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Formation of a disulfide protein conjugate of the SH-group-containing metabolite (M-I) of esonarimod (KE-298) and its elimination in rats

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Abstract

The reactivity of the thiol moiety of the active main metabolite (M-I) of esonarimod (KE-298), a novel anti-rheumatic agent, was investigated in rats. After repeated oral administration of ^{14}C-KE-298, the radioactivity decreased rapidly and no tendency towards accumulation was found, in marked contrast to other common SH-group-containing drugs. At 30 min after intravenous administration of ^{14}C-M-I to rats, the concentration of the ^{14}C-M-I plasma protein conjugate in plasma was extremely low at 0.143 nmol mL^{-1} (0.66% of total plasma radioactivity). The ^{14}C-M-I plasma protein conjugate that formed in rat plasma was mixed disulfide with plasma protein. After intravenous administration of synthetic ^{14}C-M-I plasma protein conjugate to rats, the radioactivity in plasma decreased rapidly, with the terminal half-life at 6.90 h. In-vitro, the ^{14}C-M-I plasma protein conjugate was readily dissociated by the endogenous thiol compounds, cysteine and glutathione. These results suggest that the reactivity of the thiol moiety of M-I is extremely low. Furthermore, the ^{14}C-M-I plasma protein conjugate decreased rapidly in-vivo, which would be related to interaction with endogenous thiol compounds. These properties of M-I are principally responsible for the zero accumulation in rat tissues. KE-298 could therefore be expected to have reduced adverse effects compared with other SH-group-containing anti-rheumatic drugs.

Introduction

KE-298 ((±)2-acetylthiomethyl-4-(4-methylphenyl)-4-oxobutanoic acid) is a novel anti-rheumatic agent with immunomodulatory effects produced by suppressing the production of inflammatory cytokines (Kameo et al 1988; Nagai et al 1996). KE-298 contains a thioester moiety, which is readily deacetylated into the active metabolite, M-I, in rats (Yoshida et al 1996). M-I exists as a tautomeric equilibrium of the thiol form and hydroxytetra-hydrothiophen form (Figure 1).

Some anti-rheumatic drugs with immunomodulatory effects contain the thiol moiety, for example D-penicillamine (PEN) (Otomo et al 1981; Nakaike et al 1985) and bucillamine (Fujimura et al 1980; Yamauchi et al 1985), and they can decrease the activity of rheumatic arthritis over a long period. In addition, it is expected that their application at an early stage of arthritis would be more effective (Wilske & Healey 1989). However, these drugs have adverse effects, including skin rash, loss of taste, proteinuria and chronic renal failure (Suda et al 1993; Iizasa et al 1995). A factor which could explain these adverse effects is tissue accumulation caused by disulfide conjugation between the thiol moiety of the drug and tissue protein, and

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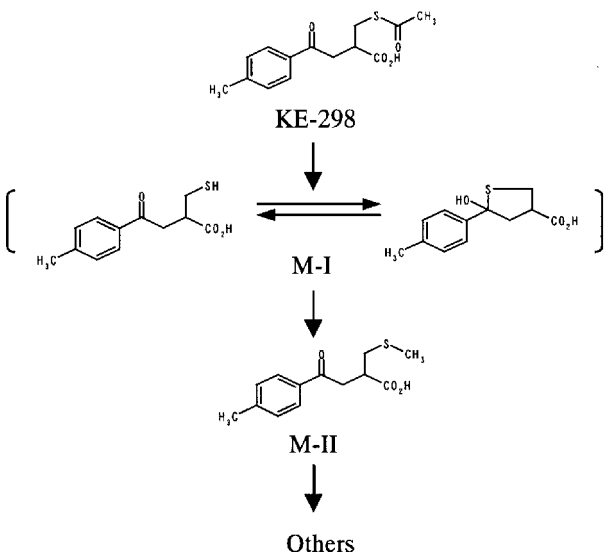

Figure 1 Putative metabolic pathway of KE-298.

immunotoxicity induced by the conjugates as a hapten (Park et al 1987; Narazaki et al 1998). The toxicity of KE-298 was found to be low in experimental animals (Yoshida et al 1996). In the present study, to clarify the properties of the thiol moiety of M-I, the formation and elimination of the protein conjugate of M-I was investigated in rats. We discuss the efficacy of KE-298 in comparison with other SH-group-containing drugs.

Materials and Methods

Chemicals

^{14}C-KE-298 (184,222 MBq mmol^{-1}) was obtained from Amersham Co., Ltd (Arlington Hights, IL). The radiochemical purity of ^{14}C-KE-298 exceeded 98%, as determined by high-performance liquid chromatography (HPLC) and thin-layer chromatography. Unlabelled KE-298 and authentic metabolites were synthesized at Taisho Co., Ltd (Saitama, Japan). All other reagents and solvents were obtained commercially and were of extra pure grade or HPLC grade.

Animals

Seven-week-old male Wistar rats were purchased from Japan SLC Inc. (Shizuoka, Japan). The rats were acclimated to the conditions of our animal facility (temperature 23±3°C; relative humidity 50±20%; 12-h light–dark cycle), and standard laboratory diet (MF, Oriental Yeast Co., Ltd) and sterile water were available *ad libitum*. All animals were used in the experiments after more than 7 days of pre-breeding. The animals were fasted overnight before dosing and for up to 4 h after dosing, except for the study on tissue distribution after repeated oral administration. The rats were grouped, three or four rats per group. The animal experiments were carried out in accordance with the Guidelines for Animal Experimentation in Taisho Pharmaceutical Co., Ltd.

Tissue distribution after repeated oral administration

^{14}C-KE-298 prepared as a suspension in 5% gum arabic solution was administered orally by gastric intubation in a dose of 5 mg kg^{-1} once daily for 21 days. At 20 min, 24 h or 21 days after dosing with ^{14}C-KE-298, the rats were anaesthetized with ether and blood samples were collected from the femoral aorta into heparinized containers. Plasma was obtained immediately by centrifugation (3000 rev min^{-1}, 4°C, 10 min). The liver, kidney, lung, aorta and skin were excised and weighed. Tissues (except aorta) were homogenized with ice-cold physiological saline to yield a 20% homogenate.

Preparation of ^{14}C-M-I from ^{14}C-KE-298

^{14}C-KE-298 was dissolved in EtOH solution and adjusted to pH 9–11 with 0.4 M NaOH. After standing for 10 min at room temperature, ^{14}C-M-I was obtained from ^{14}C-KE-298. The radiochemical purity of ^{14}C-M-I exceeded 95%, as determined by HPLC. The HPLC apparatus used included a pump (305, 306; Gilson), UV detector (206PHD; Linear), radioactive monitor (Ramona 90; Raytest), column oven (E5CS; Omron) and TSK gel ODS-120A (150 mm×4.6 mm i.d.; Tosoh). The mobile phase was a solution of 0.1 M $AcONH_4$ aq./MeOH (3:2) at a flow-rate of 0.8 mL min^{-1}. Column temperature was set at 40°C and UV detection was performed at 252 nm.

In-vivo formation of ^{14}C-M-I plasma protein conjugate

^{14}C-M-I (EtOH solution, 4.68 μmol mL^{-1}) was administered into the femoral vein at a dose of 6 μmol kg^{-1}. Plasma samples (300 μL) were collected at 5, 10, 15, 30, 60 and 120 min after administration. *N*-ethylmaleimide

(final concn 10 mmol L^{-1}) was added to each sample and the preparation was left to stand at room temperature for 5 min. Plasma samples were then transferred to tubes (centriprep-10, molecular weight cut-off at 10000; Amicon) and ultrafiltrated in 3 vols of 1% sodium dodecylsulfate (SDS) solution (3000 rev min^{-1}, 4°C, 3 times). The samples were further ultrafiltrated in 3 vols of 0.9% NaCl solution and then 3 vols of MeOH was used for extraction. The radioactivity in the protein precipitate was determined as the amount of ^{14}C-M-I plasma protein conjugate.

Preparation of synthetic ^{14}C-M-I plasma protein conjugate

^{14}C-M-I (1.5 μmol) was incubated with rat plasma (15 mL) at 37°C. At 6 h after the start of the reaction, ^{14}C-M-I (1.5 μmol) was again added to the reaction mixture, which was incubated for 24 h, and then ultrafiltrated in SDS solution (final concn 1.5 mmol L^{-1}) and 0.9% NaCl solution to exclude non-conjugate radioactivity. The radiochemical purity of ^{14}C-M-I plasma protein conjugate exceeded 96%. The conjugate solution was stored at –20°C until experiments.

Identification of the structure of ^{14}C-M-I plasma protein conjugate

Synthetic ^{14}C-M-I plasma protein conjugate (final concn 0.1 mmol L^{-1}) was treated with the disulfide reducing agent dithiothreitol (final concn 5 mmol L^{-1}) in 0.1 mol L^{-1} phosphate buffer (pH 7.4, total 1 mL). After incubation at 37°C for 1 h, the reaction mixture was extracted 4 times with 3 vols of MeOH. Radioactivity in the protein precipitate was determined as the amount of ^{14}C-M-I plasma protein conjugate. The supernatant fraction was concentrated under N_2 gas and analysed immediately by HPLC.

In-vivo elimination of ^{14}C-M-I plasma protein conjugate

Synthetic ^{14}C-M-I plasma protein conjugate was administered into the femoral vein at a dose of 0.3 μmol kg^{-1}. Plasma samples (200 μL) were collected from the jugular vein under ether anaesthesia. *N*-ethylmaleimide (final concn 10 mmol L^{-1}) was added to each plasma sample, followed by extraction with MeOH. Radioactivity in the protein precipitate was determined as the amount of conjugate. The terminal half-life ($T_{\frac{1}{2}}$) of the elimination phase was calculated by the least square method.

In-vitro dissociation of ^{14}C-M-I plasma protein conjugate by endogenous thiol compounds

Synthetic ^{14}C-M-I plasma protein conjugate (final concn 0.05 mmol L^{-1}) was incubated for 1 h with cysteine or glutathione (final concn 0–6 mmol L^{-1}) in 0.1 mol L^{-1} phosphate buffer (pH 7.4, total 1 mL) at 37°C. *N*-ethylmaleimide (final concn 5 mM) was then added to the reaction mixture, followed by extraction with MeOH. Radioactivity in the extracted fraction was determined as the dissociated amount of ^{14}C-M-I plasma protein conjugate.

Measurement of radioactivity

Plasma and tissues were solubilized with 0.5–1 mL Soluene-350 (Packard), decolourized by adding H_2O_2 (0.5 mL) and isopropanol (0.5 mL), and neutralized with 1 N HCl (0.5–1 mL). Each sample was mixed with a liquid scintillation cocktail (10 mL). Radioactivity was counted using a liquid scintillation counter (LS6000TA; Beckman) for 1 min. The limit of radioactivity detection was determined twice at the background level. If the measured radioactivity was less than twice the background level, the radioactive concentration was taken as being under the limit of detection.

Statistical analysis

Data was expressed as mean $\pm$ s.d. Statistical analysis was performed using the unpaired Student's *t*-test (SAS/STAT program). Differences were considered statistically significant at $P < 0.05$.

Results and Discussion

KE-298 contains an acetylthio group that is readily deacetylated into the SH-group-containing metabolite (M-I) in rats (Yoshida et al 1996). Common SH-group-containing drugs such as PEN, bucillamine and captopril have a tendency to accumulate in tissues and this can cause various adverse effects (Park et al 1987). Although M-I, a main metabolite of KE-298, contains the thiol moiety, tissue radioactivity at 20 min after administration of ^{14}C-KE-298 (5 mg kg^{-1}) to rats decreased rapidly up to 24 h, together with a decrease of plasma radioactivity (Table 1). After 21 days of repeated administration, the radioactivity in kidney and skin increased compared with cases of single administration. However, the rate of increase was extremely low and a tendency towards accumulation was not observed in any tissue examined.

Table 1 Tissue concentration of radioactivity after single or repeated oral administration of ^{14}C-KE-298 (5 mg kg^{-1}) to rats.

Tissue	Concentration of radioactivity (μg eq./ml or g)			
	Single		21 days	
	20 min	24 h	20 min	24 h
Plasma	10.94±2.40	0.05±0.01	11.81±1.03	0.06±0.01
Liver	2.75±0.40	0.07±0.01	3.23±0.08	0.19±0.03†
Kidney	9.10±1.81	0.14±0.03	12.77±0.82*	0.38±0.03†
Lung	2.02±0.52	ND	2.81±0.43	0.09±0.01[a]
Skin	0.69±0.19	ND	1.14±0.08*	0.08±0.02[a]
Aorta	1.02±0.42	ND	0.70±0.71	ND

Each value represents the mean±s.d. of three rats. ND, not detected. *$P < 0.05$, †$P < 0.05$, significantly different compared with the values at 20 min and 24 h, respectively. [a]Significance values were not calculated.

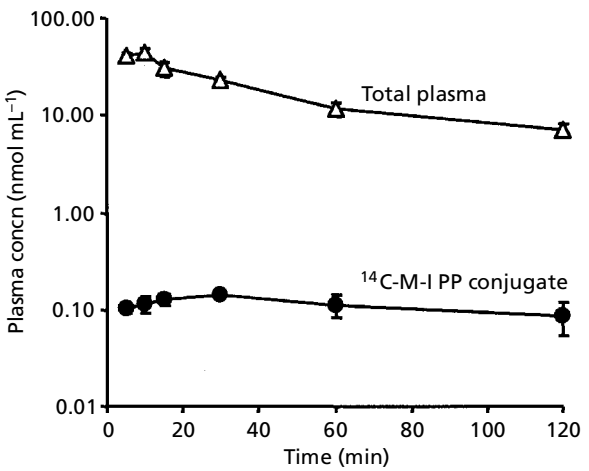

Figure 2 Formation of ^{14}C-M-I plasma protein (PP) conjugate after intravenous administration of ^{14}C-M-I (6 μmol kg^{-1}) to rats. Each point represents the mean±s.d. of three rats.

After intravenous administration of ^{14}C-M-I (6 μmol kg^{-1}) to rats, the plasma concentration of ^{14}C-M-I plasma protein conjugate was extremely low compared with the total plasma radioactivity (Figure 2). Plasma concentration of ^{14}C-M-I plasma protein conjugate increased slightly up to 30 min after administration; however, the value was low (0.143 nmol mL^{-1}) at 30 min (0.66% of total plasma radioactivity) and the concentration decreased by 0.087 nmol mL^{-1} at 120 min. In the case of ^{14}C-PEN, most of the plasma radioactivity was ^{14}C-PEN plasma protein conjugate at 2 h after oral administration of ^{14}C-PEN to rats (Nozu et al 1977). At

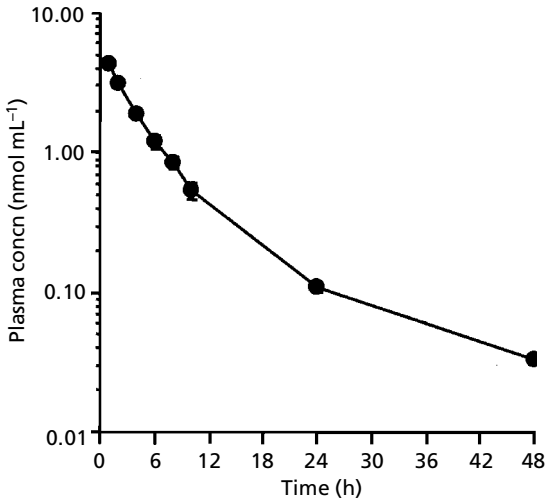

Figure 3 Elimination of ^{14}C-M-I plasma protein conjugate in plasma after intravenous administration of synthetic ^{14}C-M-I plasma protein conjugate (0.3 μmol kg^{-1}) to rats. ^{14}C-M-I plasma protein conjugate was synthesized by incubating with ^{14}C-M-I in rat plasma at 37°C. Each point represents the mean±s.d. of three rats.

2 h after intravenous administration of ^{14}C-captopril to rats, approximately 30% of plasma radioactivity existed as ^{14}C-captopril plasma protein conjugate (Park et al 1982). These results clearly show that the reactivity of the thiol moiety of M-I is less than that of the other drugs tested in-vivo.

It has been reported that the PEN plasma protein conjugate that formed in rat plasma is a mixed disulfide linkage with plasma albumin (Nozu et al 1977). We investigated the linkage form using ^{14}C-M-I plasma protein conjugate synthesized in-vitro in rat plasma, because the formation of ^{14}C-M-I plasma protein conjugate was extremely low in rats. Radioactivity of ^{14}C-M-I plasma protein conjugate (0.1 mmol L^{-1}) had a 95.66% dissociation rate with the disulfide reducing reagent dithiothreitol (5 mmol L^{-1}). Most of the dissociated radioactivity was recognized as M-I in the HPLC analysis. In the preliminary study using SDS-PAGE analysis, the plasma protein fraction forming the disulfide linkage was mainly albumin (data not shown). It follows that only a small amount of ^{14}C-M-I plasma protein conjugate would be the mixed disulfide with albumin in rats.

Since ^{14}C-KE-298 did not accumulate after repeated administration, the dissociation of ^{14}C-M-I plasma protein conjugate was investigated. After intravenous administration of synthetic ^{14}C-M-I plasma protein conjugate (0.3 μmol kg^{-1}), the radioactive concentration in plasma decreased rapidly (Figure 3). The con-

Table 2 Dissociation of ^{14}C-M-I plasma protein conjugate by cysteine and glutathione in-vitro.

Cysteine or glutathione Concn (mM)	% of ^{14}C-M-I plasma protein conjugate	
	Cysteine	Glutathione
0.5	92.33±0.31	86.69±0.44*
1	85.42±0.90	75.92±1.35*
3	49.17±4.30	28.10±0.44*
6	27.44±0.50	13.16±0.41*

^{14}C-M-I plasma protein conjugate (0.05 mmol L^{-1}) was synthesized by incubating with ^{14}C-M-I in rat plasma protein at 37°C for 1 h. ^{14}C-M-I plasma protein (0.05 mmol L^{-1}) was incubated at 37°C for 1 h. Each value represents the mean±s.d. of three experiments. *$P < 0.05$ significantly different compared with the value of cysteine.

centration of ^{14}C-M-I plasma protein conjugate was 4.32 nmol mL^{-1} at 1 h and 0.11 nmol mL^{-1} at 24 h, and the $T_{\frac{1}{2}(1-24\,h)}$ was 6.9 h. In the case of ^{14}C-PEN plasma protein conjugate, the $T_{\frac{1}{2}}$ of the plasma radioactivity was 3.48 days, which did not significantly differ from the $T_{\frac{1}{2}}$ of rat albumin (3.03 days; Joyce et al 1988). These findings suggest that the ^{14}C-M-I plasma protein conjugate was rapidly dissociated in-vivo.

Endogenous thiol compounds, cysteine and glutathione, have significant functions related to detoxification, defence against injury to cells from free radicals and active metabolites, in addition to involvement in the reduction of disulfide conjugates (Yeung et al 1983). In this study, ^{14}C-M-I plasma protein conjugate (0.05 mmol L^{-1}) was readily dissociated by the endogenous thiol compounds, cysteine and glutathione (Table 2). The physiological concentration of cysteine and glutathione in rat plasma has been reported to be 186 and 5 μmol L^{-1}, respectively (Yeung et al 1983). Since the plasma concentration of ^{14}C-M-I plasma protein conjugate was less than 0.15 μmol L^{-1} after administration of ^{14}C-M-I to rats, the concentration was much lower than that of cysteine and glutathione in-vivo. The ^{14}C-M-I plasma protein conjugate might be sufficiently dissociated by non-enzymatic disulfide interchange. In addition, it has been reported that glutathione reductase dissociates the disulfide binding (Srivastava & Beutler 1973; Yeung et al 1983). There is a possibility that M-I plasma protein conjugate is dissociated by an in-vivo enzymatic reaction as well as the non-enzymatic reaction.

Many drugs containing the thiol moiety cause common adverse effects. One reason may be tissue accumulation caused by the disulfide conjugate, and immunotoxicity is induced by conjugates such as a hapten (''hapten hypothesis''; Park et al 1987). The significant points of adverse effects based on ''hapten hypothesis'' are: (i) the relative rate of the formation and dissociation of the M-I plasma protein conjugate; and (ii) the intensity of immunogenicity of the conjugate. Although ^{14}C-M-I formed a disulfide conjugate with plasma protein, the concentration was extremely low. In addition, the ^{14}C-M-I plasma protein conjugate readily dissociated in-vivo, perhaps owing to the interaction of endogenous thiol compounds. These properties of ^{14}C-M-I may explain the lack of accumulation of radioactivity in tissues after oral administration of ^{14}C-KE-298 to rats. To further clarify the efficacy of KE-298, it would be interesting to correlate the immunogenicity of the conjugate to the rate of adverse effects.

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