Retention Mechanism of Imidazoles in Connective Tissue. IV.\textsuperscript{1)}
Identification of a Nucleophilic Imidazolone Metabolite in Rats

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Formation of a nucleophilic 4(5H)(or 5(4H))-imidazolone structure has been postulated from in vitro studies to be one of the causative elements involved in the retention of drugs with imidazole moiety in connective tissue. To confirm this, we searched for the imidazole-related metabolite in rats after intravenous dosing of 2-methylimidazole (2MI; \textsuperscript{14}C-labeled and unlabeled form, 3 and 300 \textmu mol/kg body weight) as a model compound. The excreted urine, the major route of elimination of the compound, was collected and analyzed using the HPLC/MS system with a counterion effect for metabolite separation. 2-Methyl-4(5H)(or 5(4H))-imidazolone (2MIone) was identified as a urinary metabolite by chromatographic and mass-spectral inspection with the corresponding authentic standard. Pretreatment of rats with either SKF-525A (50 mg/kg, i.p.) or cimetidine (200 mg/kg, i.p.) significantly increased the excreted amount of 2MIone in urine and the irreversible binding of 2MI equivalents in the aortic tissue, whereas both factors were reduced by pretreatment with triethylentetramine dihydrochloride (150 mg/kg/d for 5 d, s.c.). These results support the aforementioned deduction, and also raise the possibility that a cytochrome P450-independent, copper-related metabolic reaction might be involved in the imidazolone formation in vivo.

Key words  imidazolone; covalent binding; connective tissue; nucleophilic metabolite; rat

There are many drugs or drug candidates that have an imidazole moiety, and a number of them have been reported to be retained in considerable amount in the connective tissue after dosing to laboratory animals.\textsuperscript{2–3}) The mechanism of the retention, however, which potentially involves some toxicological significance, remains to be elucidated.

Investigations to identify this mechanism have been conducted in our laboratory using \textsuperscript{14}C-labeled compounds with a simple structure: imidazole and its 2-methyl derivative (2MI). A previous study in rats indicated that the in vivo retention of imidazole-containing drugs is largely attributable to irreversible binding between the imidazole moiety and elastin, a major structural macromolecule of extracellular matrix, and that their interaction might be mediated through cytochrome P450-independent biotransformation.\textsuperscript{4)} In vitro studies with cupro-ascorbate-dependent chemical oxidation have provided mechanistic information that monoxygenated 4(5H)(or 5(4H))-imidazolone product could react nucleophilically with aldehydes intrinsic to connective-tissue proteins and yield a covalent adduct via aldol-type condensation.\textsuperscript{1,2)} This reaction is shown in Fig. 1.

This paper describes the identification of an imidazolone-related metabolite in the excreted urine of rats after intravenous dosing of 2MI as a model compound. Results of a study using cytochrome P450-inhibitory and de-coppering agents are also given to provide insight into the metabolite’s biotransformation.

MATERIALS AND METHODS

Chemicals and Reagents  [Ring-C2,\textsuperscript{14}C]2MI (51.2 \textmu Ci/ \textmu mol) was purchased from Nemoto and Co., Ltd. (Tokyo). Its radiochemical purity was more than 97% by TLC analysis. The specific activity of the dosing solution was adjusted through dilution with unlabeled 2MI, obtained with more than 98% purity from Wako Pure Chemical Industries, Ltd. (Osaka). Authentic reference compounds of 2-methyl-4(5H) (or 5(4H))-imidazolone (2MIone) and 2-hydroxymethylimidazolone (2(OH)MI) were synthesized in our research center.\textsuperscript{1)} Cimetidine (CIM; Sigma Chemical Co., St. Louis, MO, U.S.A.) and SKF-525A (Funakoshi Co., Ltd., Tokyo) were used as inhibitors of cytochrome P450-mediated metabolism. Triethylentetramine dihydrochloride (TETA) was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WIS, U.S.A.) and used as a de-coppering agent. All other chemicals and reagents were of analytical grade or better and, unless otherwise indicated, were obtained from Wako Pure Chemical Industries, Ltd.

Animal Treatments  Male Wistar rats weighing 180–200 g, obtained from Japan SLC, Inc. (Shizuoka) and

\[ \text{Imidazolones} \]

\[ \text{Aldol-type condensation} \]

\[ \text{R CH \sim \text{Protein}} \]

Fig. 1. A Proposed Reaction for Covalent Binding of Imidazoles with Connective-Tissue Proteins

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allowed a standard laboratory diet and water ad libitum, were given a single intravenous dose of [14C]2MI (3 μmol/150 μCi/kg body weight) or unlabeled 2MI (300 μmol/kg, for mass-spectrometric analysis) as a solution of isotonic saline. SKF-525A (50 mg/kg) and CIM (200 mg/kg) were injected intraperitoneally into rats as a solution of isotonic saline and with a suspension with 5% gum arabic, 0.5 and 1 h before [14C]2MI dosing, respectively. TETA (150 mg/kg) in isotonic saline was given subcutaneously to the animals daily for 5 consecutive days before [14C]2MI dosing, which is thought to be an effective treatment for in vivo copper sequestration.9)

**Collection of Biological Samples** Immediately after the 2MI dosing, rats were individually placed in metabolism cages, and urine was collected in an ice-cold container as the following three fractions: 0—4, 4—8, 8—24 h. Within 24 h from the beginning of collection of each fraction, the sample was taken for analysis instead of freezing, to avoid such artificial change of a metabolite as described later.

The groups of rats which had received the 14C-labeled compound were anesthetized with diethyl-ether at 24 h post-dose, and aortas of the thoracic-abdominal portion (just below the opening of the left subclavian artery—above the common iliac artery) were obtained anatomically and used for examination of irreversible binding of radioactivity.

**Determination of Irreversibly Tissue-Bound Radioactivity** The radioactivity bound irreversibly to aortic tissue was determined as the difference in tissue homogenate resistant to washing in a series of trichloroacetic acid and methanol, as described previously.6)

**Radiometric Assay** A scintillator (Insta-gel or Hionic-fluor; Packard Instruments Co., Downers Grove, IL, U.S.A.) was added directly to each sample diluted appropriately with water, and radioactivity was determined in a Beckman LS6000TA liquid scintillation counter (Fullerton, CA, U.S.A.).

**HPLC Analysis** A Gilson model 305 system (Middleton, WI, U.S.A.) equipped with a radioisotope and a UV detector was employed. A C18-silica-packed column (Sumipack ODS A212, 5 μm, 6.0 mm i.d.×150 mm, Sumika Chemical Analysis Service, Osaka) was maintained at 50°C and subjected to the following gradient elution system at a flow rate of 1 ml min⁻¹: a mobile phase of water/methanol containing 0.1% (w/v) perfluoroketanoic acid (Kanto Chemical, Tokyo) as an ion-pairing reagent, a linear ramp from 0 to 13% methanol over 20 min with a hold for 20 min, followed by a further linear ramp up to 60% over 10 min with a hold for 10 min. Urine samples (ca. 50 μl) were injected into the above apparatus through a 0.45 μm membrane filter. For radioactivity quantification, the HPLC effluent was appropriately fractionated, mixed with a Flo-Scint II scintillation cocktail (Packard), and then analyzed using a Beckman LS6000TA system. The total recovery of chromatographed 14C material was over 96%.

**Mass-Spectrometric Analysis** Thermospray liquid chromatography/tandem mass spectrometry (TSP LC/MS/MS) was performed on a Finnigan MAT TSQ70 triple-stage quadrupole mass spectrometer (San Jose, CA, U.S.A.). HPLC separation of the samples was conducted with a Shimadzu LC9A liquid chromatograph (Kyoto) under the same conditions as described above, and a post-column addition of 0.6 M ammonium acetate to the mobile phase was performed at a flow rate of 0.2 ml min⁻¹ for efficient ionization of analytes. Operating conditions of the spectrometer were set as follows: ion source temperature, 250°C; vaporizer temperature, 85°C repeller voltage, 20 V; collision gas, argon at a pressure of 10⁻³ Torr; collision energy, 40 eV.

**Statistical Analysis** Statistical comparisons between control and treatment groups were performed using the unpaired t-test. Differences were considered to be significant when p<0.05.

**RESULTS AND DISCUSSION**

**Identification of 2MIone as a Urinary Metabolite** Following the intravenous administration of [14C]2MI at a dose of 3 μmol/kg to rats, the compound-related radioactivity was principally excreted in urine, which amounted to ca. 71.3, 77.6, and 78.1% of the dose within 4, 8, and 24 h, respectively. Thus the metabolite profile was examined on the urine sample. A radio-HPLC profile of the 0—4 h urine is shown in Fig. 2. Four radioactive peaks with retention times of 4, 17, 21, and 30 min were detected; their excretion accounted for ca. 41, 2.6, 1.3, and 60.4% of the dose, respectively. The last-eluted peak was identical to that of authentic 2MI on UV detection at 210 nm. When the same urine sample was processed to freezing and then thawing, a decrease of the peak with a retention time of 17 min was observed and, instead, a newly formed peak eluted at 39 min was detected on HPLC (chromatogram not shown). This artificial product was not detected during the sample storage on ice up to at least 24 h (including the period of urine collection). Further, when the rat blank urine samples spiked with [14C]2MI at final concentrations ranging from 10 μg to 1 mg were prepared and allowed to stand on ice for 24 h, no product was observed on HPLC at any designated concentration. This confirms that 2MI itself is chemically stable under a condi-

![Fig. 2. Radio-HPLC Profile of Rat Urine (0—4 h) after Intravenous Administration of [14C]2MI (3 μmol/kg)](image-url)
Fig. 3. Total Ion Chromatogram of Rat Blank Urine (a) and of Urine Fraction (0–4 h) of the Rat Dosed with 2MI (300 μmol/kg) (b). Two peaks 1 and 2, not present in blank urine, were observed.

Fig. 4. Mass-Spectrometric Observation of Peak 1
(a) Mass spectrum of peak 1. (b) Product ion mass spectrum of m/z 99 from peak 1.

tion of the urine collection.

Further analysis was conducted using TSP LC/MS/MS. The urine fraction of 0–4 h after intravenous administration of 2MI at a dose of 300 μmol/kg to rats was used. Total ion chromatograms of this sample and blank urine are shown in Fig. 3. Two peaks 1 and 2, absent in blank urine, were observed. The chromatographic behavior and mass-spectral data of peak 2 proved to be identical with those of authentic 2MI. The mass spectrum of peak 1 displayed a protonated molecular ion at m/z 99 (Fig. 4a), 16 mass units greater than that of 2MI, suggestive of a monoxygenated metabolite from 2MI. Collisional activation of the 99 ion at an energy of -40 eV gave fragment ions at m/z 42 (base peak) and 30 (intensity of 43.4% of the base peak) (Fig. 4b). These fragments have been found to be characteristic of the 2Mlone structure.11 Authentic 2MIone showed the same mass fragmentation and retention time on HPLC as those of the metabolite of peak 1. This identity is also supported by the aforementioned artificial product formation, which is possibly due to a nucleophilic reaction of 2MIone with an endogenous electrophile in the concentrated urine during freezing and/or thawing process, although no structural information is available for the product except that it showed a possible quasi-molecular ion at m/z 244. Ions corresponding to the 13C peaks with retention times of 4 and 21 min were not clearly observed in the mass-spectrometric analysis because
of the presence of interfering ions from endogenous substances. However, the latter \(^{14}\)C metabolite was co-eluted with authentic 2(OH)MI, suggesting the possible presence of 2(OH)MI as a urinary metabolite. The former \(^{14}\)C peak decreased by pretreatment of rats with either SKF-525A or CIM, suggesting that its component might result partially from cytochrome P450-mediated metabolism.

Oxidation in the imidazole ring is a frequent feature in the mammalian metabolism of several imidazole/imidazoline-containing compounds,\(^9\)\,\(^\ldots\)\(^13\) and a few reports have dealt with the (4(5H)\,(or 5(4H))-imidazolone formation as a possible intermediate step followed by further oxidation or scission of the ring structure, as is the case with midaglizole.\(^11\)

However, as far as we are aware, no information has been available concerning the identifiable detection of the imidazolone analogue as a metabolite. This may be either because the subsequent ring modification largely takes place in vivo, or if the imidazolone analogue occurs in the biological fluids even in small amounts, it might undergo such an artificial product formation as shown herein through sample preparation. The present results demonstrated that 2MIone, a compound with nucleophilicity,\(^1\) is actually generated from 2MI in rats, although its urinary excretion was only a small proportion of the dose (approximately 3\% of the 3 \(\mu\)mol/kg dose in 0—24-h urine).

### Pretreatment Effect of Cytochrome P450-Inhibitory and De-coppering Agents

An attempt was made to determine whether the in vivo 2MIone formation, together with irreversible binding of 2MI equivalents in the aortic tissue, can be altered by pretreatment of rats with the above agents. The data on the \(^{14}\)C2MI dosing (3 \(\mu\)mol/kg) are listed in Table 1. Pretreatment with either SKF-525A or CIM significantly increased the excreted amount of 2MIone in urine (0—24 h) and also the tissue binding of 2MI equivalents at 24 h post-dose, as compared with the corresponding control levels (non-pretreatment), whereas they were reduced by TETA pretreatment.

These findings are compatible with our previous proposi-