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ANTI-OXIDATIVE PROFILE OF LOBENZARIT DISODIUM (CCA)

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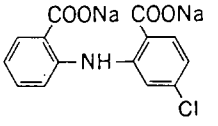
Abstract—The effects of lobenzarit disodium (CCA) on various species of activated oxygen were investigated in chemiluminescence experiments. CCA showed a quenching effect against hydroxyl radicals generated by Fenton reaction. The inhibition of CCA was much more intense than that of mefenamic acid which is an anti-inflammatory drug and an analogous compound to CCA. CCA also showed a quenching effect against singlet oxygen generated in enzymatic systems. However, CCA had no effect against superoxide anion radicals generated in the xanthine oxidase–hypoxanthine system. As a model of lipid peroxidation and protein alteration induced by activated oxygen, we examined the auto-oxidation of linolenic acid and the UV irradiation of immunoglobulin G (IgG). CCA inhibited the production of lipid peroxide; however CCA did not show a direct quenching action against lipid radicals which had been previously generated. CCA also inhibited the IgG alteration induced by UV irradiation. These results indicate that CCA has anti-oxidative actions with specificity for activated oxygen species and that CCA protects against lipid and protein damage induced by activated oxygen.

Lobenzarit disodium (CCA, disodium 4-chloro-2,2′-iminodibenzoate) was synthesized as an electron-donating and anti-oxidative compound, developed as an anti-rheumatic agent [1] and used to treat rheumatoid arthritis [2]. It appears to exert this therapeutic effect by modulating an immunological process [3], although the precise mechanism of the anti-rheumatic action remains unknown. In animal studies, CCA has been reported to suppress the development of adjuvant arthritis in rats [4], and also to prevent the spontaneous development of autoimmune kidney disease [5, 6]. Furthermore, CCA has been shown to be a compound possessing a property as an immunomodulator that can modify T cell functions [7–10].

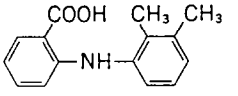
Activated forms of oxygen such as superoxide anion radicals (O_2^-), hydroxyl radicals and singlet oxygen ($^1O_2$) are implicated in several harmful biological processes and they also play an important role in several biological responses. However, the relationship between the anti-oxidative action and the anti-rheumatic effect remains to be established. In this paper, the anti-oxidative profile of CCA was investigated in chemiluminescence experiments and was compared with the analogous anti-inflammatory compounds, mefenamic acid (Mef) and flufenamic acid (Flu), the structure of which are shown with CCA in Fig. 1. A recent review has reported the ability of luminol-dependent chemiluminescence in phagocytes [11] and the results obtained here support its usefulness in model systems.

MATERIALS AND METHODS

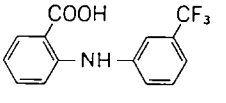
Materials. CCA was synthesized in Chugai Pharmaceutical Co. Ltd (Tokyo, Japan). Mef, Ful, luminol (5-amino-2,3-dihydrol-1,4-phthalazinedione), xanthine oxidase (XOD), hypoxanthine (HX), superoxide dismutase (SOD), lactoperoxidase



CCA



Mefenamic acid



Flufenamic acid

Fig. 1. Chemical structure of lobenzarit disodium (CCA) and the analogous anti-inflammatory compounds, mefenamic acid and flufenamic acid.

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(LPO), chloroperoxidase (CPO), deuterium oxide (D_2O, 99.8% pure), L-α-lecithin, linolenic acid, human immunoglobulin G (IgG) and protein molecular weight markers for chromatography were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The measurements for lipid peroxide were performed with Lipoperoxide-Test-Wako (Wako Chemicals, Tokyo, Japan) [12]. All other chemicals were of reagent grade.

Chemiluminescence measurements. All chemiluminescence measurements were performed on a photon counting system (Hamamatsu Photonics, Hamamatsu, Japan) that was constructed in our laboratory with standard NIM modules (NAIG, Tokyo, Japan). Time courses of chemiluminescence, in which photon signals were normally integrated every 5 sec, were recorded with a process memory (NAIG model E-562) equipped with an on-line Apple II computer (Apple, Cupertino, CA, U.S.A.).

HPLC measurements. Separation of IgG samples was performed by high performance liquid chromatography (HPLC, Jasco model BIP-1, Tokyo, Japan) with a TSK G-3000 SWXL column (Toyo Soda, Tokyo, Japan) using a solvent consisting of a phosphate buffer (67 mM KH_2PO_4/K_3HPO_4 + 0.1 M KCl, pH 7.4) [13]. The elution of altered IgG was monitored using a fluorometer (Hitachi model F-1000, Tokyo, Japan) operated with excitation and emission of 360 and 454 nm, respectively. It was also monitored simultaneously with an UV monitor (Hitachi model 655A-20) operated at 280 nm.

Reaction conditions. The anti-oxidative action of CCA against activated oxygen was investigated in the following reaction system.

(1) A hydroxyl radical was generated from the Fenton system in dimethyl formamide (DMF). The reaction mixture consisted of 50 μM $FeCl_2$ and 113 μM luminol. The reaction was started by adding 3% H_2O_2. The added volume of H_2O_2 equalled a quarter of the reaction mixture. The measurement was performed at room temperature as soon as the reaction was started.

(2) O_2^- was generated from the XOD–HX system in 100 mM HEPES buffer at pH 7.4. The reaction mixture consisted of 0.4 mM HX, 0.1 mg/mL XOD (1.1 units/mg protein; from buttermilk) and 113 μM luminol.

(3) $^1O_2$ was generated from the LPO–H_2O_2–Br^- and CPO–H_2O_2–Cl^- systems in H_2O and D_2O [14, 15]. The former consisted of 0.1 mg/mL LPO, 0.1% H_2O_2, 20 mM NaBr, 113 μM luminol and 100 mM acetate buffer at pH 4.5. The latter consisted of 0.02 mg/mL CPO, 0.3% H_2O_2, 20 mM KCl, 113 μM luminol and 100 mM potassium phosphate buffer at pH 2.85.

Direct action against hypochlorite ions was also investigated by the chemiluminescence in 113 μM luminol solution. The reaction was started by adding sodium hypochloride (270 μM final concentration). The testing compound was solved in dimethyl sulfoxide (DMSO) and added to the reaction mixture.

The lipid peroxide was generated from the lipid peroxidation induced by the Fenton reaction described above and was also generated spontaneously from the autoxidation in DMF. The former consisted of 30 mg/mL L-α-lecithin, 20 μM $FeCl_2$, 3% H_2O_2 and 40% ethanol. The reaction was started by addition of H_2O_2 at room temperature. The latter consisted of 18 mM linolenic acid and the auto-oxidative reaction was performed aerobically in a water bath at 37°. Before the reaction, the linolenic acid solution was kept on ice and then added to the sample cell which had already been incubated at 37°. In both reaction systems, the chemiluminescence without luminol was measured to observe the reaction process.

The alteration of IgG was induced by a Fenton-type reaction [16] and UV irradiation [17]. The reaction mixture of the former contained 5% IgG, 10 mM $CuSO_4$ and 3% H_2O_2 in 100 mM HEPES buffer at pH 7.4. The reaction was started by addition of H_2O_2 at room temperature. After 10 min, the alteration of IgG was measured as quickly as possible. The solution of the latter contained 2.5% IgG in phosphate-buffered saline. The IgG solution was irradiated at room temperature using 302 nm illumination. The UV irradiation was performed for 80 min with a 12-well flat bottom plate without cover. In both experiments, the alteration of IgG was evaluated by the HPLC method.

RESULTS

Inhibition of chemiluminescence induced by activated oxygen

Hydroxyl radicals generated from the Fenton reaction in DMF caused luminol-dependent chemiluminescence. The chemiluminescence reached the maximum intensity soon after the reaction started and then gradually declined. The chemiluminescence was reduced by 1% DMSO (control solution) to 3039 ± 366 cpm (mean ± SD) that was 7% of the intensity without DMSO. CCA inhibited the chemiluminescence further and the dose–response curve is shown in Fig. 2. Compared with CCA and the analogous anti-inflammatory compounds, Mef and Ful, the 50% inhibitory concentration (IC_{50}) of CCA was 1.6 μM and the IC_{50} of Mef and Ful were 56 and 38 μM, respectively. From this it can be seen that the inhibition of CCA was much more intense than that of the analogous compounds. The plasma con-

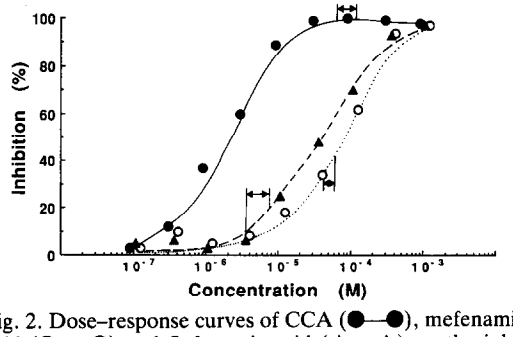

Fig. 2. Dose–response curves of CCA (●—●), mefenamic acid (○···○) and flufenamic acid (▲--▲) on the inhibition of the chemiluminescence induced by the Fenton reaction in DMF. The results are represented as the mean of triplicate. Arrows indicate the plasma concentration range of these medicines in clinical usage [17, 18].

centration of these compounds in clinical applications [18, 19] are shown in Fig. 2 for reference. In consideration of the clinical effect of CCA, the following experiments were generally performed with the 30 μM concentration of the compounds.

The inhibitory effect of CCA against $^1O_2$, O_2^- and hypochlorite ions is shown in Fig. 3. The inhibition on $^1O_2$ was investigated by luminol-dependent chemiluminescence generated in the LPO system. The generation of $^1O_2$ was confirmed by the enhancement of the chemiluminescence in heavy water. The chemiluminescence was suppressed by CCA at 30 μM concentration. The inhibition by CCA was 46% and was much more potent than that of Mef. In the CPO system, CCA suppressed the chemiluminescence in the same manner (data not shown). The inhibition of O_2^- was also investigated by luminol-dependent chemiluminescence generated in the XOD–HX system. The chemiluminescence was completely inhibited by SOD as shown in Fig. 3. However, both CCA and Mef did not affect the chemiluminescence induced by O_2^-. With regard to hypochlorite ions, CCA suppressed the chemiluminescence and Mef also suppressed it at a similar concentration.

Inhibition by CCA of lipid peroxidation

To reveal the effect of CCA on lipid peroxidation induced by hydroxyl radicals or auto-oxidation, we used the following two models. One was the L-α-lecithin peroxidation induced by the Fenton reaction and the other was the auto-oxidation of linolenic acid. The experiments were done to measure the chemiluminescence without luminol and to measure lipid peroxide production by Yagi's method [12]. For the L-α-lecithin peroxidation induced by the Fenton reaction, the chemiluminescence was abruptly induced by adding H_2O_2 and then gradually weakened. CCA showed a modest quenching effect on the chemiluminescence (Fig. 4). However, CCA at 30 μM concentration completely suppressed the lipid peroxide production by the Fenton reaction and the suppression was dose-dependent. However, Mef showed a non-significant effect on both chemiluminescence and lipid peroxide production at a similar concentration to CCA.

The chemiluminescence was induced gradually in the auto-oxidation of linolenic acid and reached a constant level after 30 min. The typical time course is shown in Fig. 5. To investigate the effect on auto-oxidation, we evaluated chemiluminescence and the lipid peroxide production with compounds after 2 hr under auto-oxidative conditions. CCA also showed a significant quenching effect on chemiluminescence and suppressed lipid peroxide generation by auto-oxidation (Fig. 5). However, the direct quenching effect was not observed by adding CCA to the solution in which chemiluminescence had previously occurred (data not shown).

Inhibition by CCA of IgG alteration

Alteration of IgG induced by activated oxygen was investigated in the following two models. One was alteration induced by a Fenton-type reaction and the other was alteration induced by UV irradiation as reported by Wickens *et al.* [16, 17]. The IgG alteration did not accompany chemiluminescence. Therefore, in order to inspect the Fenton-type reaction, we measured luminol-dependent chemiluminescence by adding 113 μM luminol and then determined the experimental conditions. Under the conditions used in this work, CCA completely quenched the chemiluminescence at 300 μM and slightly quenched it at 30 μM. In the experiment to evaluate IgG alteration, the solution without luminol was used. In the IgG alteration induced by the Fenton-type reaction, CCA

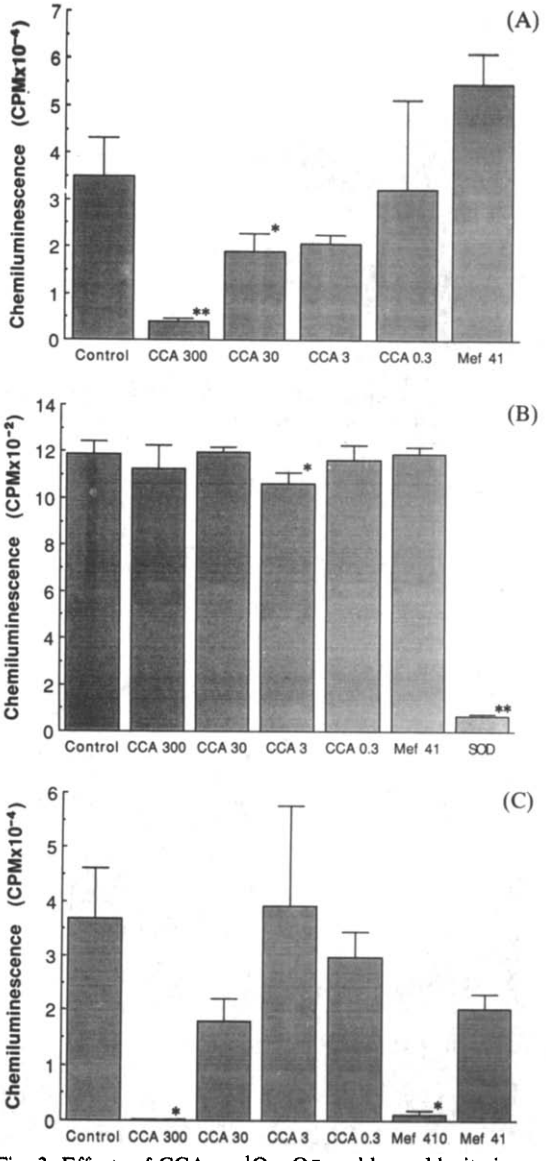

Fig. 3. Effects of CCA on $^1O_2$, O_2^- and hypochlorite ions. $^1O_2$ was generated in the lactoperoxidases–H_2O_2–Br^- system (A) and O_2^- was generated in the xanthine oxidase–hypoxanthine system (B). Sodium hypochloride (270 μM) was used as hypochlorite ions (C). The concentration of the compound is expressed by μM and SOD was used at 700 units/mL. Bars and vertical lines indicate the mean ± 1 SD of triplicate. * and ** indicate significant difference from the control at $P < 0.05$ and $P < 0.01$, respectively. See Materials and Methods for experimental details.

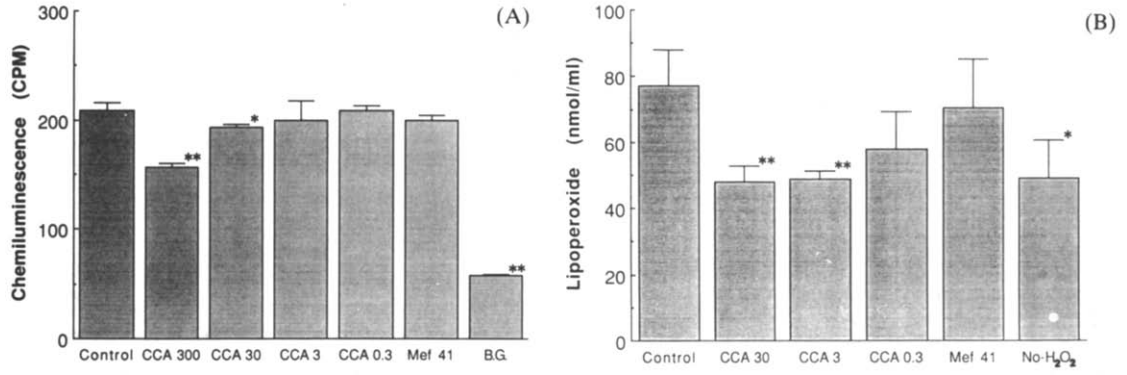

Fig. 4. Inhibition by CCA of L-α-lecithin peroxidation induced by the Fenton reaction. The chemiluminescence without luminol was measured during the first 1 min of the Fenton reaction (A) and the lipid peroxide was measured after 30 min under the Fenton reaction (B). The concentration of compound is expressed by μM. Bars and vertical lines indicate the mean ± 1 SD of triplicate (A) or quadruplicate (B). * and ** indicate significant difference from the control at $P < 0.05$ and $P < 0.01$, respectively.

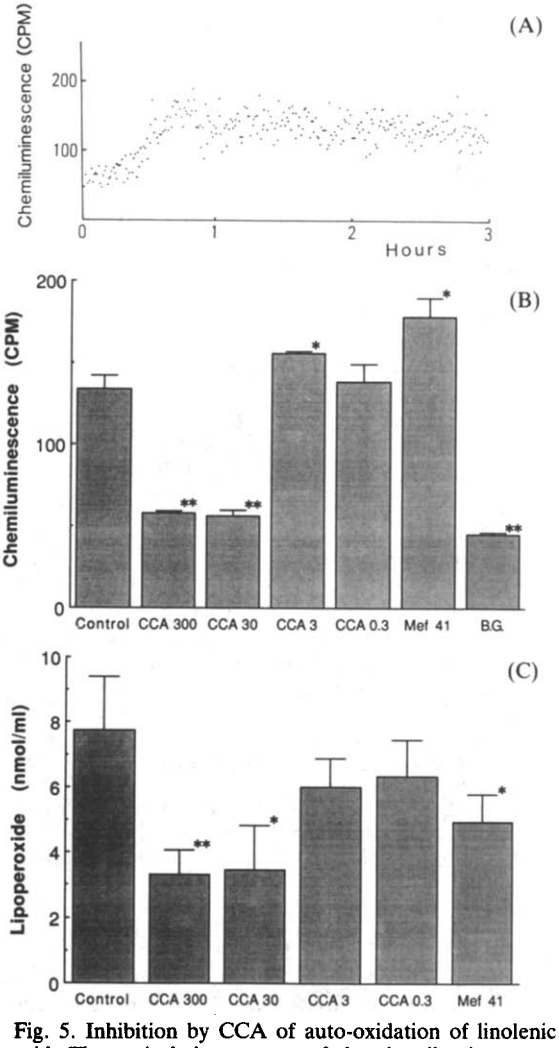

Fig. 5. Inhibition by CCA of auto-oxidation of linolenic acid. The typical time course of the chemiluminescence without luminol was shown (A). The chemiluminescence was integrated for 2 min after 2 hr (B) and the lipid peroxide was measured after 2 hr under air at 37° (C). The concentration of compound is expressed by μM. Bars and vertical lines indicate the means ± 1 SD of triplicate (B) or quadruplicate (C). * and ** indicate significant difference from the control at $P < 0.05$ and $P < 0.01$, respectively.

suppressed both IgG aggregation and fluorescent alterations in a dose-dependent manner (Table 1). However, Mef did not show the suppression of IgG alteration even at 410 μM concentration.

The UV irradiation also caused IgG alteration, both aggregation and fluorescent alterations. The aggregation increased depending on the irradiation period and the content of aggregated IgG was over 20% at 80 min. The fluorescent IgG appeared in both monomeric and aggregated IgG and the fluorescence per aggregated IgG was more emitting than that per monomeric IgG at the 80 min irradiation. The effect of compounds on this IgG alteration is shown in Table 2. The IgG aggregation decreased from 25.4 to 21.7% by using the solvent control, 1% DMSO. CCA and Mef had absorption at 302 nm and the inhibition was calculated to compensate for the reduced irradiation caused by their absorption. CCA inhibited aggregation and fluorescent alterations at 3 μM or more. The inhibition by CCA was dose-dependent and was much more intense than that of Mef, similar to the inhibition of lipid peroxidation. CCA at 30 μM concentration prevented IgG alteration more than 50%.

DISCUSSION

The effect of CCA on various species of activated oxygen was investigated in chemiluminescence experiments and compared with analogous anti-inflammatory compounds. The results indicate that the anti-oxidative property of CCA is distinctive for species of activated oxygen. That is, CCA showed inhibition against hydroxyl radicals and $^1O_2$ but not against O_2^-. Furthermore, the inhibition by CCA was much greater than that by the analogous anti-inflammatory compounds.

In the protection from activated oxygen, it is considered that hydroxyl radicals and $^1O_2$ play an important role in biological injuries. Recently, hydroxyl radicals or $^1O_2$ have been connected to biological injuries and a variety of pathways for that generation have been proposed [13–17]. From this point of view, CCA is interesting as a scavenger for hydroxyl radicals and $^1O_2$. CCA has been reported to have no

Table 1. Effect of CCA on IgG alteration induced by the Cu^{2+}–H_2O_2 system

Drug	Conc.	UV absorbance (%)		Fluorescence	
		Aggregated	Inhibition	Aggregated	Inhibition
Control	1% DMSO	4.4 ± 0.5		143 ± 27	
CCA	300 μM	1.6 ± 0.0*	85*	63 ± 0*	56*
	30 μM	2.0 ± 0.2*	73*	106 ± 43	26
	3 μM	3.3 ± 0.2	30	174 ± 16	−22
	0.3 μM	5.4 ± 2.2	−30	122 ± 38	15
Mefenamic acid	410 μM	4.4 ± 0.7	−6	164 ± 6	−15
Non-treated		1.1 ± 0.2*		ND	

The reaction mixture consisted of 5% IgG, 10 mM $CuSO_4$ and 3% H_2O_2 in 100 mM HEPES buffer at pH 7.4. The alteration of IgG was measured by the HPLC method after 10 min from the reaction start. All data were obtained from duplicate experiments.

Mean ± SD, * $P < 0.05$.

ND, not detected.

inhibitory effect on acute inflammatory reaction [20] and the anti-rheumatic mechanism remains unknown. This anti-oxidative property may support the claim that CCA is a new type of anti-rheumatic medicine. Further study will be needed to explain how the anti-oxidative profile of CCA can be used as an anti-rheumatic medicine.

Protection from injuries by activated oxygen was investigated in lipid and protein with regard to hydroxyl radicals. For lipid peroxidation, we examined Fenton reaction and auto-oxidation. The reason that we tested Fenton reaction again is to confirm that CCA terminates the chain reaction by hydroxyl radicals. In Fig. 4A, CCA shows only a modest quenching effect, whereas CCA traps hydroxyl radicals, possibly because the chemiluminescence without luminol may be induced not by hydroxyl radicals but by lipid radicals. This is supported by the fact that no chemiluminescence is observed without lipid or luminol. In both systems, CCA inhibited lipid peroxide production. CCA has been reported to be an antioxidant of lipids and biological tissues [21] and the present result supports this from another point of view. However, no direct quenching effect of chemiluminescence was observed by adding CCA after the auto-oxidative reaction reached a constant level. CCA has the inhibitory action for generating the lipid radicals but not the direct inhibition against lipid radicals generated in the auto-oxidation process.

To study effects on protein, we used the Fenton-type Cu^{2+}–H_2O_2 system [22] and UV irradiation [23]. IgG was chosen as the model protein because IgG alterations are important in rheumatoid disease [13] and involve chain reactions between aggregated IgG, neutrophil activation and activated oxygen release. Hydroxyl radicals in activated oxygen seem to play an important role in this hypothesis. Because the reactions tested here increase aggregated IgG but the reaction in the XOD–HX system reduces aggregated IgG (data not shown). In both experiments, CCA inhibited the aggregation of IgG and fluorescent change. This indicates that CCA may terminate chain reactions caused by activated oxygen and that CCA may prevent development of the disease. These results suggest that the anti-oxidative

Table 2. Effect of CCA on IgG alteration induced by UV irradiation

Drug	Conc.	UV absorbance (%)		Fluorescence	
		Aggregated	Inhibition	Aggregated	Inhibition
Control	1% DMSO	21.7 ± 0.4		727 ± 68	
CCA	300 μM	2.5 ± 0.2†	‡	15 ± 5†	‡
	30 μM	6.3 ± 0.5†	63†	112 ± 11†	79†
	3 μM	13.3 ± 0.3†	29†	418 ± 52*	41*
	0.3 μM	16.7 ± 0.1†	24	911 ± 47	−25
Mefenamic acid	410 μM	4.4 ± 0.3†	‡	112 ± 51†	‡
	41 μM	7.2 ± 0.4†	64†	342 ± 8†	44*
	4 μM	20.6 ± 0.4	4	605 ± 79	16
Non-irradiated		1.0 ± 0.4†		ND	

The reaction mixture consisted of 2.5% IgG in phosphate-buffered saline. The UV irradiation was done at 302 nm for 80 min. The alteration of IgG was measured by the HPLC method. All data were obtained from triplicate experiments. The absorption of compound was compensated in the calculation of inhibition percentage.

Mean ± SD, * $P < 0.05$, † $P < 0.01$.

‡ The compensation was not applied for the high concentration.

action of CCA is related to its direct action as an anti-rheumatic medicine in addition to that as an immunomodulator.

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