There was no change in the expression of Kim-1 compared with Control (Supplemental Fig. 4). Therefore, the effects of co-administration of CDDP and celecoxib or flurbiprofen may not be additive, but instead synergistic. Further investigation into the underlying mechanisms needs to be conducted.

We evaluated serum creatinine level to confirm CIN by another renal injury marker. Serum creatinine was increased by CDDP and celecoxib co-administration group was lower serum creatinine level than CDDP group like Kim-1 mRNA expression (Supplemental Fig. 5A). However, flurbiprofen co-administration did not increase in comparison to CDDP alone. This was thought to be due to differences in the sensitivity of kidney injury markers. As Kim-1 was more sensitivity than serum creatinine (Vaidya et al., 2006), serum creatinine may not reflect the effect of flurbiprofen co-administration. Moreover, we performed a pathological evaluation of the rat kidney by using hematoxylin-eosin staining. As shown in Supplemental Fig. 5B, there were no significant differences between all groups although it did

have strict quantification like TUNEL staining. It has been reported that low-dose CDDP does not affect renal tissue and only increases renal injury markers (Yokoo et al., 2007). In addition, increasing Kim-1 is earlier than pathological change (Han et al., 2002). As the dosage of CDDP used in this study was close to a low-dose and 2–3 times higher than the clinical dose, histological changes in the kidney may not occur and our results were consistent with a previous report. In addition, we assessed the Timp-1 and Clusterin mRNA expression, which are early kidney injury marker such as Kim-1 (Yao et al., 2007), and these markers were increased by CDDP and suppressed by the co-administration of celecoxib (Supplemental Fig. 5C). Therefore, the effect of celecoxib may increase the protective action rather than enhancing the tissue repair. However, this study was short-term and used a single injection of CDDP; if it was long-term and used multiple injections, the effect of celecoxib on the repair mechanisms may be recognized. Also, measuring urinary biomarkers may reveal new strategies.

Flurbiprofen co-administration lost more weight than CDDP alone. Low weight due to CDDP was thought to be caused by eating disorder. It has also been reported that CDDP becomes more toxic by decreasing the concentration of magnesium in the body (Solanki et al., 2014). For the above reasons, we considered that the increase in weight loss with the co-administration of flurbiprofen was due to strong eating disorder or increased toxicity of CDDP via enhancement of renal injury and accompanying hypomagnesemia. Also, we used

only male rats in this study because it has reported that OCT2 is more expressed in male and CDDP is more distributed in the kidney (Yonezawa et al., 2005). Therefore, CIN is unlikely to occur in female rats, but it may be caused similar to male rats by co-administration of flurbiprofen if CDDP and flurbiprofen interact without transporters.

The concentrations of CDDP and NSAIDs used in the *in vitro* evaluation were almost similar, or several-fold higher, compared to the blood concentration in clinical settings. As these drugs are somewhat concentrated in the kidney, the screening results of the 17 NSAIDs are highly relevant in clinical practice. However, it will be necessary to evaluate the actual concentration of NSAIDs in the kidney and judge whether the *in vivo* dose matched the *in vitro* concentration.

5. Conclusion

We observed that specific NSAIDs could either protect or worsen CDDP-induced cytotoxicity. Among the 17 NSAIDs screened, celecoxib significantly alleviated cell injury by CDDP *in vitro* via antioxidant effects and autophagy. Additionally, celecoxib significantly attenuated CIN. Further clinical studies are crucial to evaluate the nephroprotective efficacy of celecoxib in CIN.

Declaration of interest

None declared.

Acknowledgements

This work was supported by the Japan Society for the Promotion of Science (JSPS; KAKENHI Grant Number JP19J11051, 19K16437 and 16H00487). We would like to thank Editage (www.editage.jp) for English language editing.

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Figure Legends

Fig. 1: Transporter expression on NRK-52E cells and the characteristics of NRK-52E

cells treated with CDDP. (A) Upper picture is the apical side and lower picture is the basolateral side of renal transporters by RT-PCR. L means left sample used rat kidney cortex's cDNA and R means right sample used NRK-52E cells' cDNA. (B) Cells were incubated with CDDP (0–30 μ M). (C) Clusterin mRNA was normalized to Actin. Cells in the CDDP group were incubated with CDDP (10 μ M) for 48 h. The expression level of the Control group was arbitrarily set at 1.0. $^{***}P < 0.01$ compared with the Control group; an unpaired Student's t-test. Data are presented as means with S.E.M., for three independent experiments.

Fig. 2: Effect of NSAIDs on CDDP-induced cytotoxicity and Clusterin mRNA levels in

CDDP-treated NRK-52E cells. (A) The values were the ratio of IC₅₀ of co-addition of CDDP and NSAIDs to IC₅₀ of CDDP alone. The concentration of NSAIDs co-addition with CDDP was defined as the concentration at which no effect on cell viability was observed when treated with NSAIDs only (described Table 1). Clusterin mRNA levels were altered following incubation with CDDP (10 µM) and (B) celecoxib (50 µM) or (C) flurbiprofen (200 µM) for 48 h. Clusterin mRNA was normalized to Actin. The expression level of the Control group was arbitrarily set at 1.0. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 compared with the Control group, ††*P* < 0.01 and †††*P* < 0.001 compared with the CDDP group, ‡‡*P* < 0.01 and ‡‡‡*P* < 0.001 compared with the celecoxib or flurbiprofen group; Tukey's post-hoc test. Data are presented as means with S.E.M. for three independent experiments.

Fig. 3: Effect of NSAIDs on mRNA levels of antioxidant markers in CDDP-treated

NRK-52E cells. (A) Ho-1, (B) Sod1 and (C) Nrf2 mRNA were normalized to Actin. Cells were incubated with CDDP (10 µM) and each NSAID (concentration is shown in Table 1) for 48 h. The expression level of the CDDP group was arbitrarily set at 1.0. †*P* < 0.05, ††*P* < 0.01 and †††*P* < 0.001 compared with the CDDP group; Dunnett's test. Data are presented as means with S.E.M. for three independent experiments.

Fig. 4: Effect of NSAIDs on mRNA levels of autophagy markers in CDDP-treated

NRK-52E cells. (A) Atg5, Atg7, Lc 3 and Beclin 1 mRNA levels were altered by incubation with CDDP (10 µM), celecoxib (50 µM) and flurbiprofen (200 µM) for 48 h. The mRNAs

were normalized to Actin. The expression level of the Control group was arbitrarily set at 1.0.

$^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the Control group, $^{\dagger}P < 0.05$ and $^{\dagger\dagger\dagger}P < 0.001$

compared with the CDDP group, $^{\ddagger}P < 0.05$, $^{\ddagger\ddagger}P < 0.01$ and $^{\ddagger\ddagger\ddagger}P < 0.001$ compared with the

CDDP + celecoxib group; Tukey's post-hoc test. (B) Atg5 mRNA was normalized to Actin.

Cells were incubated with CDDP (10 μ M) and each NSAID (concentration is shown in Table

1) for 48 h. The expression level of the CDDP group was arbitrarily set at 1.0. $^{\dagger\dagger\dagger}P < 0.001$

compared with the CDDP group; Dunnett's test. Data are presented as means with S.E.M. for

three independent experiments.

Fig. 5: Effect of celecoxib and flurbiprofen on rats treated with CDDP. (A) Kim-1 and

Actin mRNA expression by RT-PCR, and (B) mRNA was quantified by ImageJ analysis

software. Kim-1 was normalized to Actin. The expression level of the Control group was

arbitrarily set at 1.0. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the Control group, $^{\dagger}P < 0.05$

and $^{\dagger\dagger}P < 0.01$ compared with the CDDP group, $^{\ddagger\ddagger\ddagger}P < 0.001$ compared with the CDDP +

celecoxib group; Tukey's post-hoc test. Data are presented as means with S.D., n = 5–7 per

group.

Table 1. MTT assay of NSAIDs alone and setting of concentration for co-addition of CDDP

NSAIDs	IC ₅₀ (μM)	Concentration of co-addition (μM)
Aspirin	> 200	200
Celecoxib	63.4 ± 1.5	50
Diclofenac sodium	> 200	200
Etodolac	> 200	200
Flufenamic acid	> 200	200
Flurbiprofen	> 200	200
Ibuprofen	> 200	200
Indomethacin	> 200	25
Ketorolac tromethamine	> 200	200
Lornoxicam	> 10	10
Mefenamic acid	> 200	200
Meloxicam	> 80	80
Naproxen	> 10	10
Oxaprozin	> 200	200
Piroxicam	> 200	200
Rofecoxib	> 25	25
Zaltoprofen	> 200	200

Cells were incubated with each NSAID. MTT assay was evaluated until each saturation concentration was established. Concentration of the

NSAID co-added with CDDP was defined as no effect on NRK-52E cells within the range of 95–120% cell viability when treated with NSAIDs

alone. Data are presented as means $\pm$ S.E.M., of three independent experiments.

Table 2. Correlations between cell viability and antioxidant or autophagy markers

		R	<i>P</i> value
IC ₅₀ of MTT assay	Ho-1 mRNA level	0.505	0.0385 ^c
	Sod1 mRNA level	0.758	< 0.001 ^a
	Nrf2 mRNA level	0.685	0.00242 ^b
	Atg5 mRNA level	0.898	< 0.001 ^a

IC₅₀ of MTT assay is shown in Fig. 2A, and Ho-1, Sod1, Nrf2 and Atg5 mRNA level are shown in Fig. 3A, Fig. 3B, Fig. 3C and Fig. 4B

respectively. R means correlation coefficient.

^a $P < 0.001$ compared between IC₅₀ of MTT assay and each mRNA level; Pearson correlation coefficient.

^b $P < 0.01$ compared between IC₅₀ of MTT assay and each mRNA level.

^c $P < 0.05$ compared between IC₅₀ of MTT assay and each mRNA level.

Table 3. Variation of body and kidney weight in rats

	Body weight (g)			Kidney weight (g)	Kidney weight/body weight
	Day 1 (baseline)	Day 5	Day 5 – Day 1	Day 5	Day 5
Control	193.9 ± 4.7	219.4 ± 4.5	25.5 ± 1.3	2.08 ± 0.09	0.0095 ± 0.0005
CDDP	191.3 ± 8.8	184.4 ± 8.5	-7.0 ± 4.6 ^a	1.75 ± 0.17 ^b	0.0095 ± 0.0008
CDDP + celecoxib	194.9 ± 5.7	221.0 ± 5.3	26.2 ± 2.2 ^c	1.99 ± 0.09	0.0090 ± 0.0003
CDDP + flurbiprofen	191.8 ± 10.8	162.0 ± 17.6	-29.8 ± 15.5 ^{a, d, e}	1.62 ± 0.21 ^{a, f}	0.0100 ± 0.0008

Body weight at baseline and day 5 was measured. Variation of body weight from baseline was compared between groups. Kidney weight was total right and left kidney. Data are presented as means ± S.D., n = 5–7 per group.

^a $P < 0.001$ compared with Control group; Tukey's post-hoc test.

^b $P < 0.01$ compared with Control group.

^c $P < 0.001$ compared with CDDP group.

^d $P < 0.01$ compared with CDDP group.

^e $P < 0.001$ compared with CDDP + celecoxib group.

^f $P < 0.01$ compared with CDDP + celecoxib group.

Apical Transporter



Mate1

Mdr1a

Mrp2

Mrp4

Sglt2

Pept2

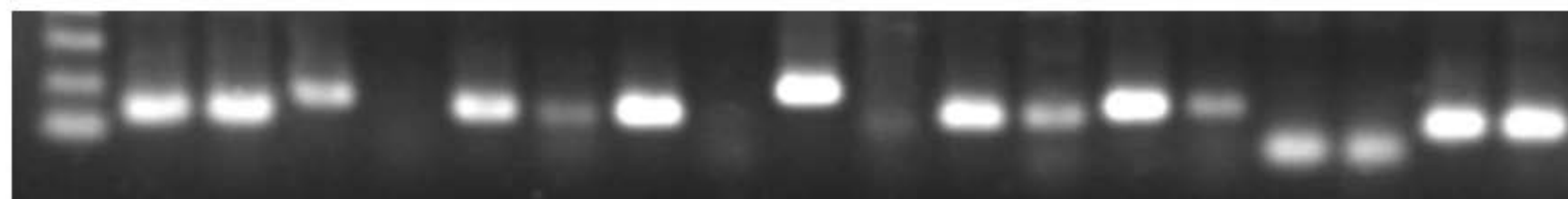
Octn1

Octn2

Urat1

Bcrp

Basolateral Transporter



Glut1

Glut2

Glut9

Oat1

Oat3

Oct1

Oct2

Ctr1

Actin

L R

Fig. 1A

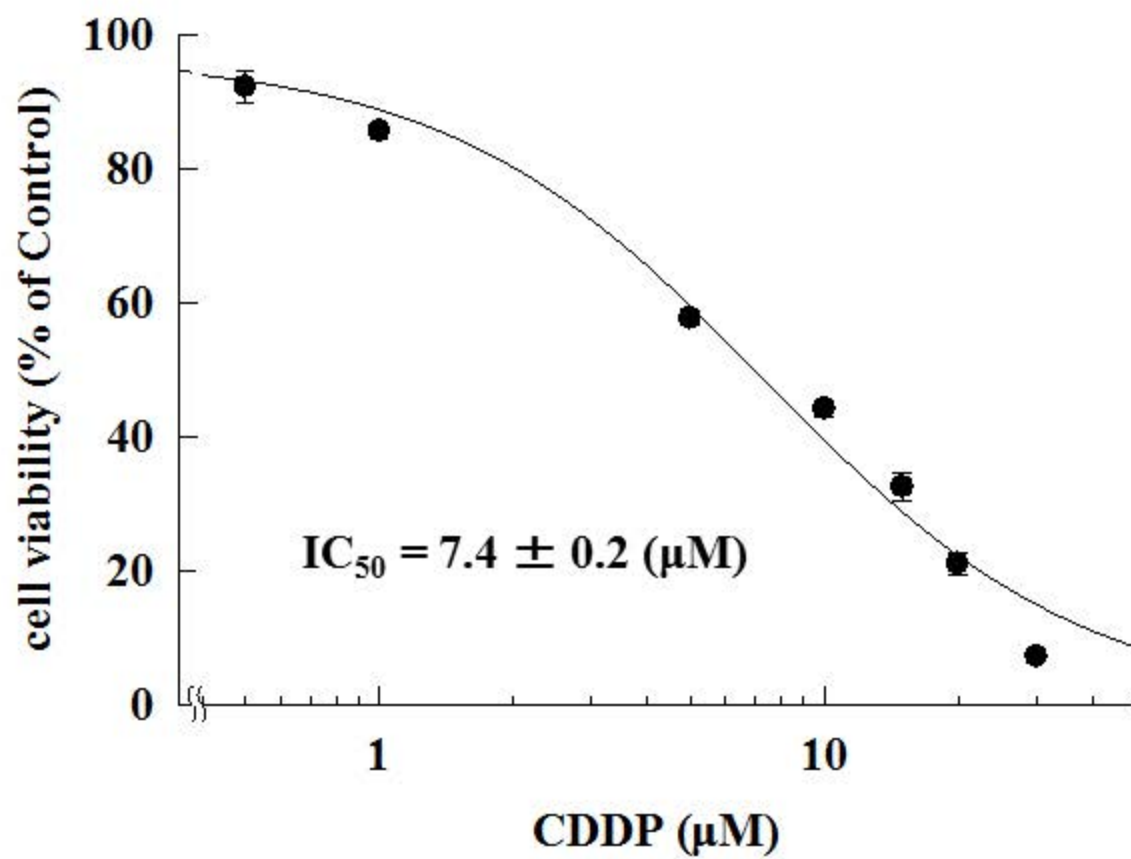


Fig. 1B

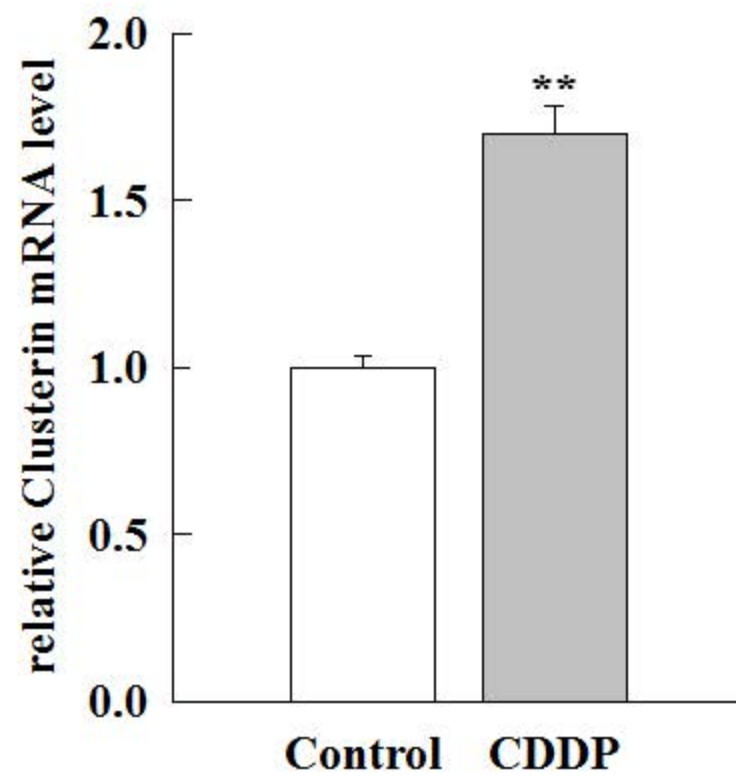


Fig. 1C

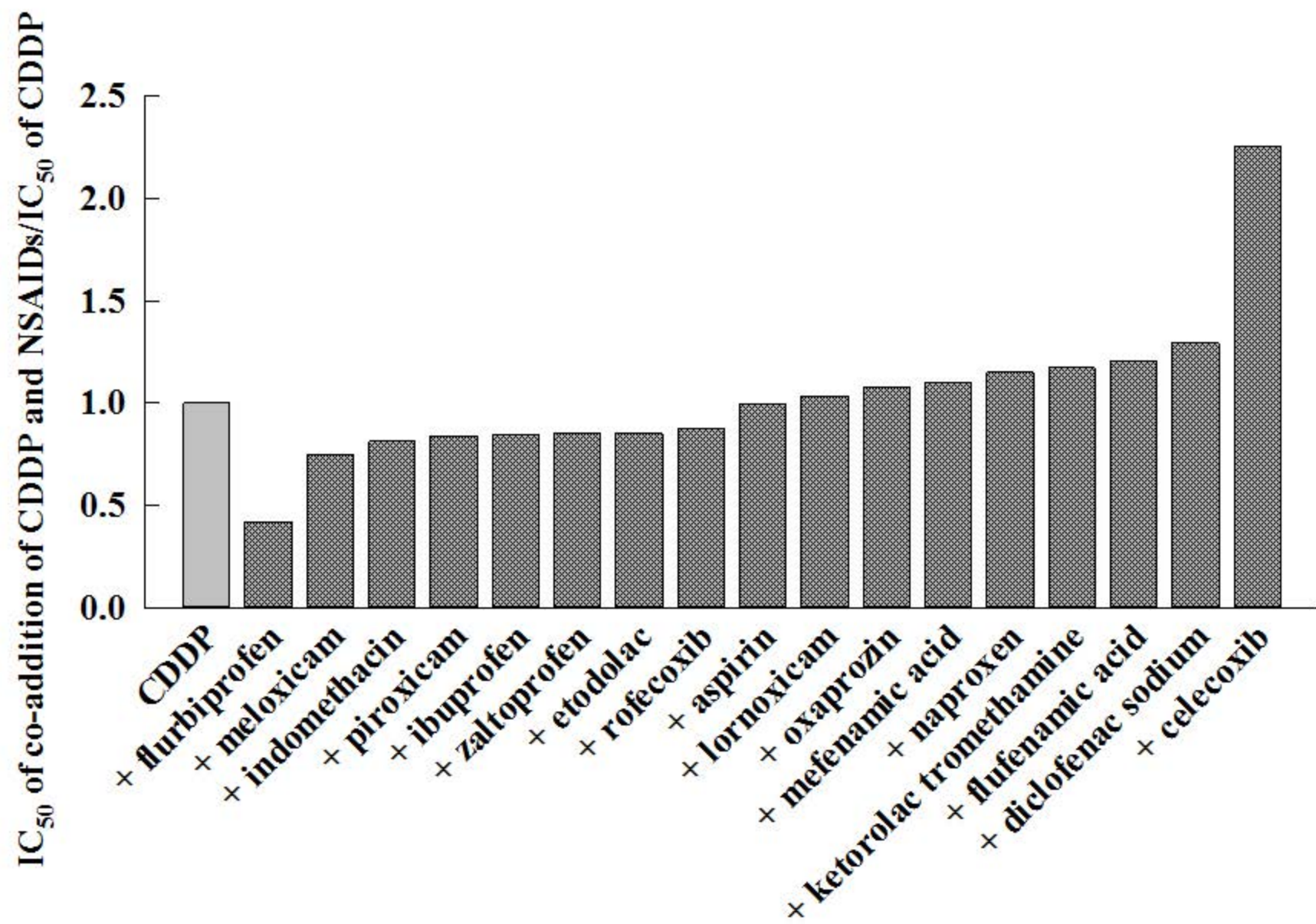


Fig. 2A

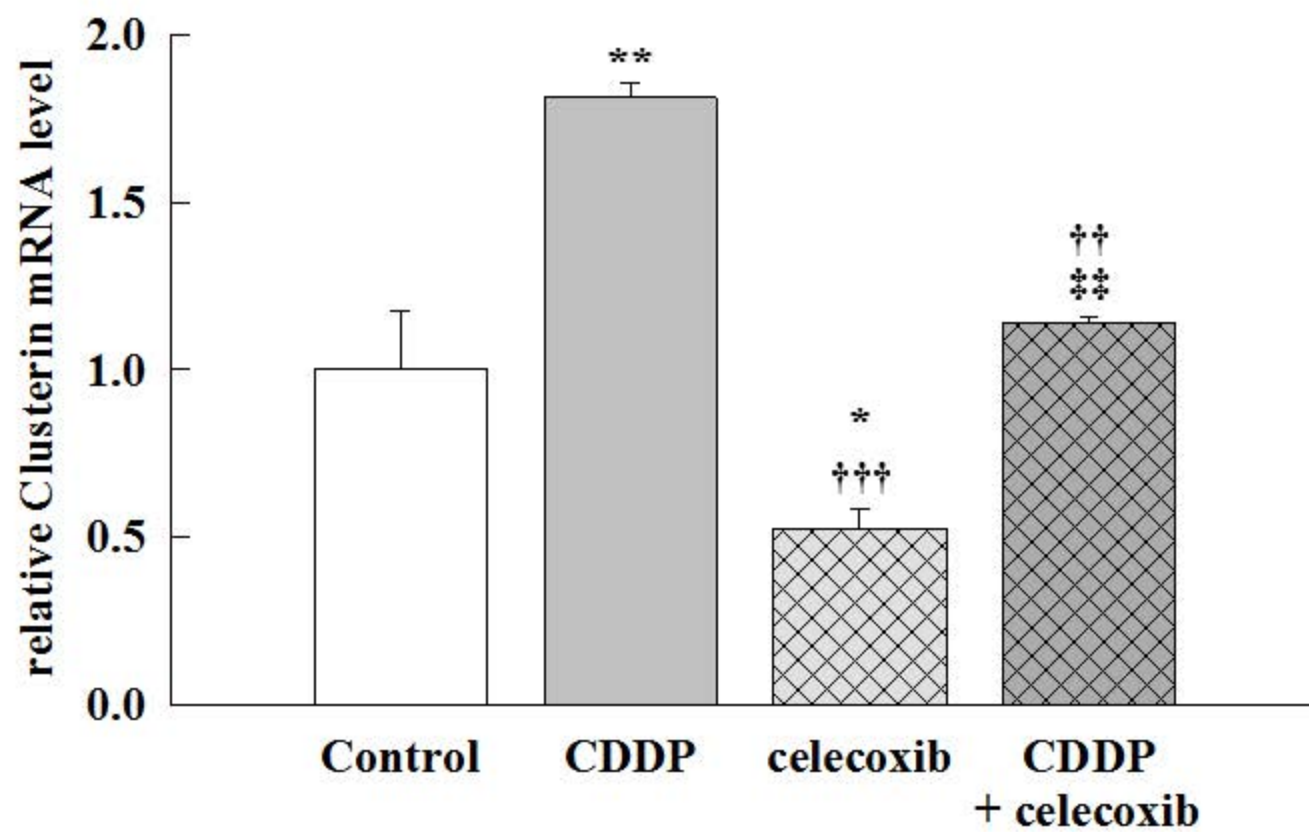


Fig. 2B

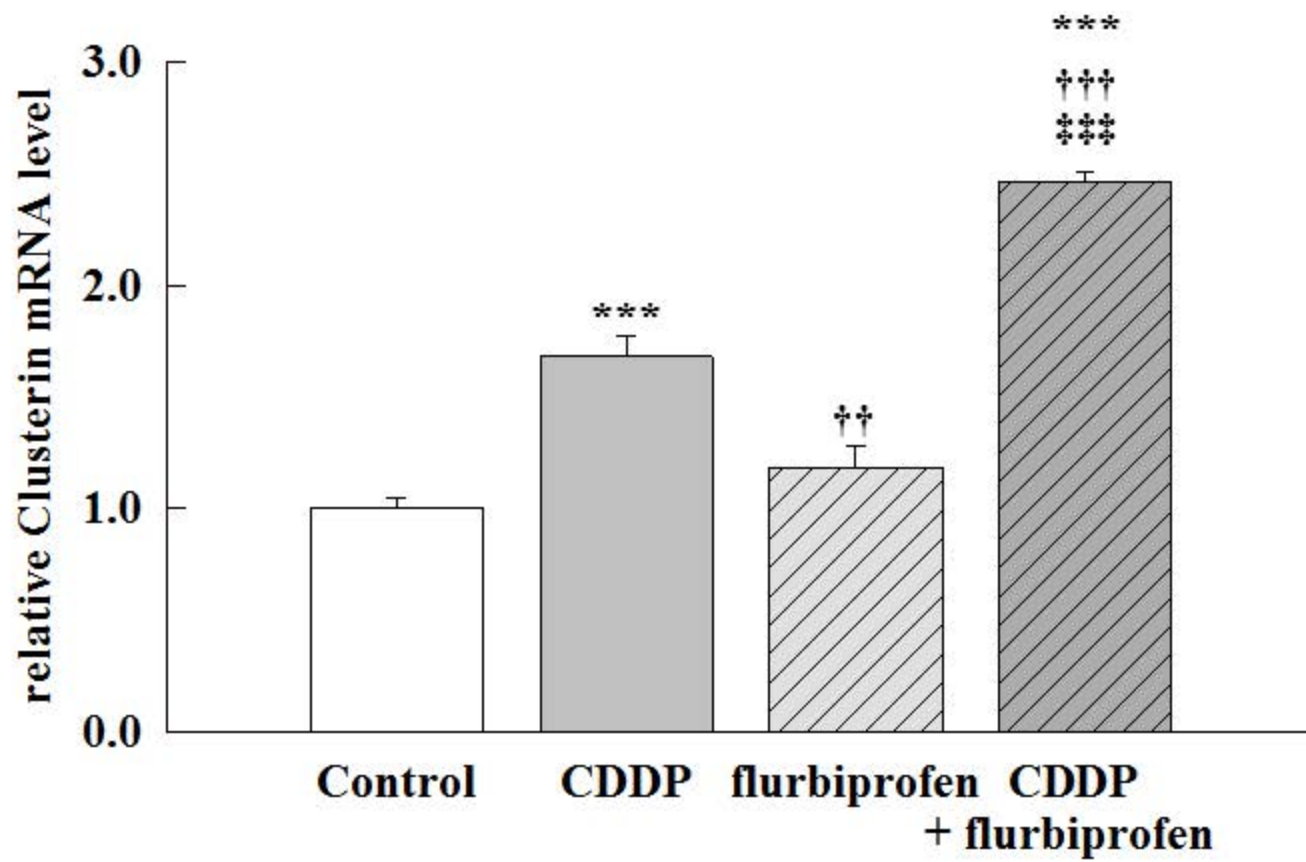


Fig. 2C

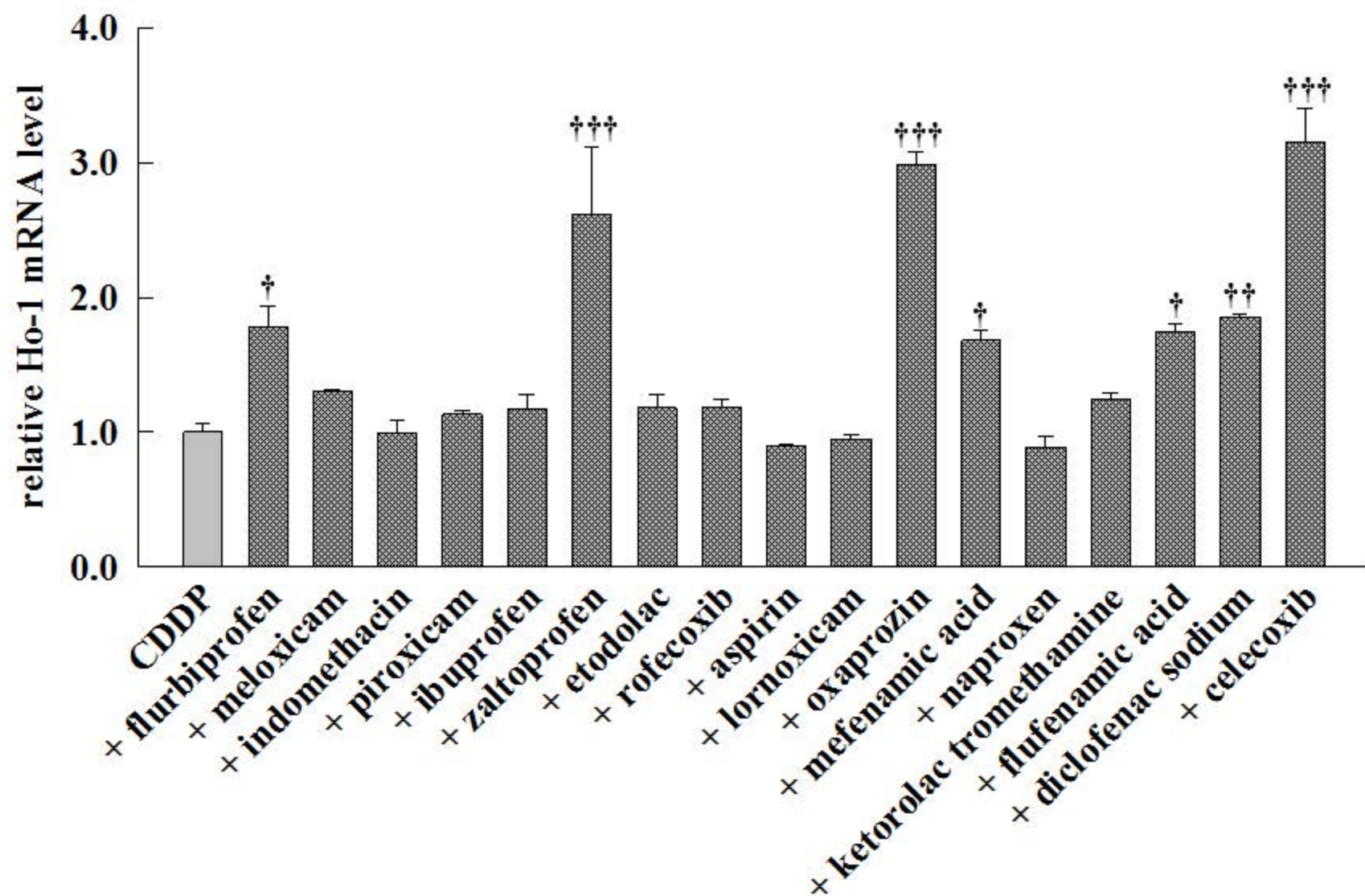


Fig. 3A

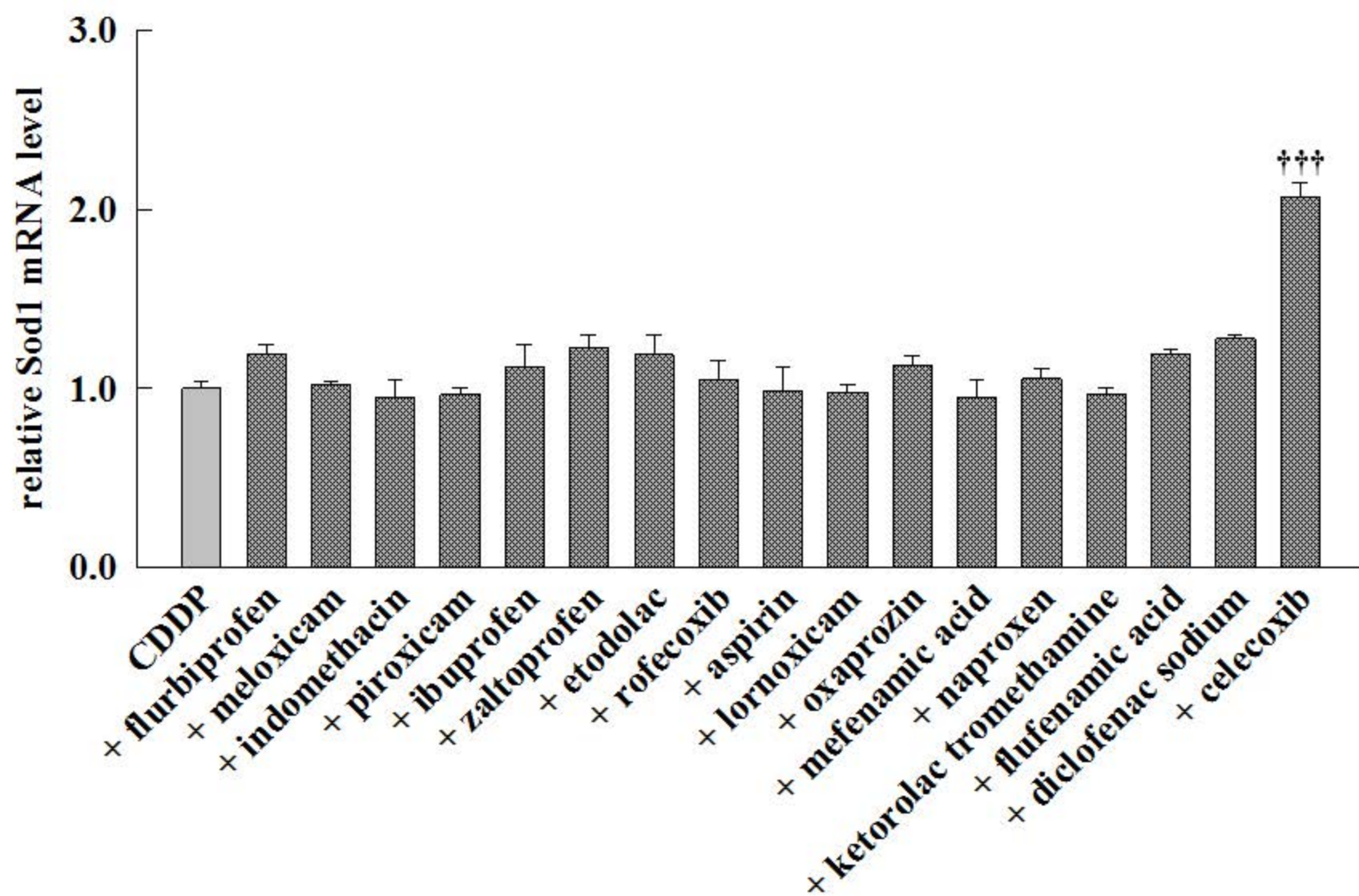


Fig. 3B

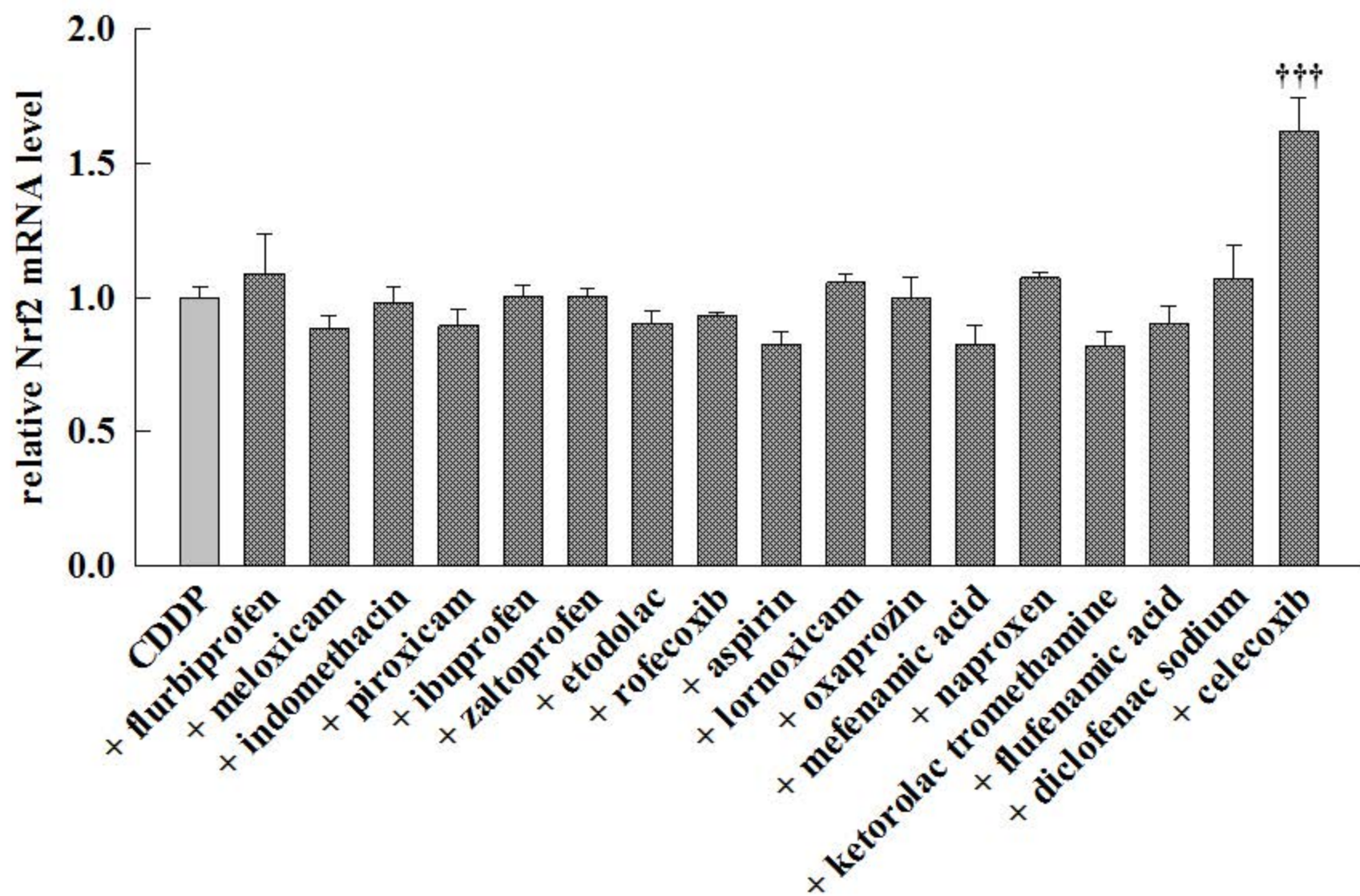


Fig. 3C

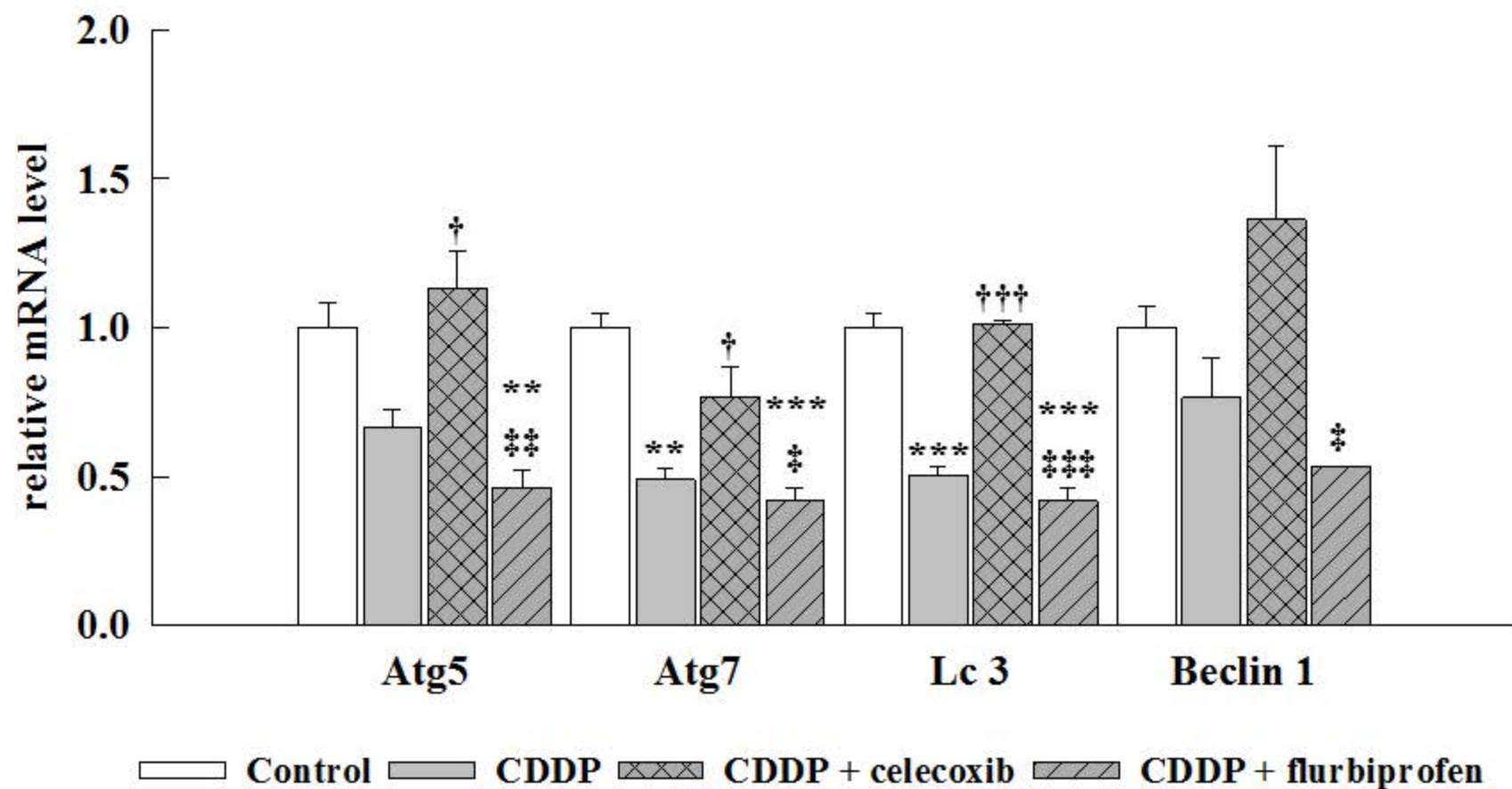


Fig. 4A

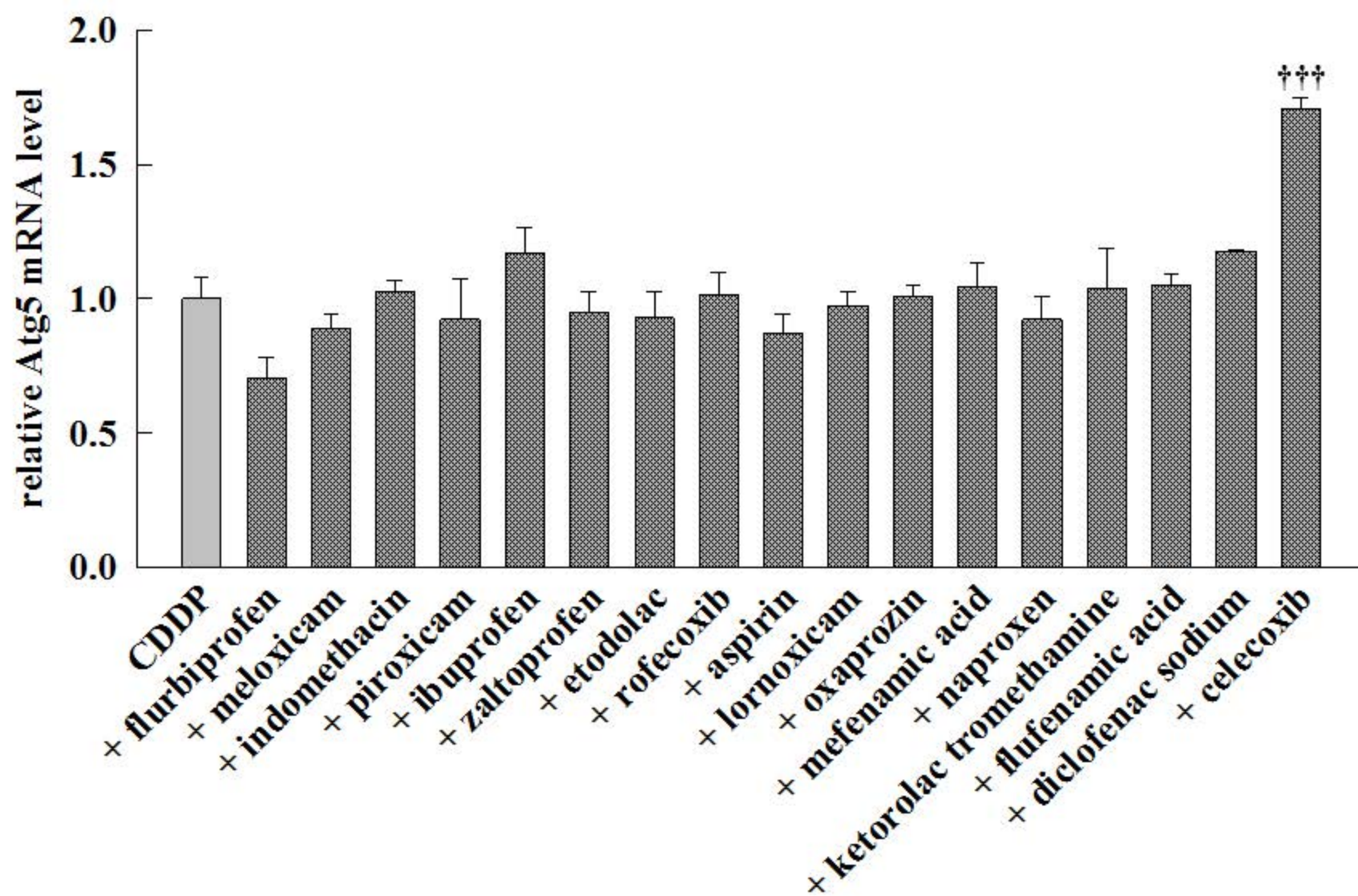


Fig. 4B

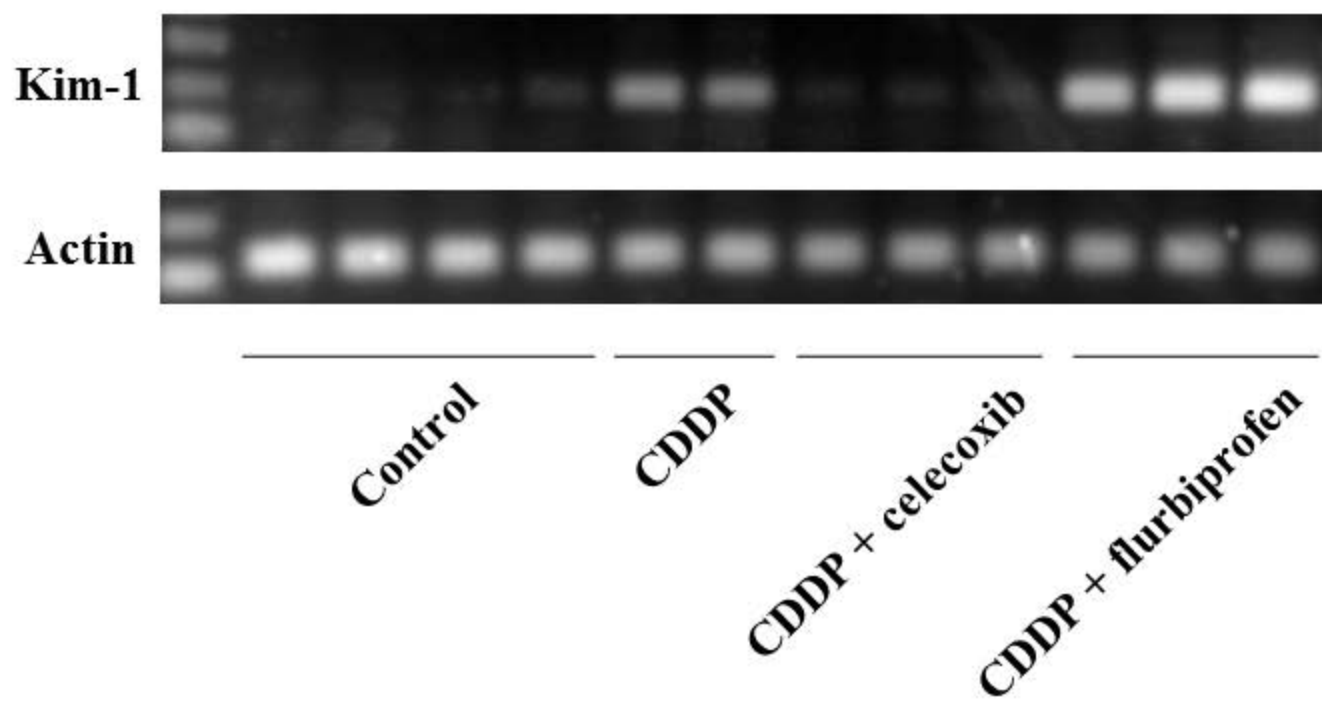


Fig. 5A

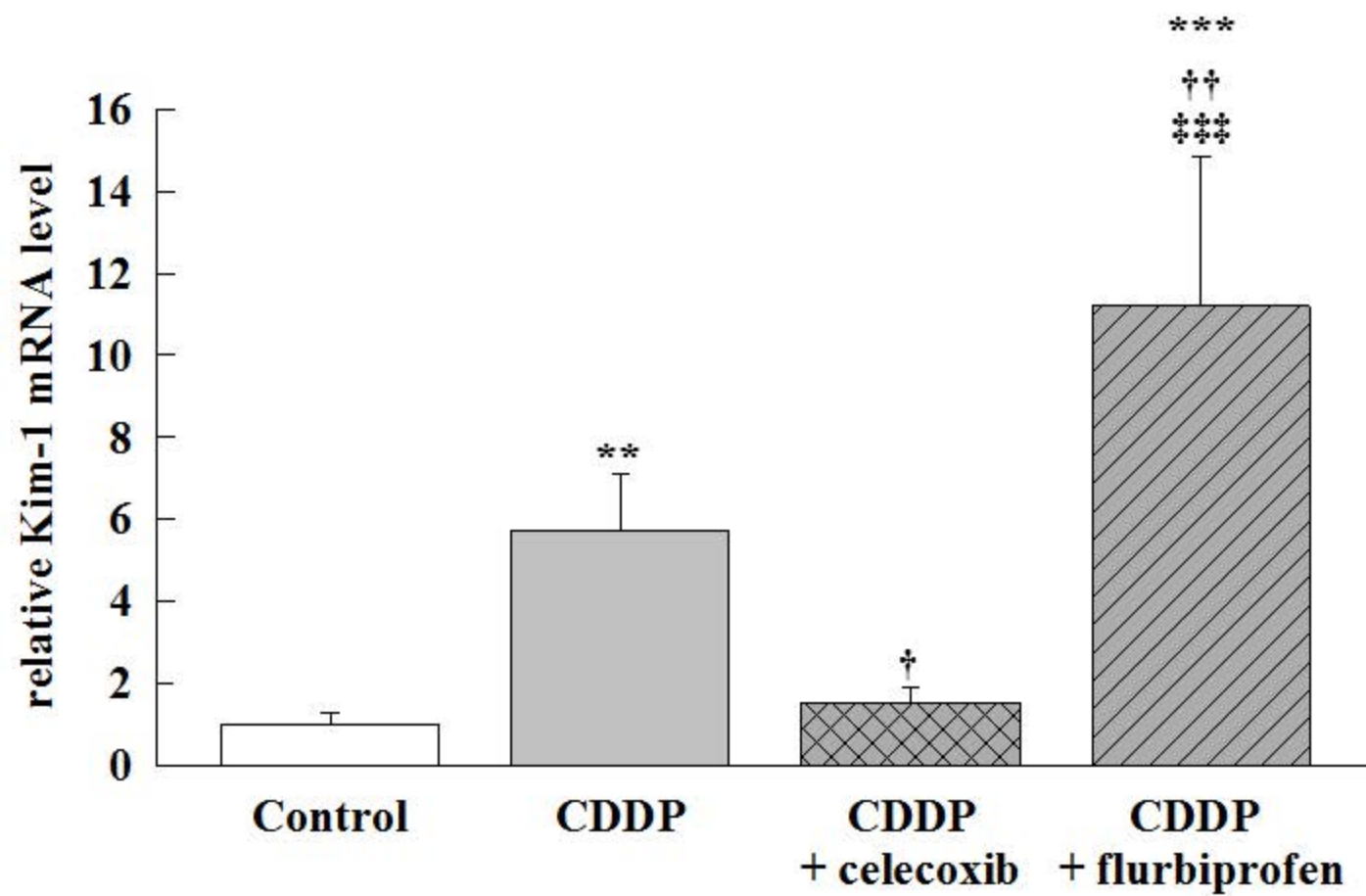


Fig. 5B