

Copper-Aspirin Complex Inhibits Cyclooxygenase-2 More Selectively than Aspirin

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The antiinflammatory effects of the copper-aspirin complex (Cu-Asp) were more potent than that of Asp in rats or mice with fewer classic adverse effects. The aim of this study was to determine the cause by evaluating Cu-Asp selective inhibition on cyclooxygenases (COX). COX-1 inhibition was evaluated based on 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-PGF $_{1\alpha}$) in an endothelial cell model, and COX-2 inhibition was based on prostaglandin E $_2$ (PGE $_2$) in a macrophage model. Radioimmunoassay (RIA) was applied to determine 6-keto-PGF $_{1\alpha}$ in resting human umbilical vein endothelial cell line (ECV304), and PGE $_2$ in activated macrophages. The results showed that the inhibition of 6-keto-PGF $_{1\alpha}$ yield by Cu-Asp (3 to 0.01 mM) was markedly weaker than that by aspirin (Asp); while the inhibition of PGE $_2$ yield by Cu-Asp (10 to 0.1 mM) was significantly stronger than that by Asp. Based on the inhibition on 6-keto-PGF $_{1\alpha}$ and PGE $_2$, the medium inhibitory concentration (IC $_{50}$) of Cu-Asp on COX-1 and on COX-2 was 1.03 ± 0.15 mM, and 0.32 ± 0.04 mM, respectively. The selective inhibition index on COX-2, IC $_{50}$ (COX-1)/IC $_{50}$ (COX-2), of Cu-Asp was 3.33 ± 0.89 , while that of Asp was 0.42 ± 0.12 . The results suggest that, unlike Asp, Cu-Asp is a relatively selective inhibitor of COX-2 in the present models; the selectivity of Cu-Asp is about seven-fold greater than that of Asp.

Key words—copper-aspirin complex; aspirin; prostaglandin E $_2$; 6-keto-prostaglandin $F_{1\alpha}$

INTRODUCTION

The copper-aspirin complex (Cu-Asp), tetrakis-mu-acetylsalicylato-dicopper (II), is a complex of aspirin (Asp) and copper ion (II) (Fig. 1).^{1,2} Early studies^{3,4} and our recent studies^{5,6} indicated that the antiinflammatory effects of Cu-Asp were more potent than those of Asp in rats or mice, including 1) inhibiting ear swelling induced by xylene, 2) suppressing acute paw edema produced by injecting carrageenan, 3) decreasing wet weight and dry weight of pouch granuloma elicited by turpentine and nitrogen, and 4) decreasing the content of protein in the inflammatory exudate from air pouch synovitis caused by acetic acid and reducing leukocytes in the exudates. Moreover, Cu-Asp had fewer adverse effects, *i.e.*, it seldom destroyed gastrointestinal mucosa.^{4,6,7} Similarly, other transition metal aspirinates (mononuclear and binuclear transition metal [Co(II), Cu(II), Ni(II) and Zn(II)] acetylsalicylates) shared the activity pattern in antiinflammation.^{3,8} Asp is one of the clas-

sic nonsteroidal antiinflammatory drugs (NSADs), and its antiinflammatory activity is associated with cyclooxygenase (COX) inhibition directly or indirectly.⁹

According to the results of recent research, there are at least three types of COX (COX-1, COX-2, and COX-3) involved in arachidonic acid (AA) metabolism.^{10,11} COX-1 was recognized as a constructive enzyme to maintain physiologic functions such as protecting the gastrointestinal mucosa; the activity of the COX-1 pathway can be measured by its

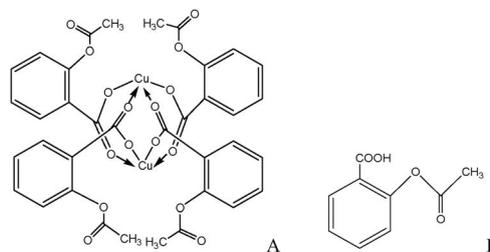


Fig. 1. Structure of Cu-Asp and Asp

A: Cu-Asp tetrakis-mu-acetylsalicylato-dicopper (II) MW: 844
B: Aspirin MW: 180

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downstream product 6-keto-PGF_{1 α} .^{12,13} COX-3 is present in the central nervous system (CNS) or neural cells.¹⁴ Both COX-1 and COX-3 have only a slight relationship with peripheral inflammation. However, COX-2 can be induced by various inflammatory stimuli, such as lipopolysaccharide (LPS),¹⁵ phorbol 12-myristate 13-acetate (PMA),¹⁶ and cytokines.¹⁷ Prostaglandin E₂ (PGE₂), a metabolite of AA through the COX-2 pathway, is thought to be one of the key molecules involved in the inflammatory process.¹⁸ The classic nonselective COX inhibitors like Asp and the newly developed COX-2 selective inhibitors are effective agents to quench, or help to quench, inflammation through the pathway. That is accepted as the main mechanism of NSAIDs.

Since the adverse effect of damage to the gastrointestinal mucosa by classical NSAIDs like Asp was believed to be caused by a physiologic PG decrease *via* COX-1 inhibition,^{19–21} while the antiinflammatory activity of Cu-Asp is more potent than that of Asp with less frequent side effects, it could be assumed that there would be difference in the inhibition of COX between Asp and Cu-Asp. However, the assumption was not confirmed. The present study was designed to evaluate the direct inhibitions of COX-1 and COX-2 based on different cell models to determine the mechanism.

MATERIALS AND METHODS

Materials Healthy ICR mice were obtained from the Animal Department, Yunnan Pharmacological Laboratories of Natural Products (certification number: 2004009). Human umbilical vein endothelial cells, ECV304, were purchased from the Shanghai Institute of Materia Medica, Chinese Academy of Sciences, China. Cu-Asp of blue crystal (Cu 14.99%, C 51.21%, and H 3.32%; purity \geq 98%) was synthesized by the Kunming Institute of Precious Metals. Asp, arachidonic acid (AA), and lipopolysaccharides (LPS) were purchased from Sigma-Aldrich Corporation (USA). RPMI 1640 culture media were manufactured by Gibco BRL (USA). Fetal bovine serum was manufactured by Hangzhou Sijiqing Biomaterial Co., Ltd. (China). Cu-Asp, CuSO₄+Asp, Asp, and CuSO₄ were dissolved in phosphate buffer solution (PBS, KCl 2.68 mM, KH₂PO₄ 1.47 mM, Na₂HPO₄ 5.81 mM, NaCl 136.9 mM) containing 1% pyridine. PGE₂ radioimmunoassay (RIA) kits and 6-keto-PGF_{1 α} RIA kits were produced by the Chinese

Academy of Jiangsu Province Blood Study Institution. Other reagents used were of analytic purity from China.

Dose Design Asp was applied as a positive control. Based on its IC₅₀ on COX-2,²² its dosage was 10, 3, 1, 0.3, and 0.1 mM. Because a mole of Cu-Asp contains two mole of copper ions and four mole of Asp,² the equivalent dosage was utilized in the present study. The dosages of Cu-Asp were equivalent respective dosages to Asp, while the dosages of CuSO₄, or CuSO₄+Asp were equivalent to those of Cu-Asp.

Culture of ECV304 Human umbilical vein endothelial cells, ECV-304, were incubated with RPMI 1640 containing 15% fetal bovine serum in a CO₂ incubator at 37°C. When the cells covered about 80% of the full area of the flask, they were digested into disrupted cells with 0.25% trypsin solution and harvested by spinning at 800 \times g for 10 min. The disrupted cells (1.0 \times 10⁵/ml, 200 μ l per well) were transplanted in to 96-well plates and incubated in the same conditions. When the cells adhered to the bottom (about 12 h), the above media were removed. The cells were rinsed twice with PBS, then exposed to Asp, Cu-Asp, CuSO₄+Asp, and CuSO₄, or exposed to 1% pyridine (blank control for Cu-Asp). After incubation in the same conditions described above for 20 min, AA (10 μ M) was added, and then incubated for another 20 min. The supernatant was collected as sample to determine levels of 6-keto-PGF_{1 α} .

Culture of Mouse Macrophages ICR mice weighing 22–24 g were injected with 1% starch broth 1 ml into the abdominal cavity. The next day, the mice were killed and disinfected with 75% ethanol. The abdominal cavity was rinsed with 2 ml of PBS three times by injection. The PBS was collected, mixed, and spun for 5 min (800 rpm) to harvest macrophages. After cell identification, macrophages (1.0 \times 10⁵/ml, 200 μ l per well) were transplanted into 96-well plates and incubated with RPMI 1640 containing 15% fetal bovine serum and Asp 1.0 mM in a CO₂ incubator at 37°C overnight. Then, the macrophages were rinsed three times and induced with LPS (10 μ g/ml) for 18 h. The macrophages in every well were rinsed three times with PBS and exposed to Asp, Cu-Asp, CuSO₄+Asp, and CuSO₄, or exposed to 1% pyridine. When the cells were incubated in the same conditions described above for 20 min, AA 10 μ M was added. After incubation for another 20 min, the

supernatant was collected to determine levels of PGE₂.

Radioimmunoassay for 6-keto-PGF_{1α} and PGE₂
Levels of 6-keto-PGF_{1α} or PGE₂ in the collected samples were determined using RIA kits according to the protocols provided by the manufacturer.

Absorption Bands of Cu-Asp, CuSO₄ + Asp, and CuSO₄
Cu-Asp, CuSO₄+Asp, or CuSO₄ were dissolved in PBS. The equivalent concentration in all specimens was the same, while the pyridine in all specimens was controlled at 1%. The absorption bands were read with a spectrophotometer from 400 nm to 800 nm with an interval of 2 nm.

Mass Spectrograms of Cu-Asp and CuSO₄ + Asp
Cu-Asp, CuSO₄+Asp, or CuSO₄ were dissolved in PBS. The equivalent concentration in all specimens was the same, while the pyridine in all specimens was controlled at 1%. The mass spectrograms were scanned with a mass spectrometer (Shimadzu, Japan) in an electrospray ionization (ESI⁺) model from 750 m/z to 900 m/z.

Statistical Analyses
Values are expressed as mean ± SD. One-way analysis of variance (ANOVA) was performed, and Hochberg's GT2 method of the *post hoc* test was performed to compare the means of different groups with that of the control group. Statistically significant differences were accepted at *p* < 0.05. The concentration was transformed to logarithmic (Lg) value in statistical graphs.

Selective inhibition index of COX-2 was calculated according to formula 1 to evaluate selective inhibition. A greater index means more selective inhibition of COX-2.

Selective inhibition index of COX-2

$$= \frac{IC_{50}(COX-1)}{IC_{50}(COX-2)} \text{ (formula 1)}$$

RESULTS

Effects of Cu-Asp on 6-keto-PGF_{1α} Biosynthesis
Asp, Cu-Asp, CuSO₄±Asp, and CuSO₄ inhibited 6-keto-PGF_{1α} biosynthesis in a concentration-dependent manner. At the highest concentration (10 mM), every group significantly inhibited 6-keto-PGF_{1α} synthesis. At other concentrations (3, 1, 0.3, and 0.1 mM), the inhibition of Cu-Asp was less powerful than that of Asp (*p* < 0.05), while the inhibitory effects of CuSO₄+Asp were similar to those of Asp (*p* > 0.05) (Fig. 2, Table 1). The IC₅₀ of Cu-Asp on COX-1 was 1.03 ± 0.15 mM (Table 1).

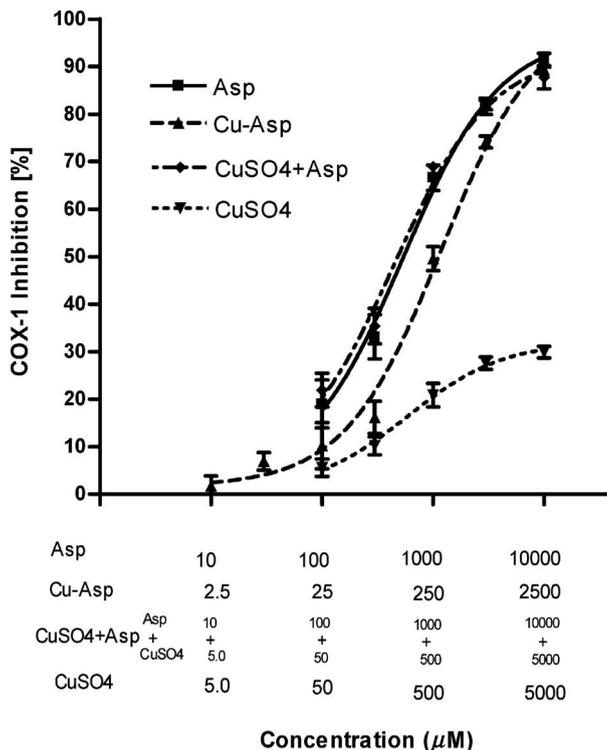


Fig. 2. Inhibition of COX-1 by Cu-Asp (mean ± S.D. *n* = 3)
Cu-Asp inhibited COX-1 less powerfully than other groups; however, the inhibition caused by CuSO₄ is the strongest. The inhibition of COX-1 is measured by the inhibition of 6-keto-PGF_{1α} yield in an endothelial cell (ECV304) model. The concentration is an equivalent dose for every group. Asp, aspirin; Cu-Asp, copper-aspirin complex

Table 1. Selective Inhibition of Asp, Cu-Asp, Cu-SO₄, and Asp + CuSO₄ on COX-1 and COX-2

Agent	IC ₅₀ for COX-1 (mM)	IC ₅₀ for COX-2 (mM)	Selective inhibition index for COX-2*
Asp	0.56 ± 0.11	1.36 ± 0.14	0.42 ± 0.12
Cu-Asp	1.03 ± 0.15 ^a	0.32 ± 0.04 ^a	3.33 ± 0.89
CuSO ₄	—	—	—
CuSO ₄ + Asp	0.55 ± 0.09	0.94 ± 0.11 ^a	0.59 ± 0.10

^a *p* < 0.05 vs Asp. *Calculated using Formula 1; IC₅₀ value of CuSO₄ for COX-1 or for COX-2 was not calculated because its maximal inhibition was less than 50%.

Effects of Cu-Asp on PGE₂ Formation
Asp, Cu-Asp, CuSO₄+Asp, and CuSO₄ inhibited PGE₂ formation in a concentration-dependent manner. The inhibitory effects of Cu-Asp were much greater than those of Asp (*p* < 0.05). Although the inhibitory effects of CuSO₄+Asp were statistically greater than those of Asp (*p* < 0.05) (Fig. 3), its IC₅₀ value for COX-2 decreased relatively slightly (Table 1) compared with the IC₅₀ value of Cu-Asp.

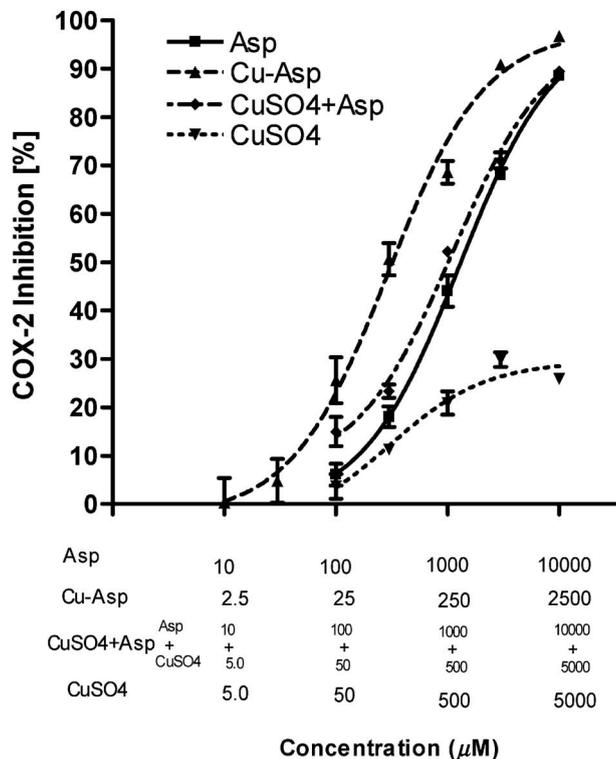


Fig. 3. Inhibition of COX-2 by Cu-Asp (mean \pm S.D. $n=3$)
Cu-Asp inhibited COX-2 more powerfully than other groups. The inhibition of COX-1 is measured by the inhibition of PGE₂ yield in a macrophage model. The concentration is an equivalent dose for every group.

In the two cell-based models, CuSO₄ only showed a mild inhibition of COX-1 and COX-2. Since the maximal inhibitory effect was less than 50%, its IC₅₀ value and selective inhibition index for COX-2 were not obtained.

Cu-Asp Transformation from Copper Sulfate and Asp To determine how Cu-Asp is transformed from copper sulfate and Asp, the absorption bands of Cu-Asp, CuSO₄+Asp, and CuSO₄ in PBS with 1% pyridine were read with a spectrophotometer, as shown in Fig. 4. Compared with that of CuSO₄, the absorbance pattern of CuSO₄+Asp is similar to that of Cu-Asp. The similarity could be caused by the transformation of Cu-Asp from CuSO₄ and Asp. This was confirmed by the mass spectrograms in the ESI⁺ model (Fig. 5) of Cu-Asp (Fig. 5 A) and CuSO₄+Asp (Fig. 5 B) showing a spike near 848 m/z, although the spike is lower in the spectrogram of CuSO₄+Asp. The results suggest that it is possible to produce Cu-Asp from CuSO₄ and Asp in water solution.

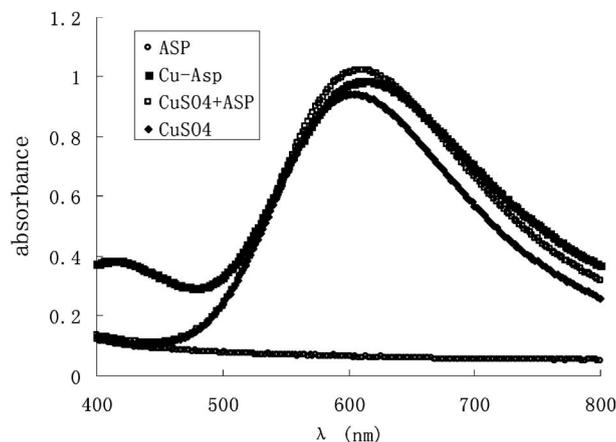


Fig. 4. Absorption Bands of Cu-Asp, CuSO₄+Asp, and CuSO₄

There are some differences among the absorbance patterns of Cu-Asp, CuSO₄+Asp, and CuSO₄ from 400 nm to 800 nm, although they have a similar λ max near 600 nm. However, the absorbance pattern of CuSO₄+Asp is very similar to that of Cu-Asp. Asp 100 μ M; Cu-Asp 25 μ M; CuSO₄+Asp: mixture of CuSO₄ 50 μ M and Asp 100 μ M; CuSO₄ 50 μ M

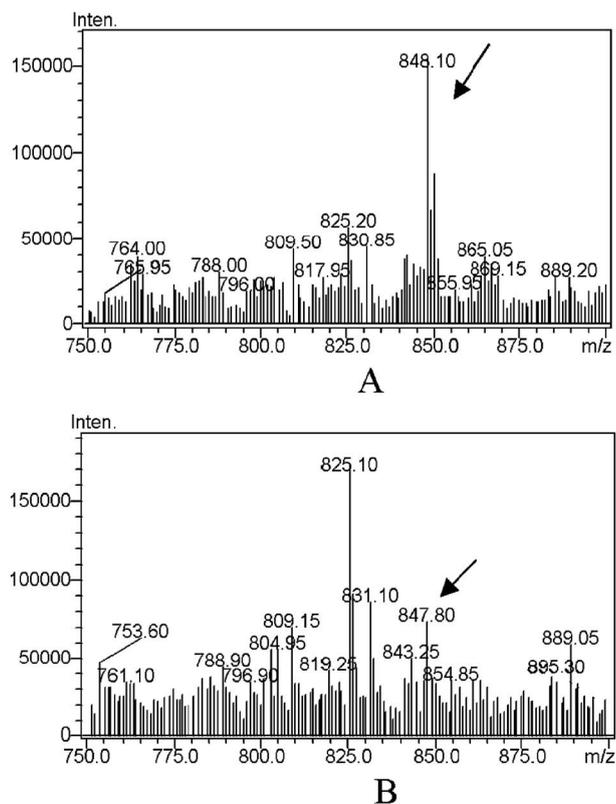


Fig. 5. ESI⁺ Mass Spectra of Cu-Asp and CuSO₄+Asp
Arrowhead indicates the spikes of Cu-Asp, which is 848.10 m/z in A and 847.80 m/z in B. A: ESI⁺ mass spectrum of Cu-Asp; B: ESI⁺ mass spectrum of CuSO₄+Asp

DISCUSSION

As a classic NSAID, the antiinflammatory effects of Asp are achieved by inhibiting PGs synthesis *via* COX

inhibition. Asp is a well-known nonselective COX inhibitor, and the IC_{50} value for COX-1 and COX-2 is 0.75 and 1.25 mM in ovine cells, respectively.²²⁾ Our early study and other studies found that Cu-Asp had an more powerful antiinflammatory effect than Asp with fewer adverse effects.^{5,6,23)} Since Cu-Asp is a derivate from Asp, the mechanism of action of Cu-Asp may be similar to that of Asp. According to the COX theory, it was assumed that Cu-Asp could also inhibit COX-2 even more selectively, and the present study verified that hypothesis.

Cu-Asp Inhibits COX-2 More Selectively Without stimulation, endothelial cells mainly expresses COX-1, and PGI_2 is one of the main products *via* the enzyme, which can be quickly transformed to the stable substance 6-keto- $PGF_{1\alpha}$.^{18,24-26)} COX-2 is an inducible enzyme mainly present in inflammatory cells including macrophages. With inflammatory stimulation, COX-2 expression is strongly upregulated.^{18,27)} In the present study, the resting human umbilical vein endothelial cell line ECV304 was employed to study COX-1, while activated macrophages were employed to study COX-2. 6-Keto- $PGF_{1\alpha}$ and PGE_2 were used as indicators to measure COX-1 and COX-2 inhibition, respectively.^{28,29)} Since the low quantity of endogenous AA could limit COX activity, 10 μ M of exogenous AA was added into the reaction system. Asp can inhibit COX directly; furthermore, Asp can inhibit COX expression.⁹⁾ To study the direct COX inhibition by Asp and Cu-Asp, rather than to study COX expression inhibition, the preincubation time in the present study was 20 min. Models based on resting endothelial cells and activated macrophages, have been established to study selective inhibition of COX.^{18,25)}

The present results showed that the IC_{50} value of Asp for COX-1 (0.56 ± 0.11 mM) or COX-2 (1.36 ± 0.14 mM) is similar to the reported results (one sample Student *t*-test, $p > 0.05$).²²⁾ The selective inhibition index of Asp for COX-2 is 0.42 ± 0.12 (< 1), which supports the fact that Asp is a nonselective inhibitor of COX.²²⁾

The results also showed that Cu-Asp inhibited COX-1 and COX-2 in a concentration-dependent manner with an IC_{50} value of 1.03 ± 0.20 mM for COX-1 and 0.32 ± 0.05 mM for COX-2. The selective inhibition index for COX-2 is 3.22 (> 1), which suggests that Cu-Asp inhibits COX-2 more selectively than Asp, and its selectivity is about 8-fold that of

Asp (Table 1). Therefore it can be deduced that the stronger antiinflammatory effects and fewer adverse effects may derive from its selective inhibition of COX-2. However, compared with classic COX-2 selective inhibitors, such as NS398 (IC_{50} value for COX-1, 75 μ M; IC_{50} value for COX-2, 1.77 μ M; selective index, 42 in humans) or celecoxib, the selectivity is not satisfactory.³⁰⁾ Nevertheless, the phenomenon can explain the antiinflammatory effects *in vivo* and give an interesting clue to synthesize the ideal Asp derivative to quench inflammation.

Putative Mechanism of Cu-Asp Inhibition of COX COX-1 and COX-2 catalyze the same reaction and their proteins are similar in stereochemical structure.³¹⁾ There are three independent folding units in COX proteins, a surface domain, a membrane banding site, and an active domain in the stereostructure. The COX active site is composed of three spiral structures in the top of the hydrophobic channel that encaves into the cellular membrane. The channel is large in the active site and forms a cavity. In the side pocket towards the main channel in COX-1, a large amino acid, isoleucine 523, can block large molecules from the entrance to the side pocket, while in COX-2, a smaller hydrophilic amino acid, valine, stands at the site, and allows large molecules to pass into the entrance. COX-2 selective inhibitors, such as celecoxib and rofecoxib, which have a large sulfanilamide side chain in their structures, can bind to the hydrophilic side pocket and show selective inhibition of COX-2. As a small molecule, Asp can easily pass into both pockets in COX and inhibits COX by acetylating serine 530.³²⁾ As a complex of Asp, the molecule Cu-Asp is larger than Asp (Fig. 1) and can not easily enter the side pocket of COX-1. In COX-2, however, Cu-Asp can pass the side pocket entrance and inhibited its active site.

In our experimental design, the amount of copper ion or Asp is equivalent to the Cu-Asp or $CuSO_4 + Asp$ group. However, the inhibition of COX-2 by $CuSO_4 + Asp$ is also more potent than that by Asp. The copper ion (II) is important in Cu-Asp. The copper ion chelated in Cu-Asp is different from free copper ions.¹⁾ In our experiments, it was found that the mixture of Asp 1 mM and $CuSO_4$ 0.5 mM in distilled water easily yields crystals with color almost identical to that of Cu-Asp crystals (Cu-Asp is not soluble in water). The hypothesis that the mixture of $CuSO_4$ and Asp could produce some Cu-Asp, since

the absorbance pattern of $\text{CuSO}_4 + \text{Asp}$ is similar to that of Cu-Asp was confirmed by the ESI^+ mass spectra. That could be a reasonable explanation for the fact that $\text{CuSO}_4 + \text{Asp}$ inhibited COX-2 more selectively than Asp but less selectively than Cu-Asp .

CONCLUSION

The antiinflammatory activity of Cu-Asp is more potent than that of Asp with fewer adverse effects *in vivo*, which is at least partly derived from its selective inhibition of COX-2. The selectivity of Cu-Asp is about seven-fold greater than that of Asp. Although more selective COX-2 inhibitors have been discovered, developed, and marketed, and although Cu-Asp may show fewer advantages in drug development, the present study at least gives a reasonable explanation for the antiinflammatory activity of Cu-Asp *in vivo* based on cell models. The results explain the antiinflammatory pharmacologic pattern of other transition metal aspinates.

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