Title

Diclofenac potentiates the antitumor effect of cisplatin in a xenograft mouse model transplanted with cisplatin-resistant cells without enhancing cisplatin-induced nephrotoxicity

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Abstract

Cisplatin (CDDP) is a well-known anticancer agent, and CDDP–induced nephrotoxicity (CIN) is one of the most serious adverse effects. Previously, we revealed that while celecoxib reduces CIN, diclofenac does not appear to enhance it. Furthermore, we reported that diclofenac additively enhances the cytotoxic effect of CDDP on CDDP-resistant A549 cells (A549/DDP cells) and their spheroids. In addition, celecoxib reduces the cytotoxic effect of CDDP on A549/DDP cells while demonstrating an anticancer effect; however, it enhanced the effect of CDDP cytotoxicity on spheroids. Therefore, we evaluated the effects of diclofenac or celecoxib on CIN and the antitumor effect of CDDP in a xenograft mouse model transplanted with A549/DDP cells. Although CDDP did not decrease tumor size and tumor weight, these parameters were significantly reduced following co-administration with diclofenac when compared with the control group. Conversely, celecoxib marginally suppressed the antitumor effect of CDDP. Moreover, CDDP increased the mRNA levels of kidney injury molecule 1 (Kim-1), a renal disorder marker, in the kidneys of xenograft mice; treatment with celecoxib and diclofenac did not impact Kim-1 mRNA levels increased by CDDP. In conclusion, diclofenac potentiated the antitumor effect of CDDP without enhancing CIN.

Keywords

Cisplatin; Diclofenac; Celecoxib; Xenograft; Nephrotoxicity; Antitumor effect

1. Introduction

Cisplatin (CDDP) is a highly potent anticancer agent widely used for treating several types of cancers, including lung cancer [1]. However, CDDP–induced nephrotoxicity (CIN) is one of the most serious adverse effects of CDDP, with CIN reportedly occurs in approximately 30% of patients [2]. Non-steroidal anti-inflammatory drugs (NSAIDs) are well-established anti-inflammatory agents that exert their effects via cyclooxygenase inhibition [3].

Previously, we performed a meta-analysis and reported that NSAIDs are a risk factor for developing CIN [4], while our basic research revealed that celecoxib and diclofenac, both NSAIDs, reduce and do not enhance CIN, respectively [5]. In addition, we have demonstrated that diclofenac additively enhances the cytotoxic effect of CDDP on A549/DDP cells, a CDDP-resistant human lung cancer cells established at our laboratory [6], and their spheroids, with cell morphology displaying characteristics of cancer stem cells (CSCs) in spheroid culture [7].

Although celecoxib reduces the cytotoxic effect of CDDP on A549/DDP cells while demonstrating an anticancer effect itself, it enhances the effect of CDDP cytotoxicity on spheroids, which are known to demonstrate an environment closer to *in vivo* conditions than typical two-dimensional cultures [8]. Consequently, co-administration with celecoxib is expected to enhance the antitumor effect of CDDP while reducing CIN. In recent years, the

search for NSAIDs that can enhance the antitumor effect of CDDP by developing a CDDP and NSAID conjugate has gained momentum, and the combination of CDDP and NSAIDs has drawn considerable attention [9,10]. However, these studies only evaluated the antitumor effect and did not consider potential side effects, especially nephrotoxicity. In addition, although NSAIDs are often used in combination with anticancer drugs in clinical practice [11], long-term administration is uncommon as NSAIDs are used for cancer pain and fever, and it is difficult to evaluate the effects of CDDP and NSAIDs in clinical studies. Therefore, in the present study, we evaluated both diclofenac and celecoxib using a xenograft model, which reflects cancer disease and is relatively close to clinical practice. Herein, the effects of diclofenac or celecoxib on the side effects and antitumor effects of CDDP were evaluated *in vivo* to optimize cancer CDDP composed chemotherapy. Therefore, to assess whether diclofenac enhances the antitumor effect of CDDP without exacerbating CIN *in vivo*, we established and examined a xenograft mouse model transplanted with A549/DDP cells.

2. Materials and methods

2.1. Chemicals

CDDP and diclofenac sodium were purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan). Celecoxib was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All other chemicals and reagents were commercially available and were of the

highest possible purity.

2.2. Animals

BALB/cAJcl-*nu/nu* mice (male, 6-week-old) were procured from CLEA Japan (Tokyo, Japan). 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."

2.3. Cell culture

CDDP-resistant human lung adenocarcinoma cells, i.e., A549/DDP cells, were cultured as previously described [6].

2.4 Establishment and treatment of a lung cancer xenograft mouse model

Briefly, A549/DDP cells, at a volume of 2×10^6 cells suspended in 100 μL RPMI-1640 (FUJIFILM Wako Pure Chemical Corp.), were subcutaneously injected into the dorsal surface of BALB/cAJcl-*nu/nu* mice. Mice were then employed in the study after the tumor volume reached 80–120 mm^3 (day 1). Tumor volume was measured using digital calipers, calculated using the formula: volume (mm^3) = A (mm) × B^2 (mm) × 0.5, where A is the longest diameter and B is the shortest diameter. The humane endpoint was set to > 1000 mm^3.

Mice were treated with CDDP (5 mg/kg in saline, intraperitoneal [i.p.]) or saline, and celecoxib (30 mg/kg in methylcellulose, peroral [p.o.]) or diclofenac (10 mg/kg in

methylcellulose, p.o.), or methylcellulose. Based on the previously described protocols with few modifications [5,12], i.p. and p.o. administration were performed on days 1, 8, 15, and 1–4, 8–11, 15–18, with the tumor volume measured once every three days and once weekly, respectively. On day 22, mice were anesthetized with sevoflurane and euthanized, and the kidneys and tumors were excised immediately. The kidney tissues and tumors were washed in saline and stored at −80°C until further analysis.

2.5. Measurement of mRNA expression

mRNA extraction, reverse transcription-polymerase chain reaction (RT-PCR), and quantitative PCR (qPCR) were performed according to a previously published method [5]. The primer sequences used for RT-PCR and qPCR are listed in Supplemental Table 1.

2.6. Statistical analysis

Statistical data analyses were performed using one-way ANOVA followed by Tukey's post-hoc test. Data were analyzed using SigmaPlot 14 (HULINKS Inc., Tokyo, Japan), and differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Effect of diclofenac and celecoxib on the antitumor effect of CDDP

CDDP did not decrease tumor size in xenograft mice transplanted with A549/DDP cells; however, compared with the control group, co-administration of diclofenac and CDDP

significantly reduced tumor size (Fig. 1A). In contrast, celecoxib did not significantly suppress the antitumor effect of CDDP. Furthermore, changes in tumor weight were similar to the variations in tumor size (Fig. 1B and Fig. 1C).

3.2. Effect of diclofenac and celecoxib on CIN

We evaluated the mRNA level of kidney injury molecule 1 (Kim-1), a renal disorder marker, and observed that CDDP increased Kim-1 expression in the kidneys of xenograft mice (Fig. 2A and Fig. 2B). Moreover, celecoxib and diclofenac did not impact the CDDP-enhanced Kim-1 mRNA level, while diclofenac and celecoxib did no influence CIN. Celecoxib co-administration increased heme oxygenase 1 (Ho-1), an antioxidant marker, when compared with the CDDP group; however, the observed variation was marginal (Fig. 2C).

4. Discussion

In the present study, co-administration of CDDP and diclofenac significantly reduced tumor size and weight, and diclofenac was found to enhance the antitumor effect of CDDP. Although co-administration of diclofenac showed a decreasing tendency when compared with CDDP alone, no significant difference was observed. Herein, mice were evaluated until day 22, and extending the evaluation period could demonstrate further improvement. However, as the tumor size in the control group approached the humane endpoint, further studies could not be performed. Based on previously reported plasma concentrations of celecoxib or diclofenac

following oral administration in mice [13,14], it is considered that both drugs sufficiently inhibit cyclooxygenase 1 and 2 at the doses employed in the present study [15]. Moreover, although celecoxib and diclofenac inhibit organic anion transporter, there appears to be no difference in transporter inhibition between these drugs, considering the strength of inhibition against organic anion transporter in the previous report and the estimated plasma concentration in the present study [16]. In the present study, it can be speculated that the effect of diclofenac on the antitumor effect mediated by CDDP does not involve cyclooxygenase or transporters.

Notably, celecoxib co-administration tended to slightly reduce the antitumor effect of CDDP, suggesting that the impact of celecoxib on enhancing CDDP resistance, observed in our previous report, might be greater than the antitumor effect of celecoxib itself [7]. Moreover, as the antitumor effect of CDDP was not enhanced by celecoxib in mice, the effect of celecoxib might afford a more substantial contribution to cancer cells than CSCs. Conversely, as the isolated mouse tumor presented a heterogeneous cancer cell population and was atypical, it can be considered that celecoxib displayed a concentration gradient in the central and peripheral parts of the tumor, with effects insufficiently exerted on the entire tumor. Therefore, celecoxib might not affect CSCs, as the precise location of CSCs in the tumor remains unclear. Although NSAIDs demonstrate non-tissue-specific effects, to our knowledge, no previous studies have reported differences in concentrations between blood and tumors. This issue could be resolved by evaluating NSAID concentrations using liquid chromatography-mass spectrometry. In

addition, this method could clarify whether celecoxib or diclofenac demonstrate a uniform effect or a concentration gradient in atypical and heterogeneous cancer cell populations, as in the present study, and will be comprehensively evaluated in the future.

Diclofenac did not enhance CIN, which was consistent with our previous *in vitro* investigation [5], indicating the relative safety of this NSAID in patients receiving CDDP. Diclofenac is used to treat cancer pain and fever during cancer treatment [17]. Therefore, the present study evaluating the safety of diclofenac for CIN can present crucial evidence for improving the quality of life of cancer patients.

In our previous report, celecoxib attenuated CIN in rats [5]; however, no CIN attenuation effect was observed in mice in the present report. Furthermore, we reported that the mechanism underlying the attenuating effect of celecoxib on CDDP–induced cytotoxicity involves an increase in antioxidant markers; however, celecoxib only marginally increased the mRNA levels of Ho-1, indicating that the influence was weak in the kidneys. There are three possible explanations for this difference. First, we observed that the expression of the renal transporters that transport CDDP varies between single and multiple CDDP administrations [18,19]. Therefore, the results of multiple CDDP administrations may differ from those following a single-dose study. Second, repeated oral administration of NSAIDs might reduce the degree of CIN as the water load increases with multiple oral doses. Hydration is crucial to prevent CIN [20], and loading a large amount of infusion is employed to promote excretion of

CDDP accumulated in the kidney; this strategy could have a greater impact than NSAID co-administration. Third, as CIN model mice received higher doses of CDDP than CIN model rats [21], it can be postulated that celecoxib effects on CIN are species-dependent. The effect of celecoxib on CIN differed between single and multiple doses, as well as in mice and rats. Moreover, clinical implications remain unclear; therefore, further verification by undertaking clinical studies is required.

In conclusion, of the two types of NSAIDs evaluated, diclofenac potentiates the antitumor effect of CDDP without enhancing CIN. Future clinical studies should attempt to determine whether this finding can be observed in clinical practice.

Author Contributions

Participated in research design: Keisuke Okamoto, Hinata Ueda, Yoshitaka Saito, and Masaki Kobayashi

Performed the experiments and sample collection: Keisuke Okamoto and Hinata Ueda

Analyzed the data: Keisuke Okamoto and Masaki Kobayashi

Contributed to the writing of the manuscript: Keisuke Okamoto, Hinata Ueda, Yoshitaka Saito, Katsuya Narumi, Ayako Furugen, and Masaki Kobayashi

Declaration of interest

The authors report no conflicts of interest.

Acknowledgments

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

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Figure Legends

Fig. 1: Effect of celecoxib and diclofenac on anticancer effects of CDDP in xenograft model mice. (A) Tumor size was measure at days 1, 4, 7, 10, 13, 16, 19, and 22. (B) The images (1 scale is 1 mm) represent excited tumors at day 22 and (C) tumor weight. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group; Tukey's post-hoc test. Data are presented as means ± standard deviation (S.D.), n = 5 per group. CDDP, cisplatin.

Fig. 2: Effect of celecoxib and diclofenac on Kim-1 and Ho-1 mRNA level in the kidneys of CDDP treated mice. (A) Representative images of Kim-1 and Actin mRNA expression by RT-PCR and (B) mRNA quantified by ImageJ analysis software. (C) Ho-1 mRNA expression was measured by real-time PCR. Kim-1 and Ho-1 mRNA levels were normalized to those of Actin. The expression level of the control group was arbitrarily set at 1.0. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group, $^{\dagger}P < 0.05$ compared with the CDDP group; Tukey's post-hoc test. Data are presented as means ± standard deviation (S.D.), n = 5 per group. CDDP, cisplatin; Ho-1, heme oxygenase-1; Kim-1, kidney injury molecule 1.

Supplemental Table 1. Mouse primer sequences

Genes	Forward sequence	Reverse sequence
Kim-1	5’-agccgcagaaaaaccctac-3’	5’-cgcttagagatgctgacttcc-3’
Ho-1	5’-agatagagcgcaacaagcag-3’	5’-agtgaggcccataccagaag-3’
Actin	5’-ctaaggccaaccgtgaaaag-3’	5’-atcacaatgcctgtggtacg-3’

Supplemental Table 2. Variation of body weight in mice

	Body weight (g)			
	Day 1 (baseline)	Day 8 - Day 1	Day 15 - Day 1	Day 22 - Day 1
control	23.4 ± 1.9	2.1 ± 1.0	2.0 ± 0.7	3.0 ± 1.0
CDDP	23.1 ± 1.0	0.8 ± 0.9	-0.2 ± 0.8**	0.2 ± 0.9**
CDDP + celecoxib	22.8 ± 1.4	1.9 ± 0.3	0.7 ± 0.8	1.6 ± 0.6
CDDP + diclofenac	23.4 ± 0.8	-0.7 ± 1.3**, ‡‡	-0.7 ± 1.2**	-1.3 ± 1.8**, ‡‡

Body weight was measured at days 1, 8, 15, and 22. Variation of body weight from baseline was compared between groups. **$P < 0.01$ compared with the control group, ‡‡$P < 0.01$ compared with the CDDP + celecoxib group; Tukey's post-hoc test. Data are presented as means ± standard deviation (S.D.), n = 5 per group. CDDP, cisplatin.

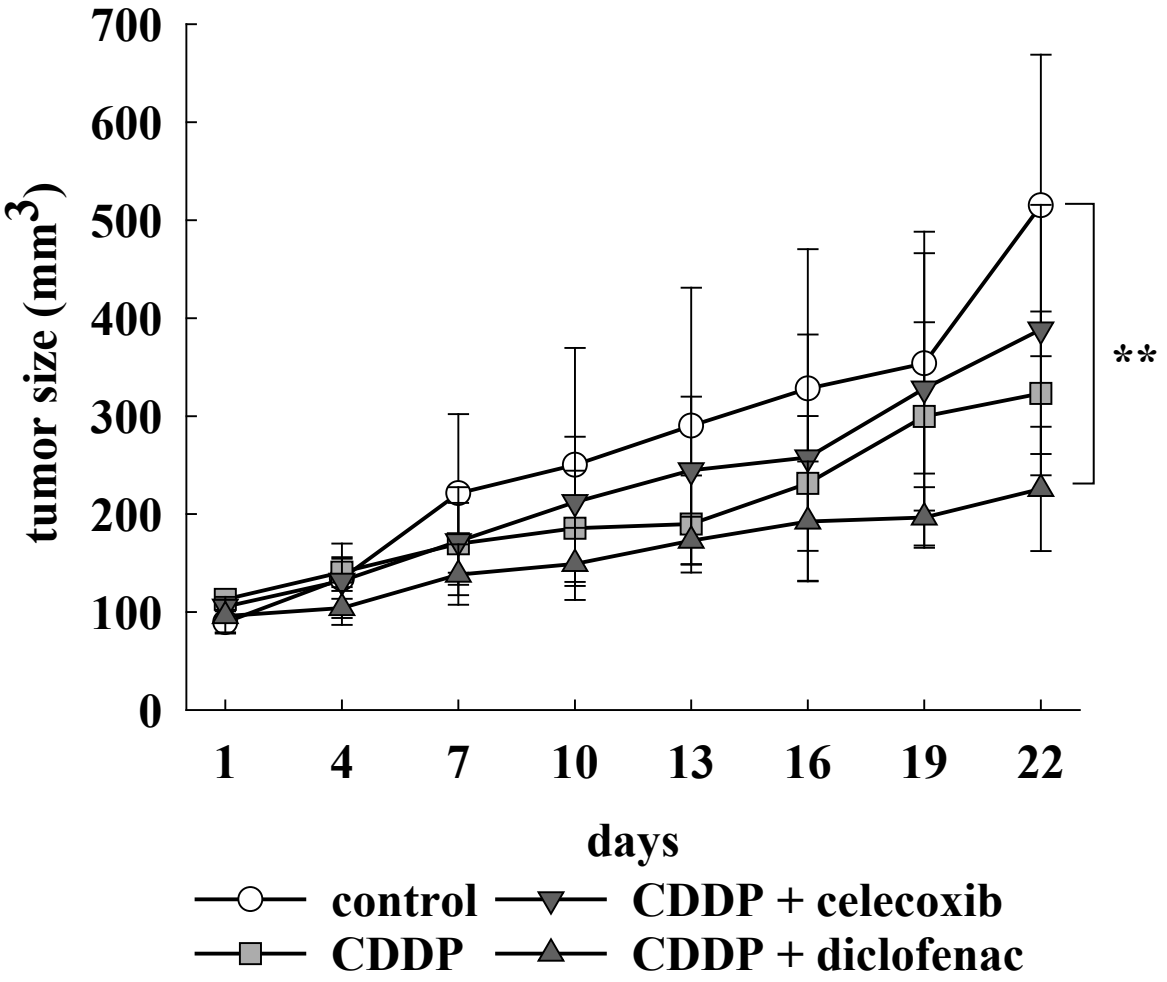
700
600
500
400
300
200
100
0
tumor size (mm3)
1
4
7
10
13
16
19
22
days
**
control
CDDP + celecoxib
CDDP
CDDP + diclofenac

Fig. 1A

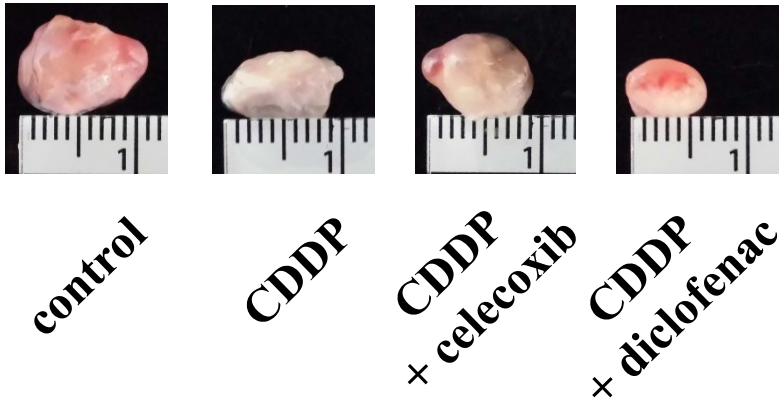

Fig. 1B

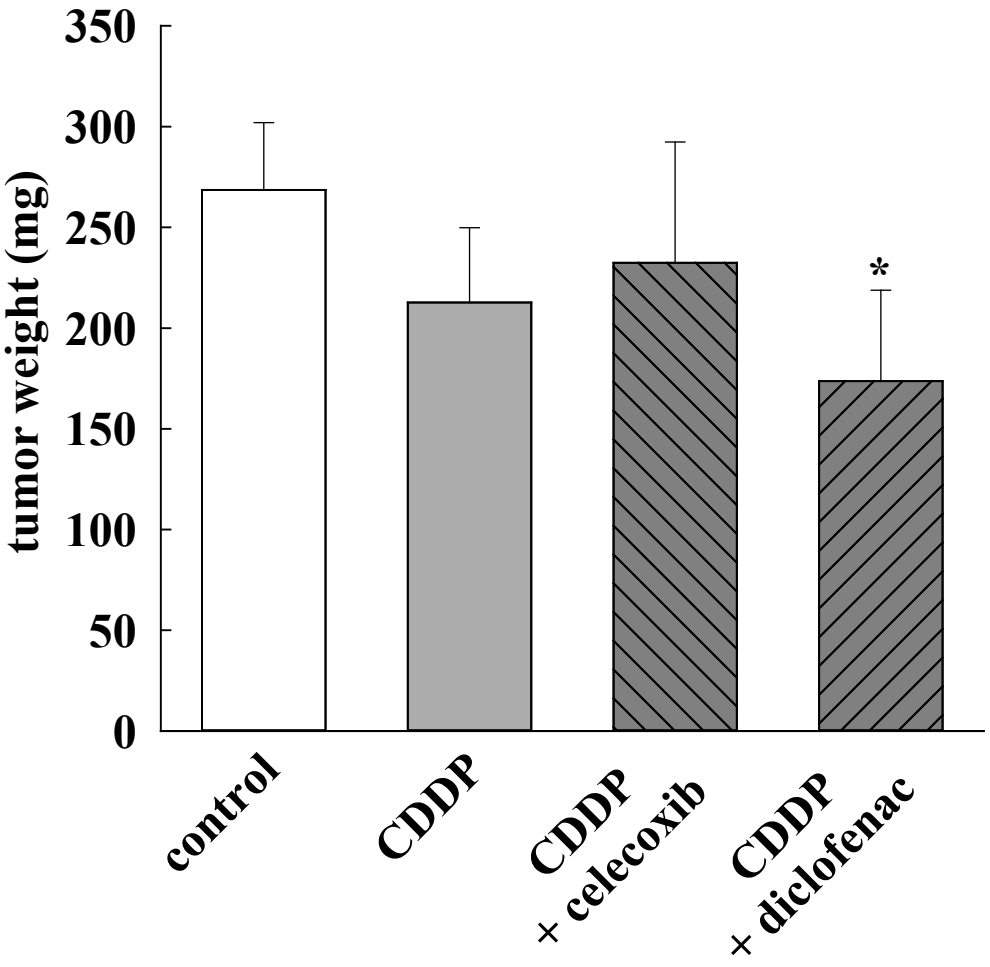
350
300
250
200
150
100
50
0
tumor weight (mg)
*
control
CDDP
CDDP
+ celecoxib
CDDP
+ diclofenac

Fig. 1C

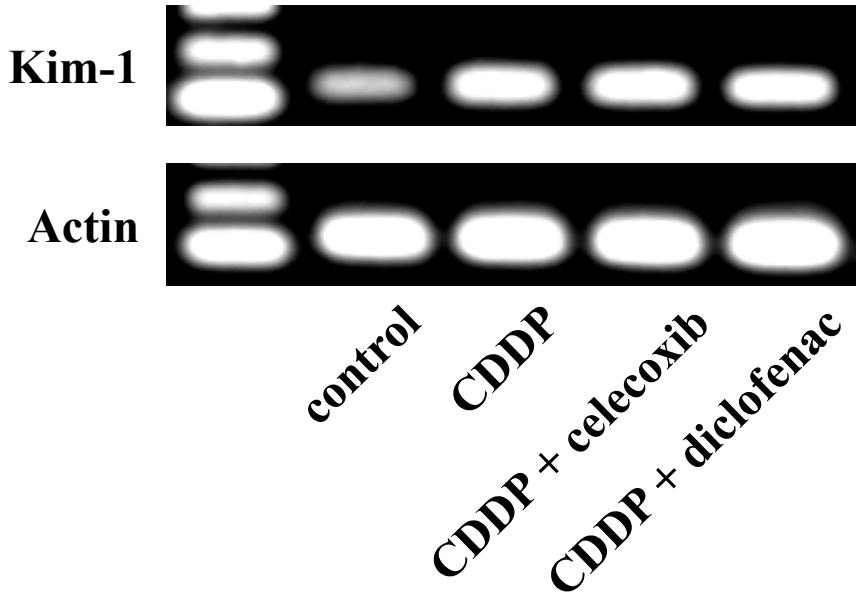

Fig. 2A

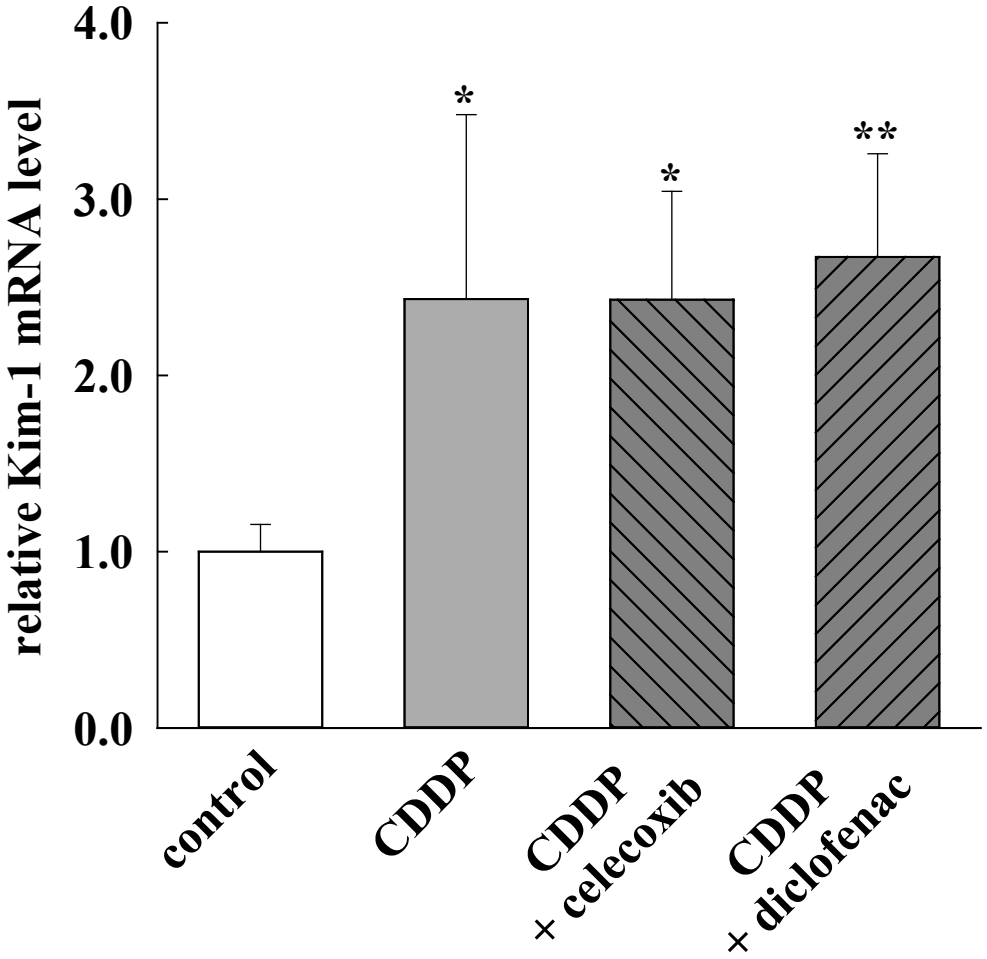
relative Kim-1 mRNA level
4.0
3.0
2.0
1.0
0.0
*
*
**
control
CDDP
CDDP + celecoxib
CDDP + diclofenac

Fig. 2B

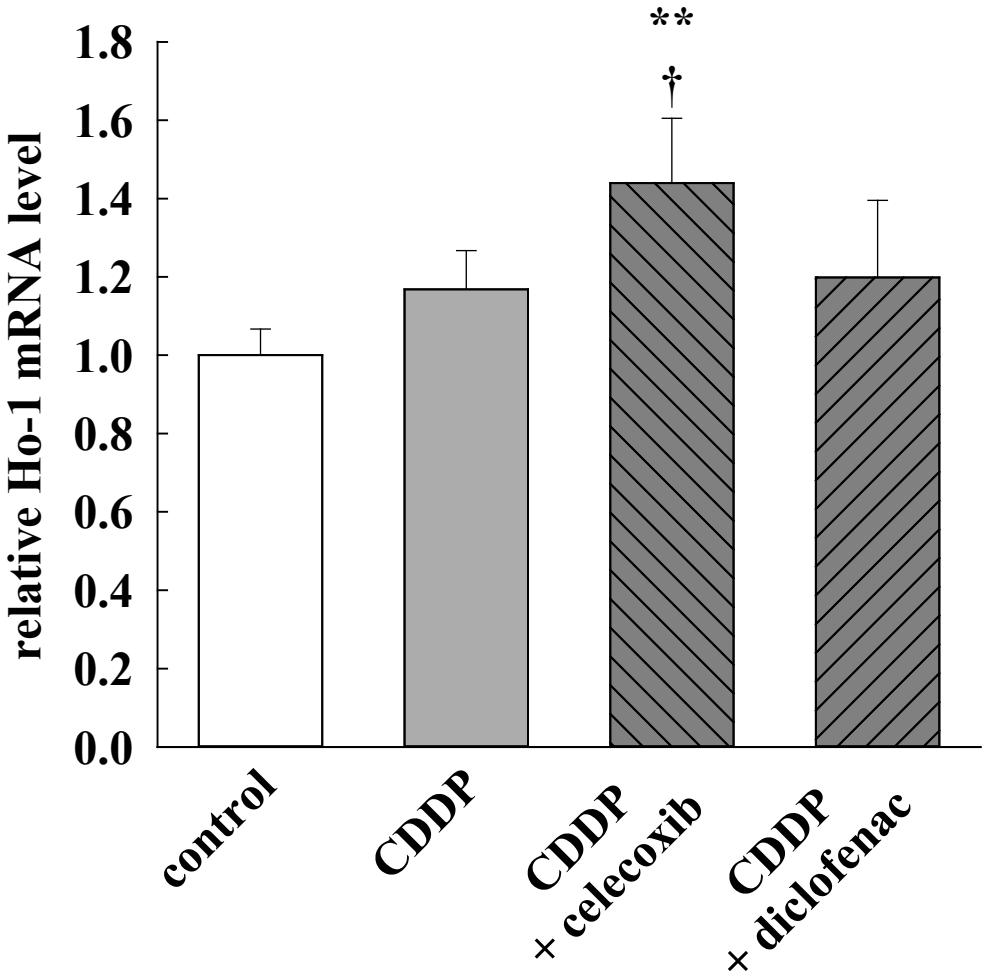
relative Ho-1 mRNA level
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
†
control
CDDP
CDDP + celecoxib
CDDP + diclofenac

Fig. 2C