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IN VITRO AND *IN VIVO* INHIBITION OF CYSTEINE PROTEINASES BY EST, A NEW ANALOG OF E-64*

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E-64 isolated from a culture of *Aspergillus japonicus* is a specific inhibitor of cysteine proteinases. E-64-c, a synthetic analog of E-64, was effective in model animals of muscular dystrophy only when it was given intraperitoneally and by means of osmotic minipump. It showed no effects due to its low absorbability from intestine when it was administered orally. EST, the ethyl ester of E-64-c, was expected to be readily absorbed through intestinal membrane, since it is more lipophilic than E-64-c.

Both EST and E-64-c have a high specificity to cysteine proteinase similar to E-64 but E-64-c was 100 to 1000 times stronger than EST in *invitro* cathepsin inhibition.

However, EST was stronger than E-64-c in cathepsin inhibition when given orally. The cathepsin B&L activities (whole activities of cathepsins B and L) in the skeletal muscle, heart and liver of hamsters were strongly inhibited soon after oral administration of 100 mg/kg body weight of EST. The inhibition continued for at least 3 h and then disappeared gradually. E-64-c was found in plasma of hamster treated with EST, but unchanged EST was not found. These results suggested that EST was converted to E-64-c, a more active form, during the permeation through intestinal membrane. The conversion of EST to E-64-c was also indicated by the absorption experiment using *in situ* loop method. EST was thus shown to be useful as an oral drug and expected to be effective in therapeutic trials using model animals.

Keywords — cysteine proteinase inhibitor; intestinal absorption; cathepsin B; oral administration

INTRODUCTION

The degradation of myofibrils in muscle atrophy has been considered to be attributable to proteinases. Studies from several laboratories have indicated that the activities of cysteine proteinases including calcium activated neutral protease (CANP)[1] and cathepsins[2] were elevated in Duchenne muscular dystrophy. It has been presumed that CANP causes the fragmentation of the myofibrils at the initial stage of muscle degradation from the observations of a focal accumulation of calcium and Z-band loss in muscular dystrophy,[3,4] and then lysosomal cathepsins complete the muscle protein degradation. Therefore, there has been a growing expectation of the possible use of specific ihibitors in treatment of Duchenne muscular dystrophy.

E-64 is a potent inhibitor of cystein proteinases isolated from a culture of *Aspergillus japonicus*.[5] It has a strong affinity only for cysteine proteinases[5,6] and reacts irreversibly with the thiol group at the active site.[7,8] Many analogs were synthesized and evaluated to obtain superior inhibitors for medical application.[6,7,9,10] E-64-c has been selected as a strong candidate due to inhibitory activity against lysosomal cathepsins and CANP.[7,10]

Hudecki *et al.* reported that intraperitoneal administration of E-64-c significantly improved the righting ability of dystrophic chickens.[11] Jasmin and Proschek showed that E-64-c administered by means of an osmotic minipump significantly impeded the progression of the necrotic changes accompanied by a marked decrease in myocardial calcium content in muscular dystrophic hamsters, UM-X7.1.[12] However, no effects were apparent when it was given orally due to its low absorbability from intestine.

Since an oral drug is desirable for long term use in chronic diseases such as muscular dystro-

* Abbreviations; EST(Ep-453): Ethyl(+)-(2*S*,3*S*)-3-[(*S*)-3-methyl-1-(3-methylbutylcarbamoyl)butylcarbamoyl]-2-oxiranecarboxylate
E-64-c (Ep-475): (+)-(2*S*,3*S*)-3-[(*S*)-3-methyl-1-(3-methylbutylcarbamoyl)butylcarbamoyl]-2-oxiranecarboxylic acid

phy, we tried to improve E-64-c in terms of absorbability and found EST, the ethyl ester of E-64-c, to be well absorbed from intestine and rapidly hydrolyzed into E-64-c. In this report the *in vitro* inhibitory spectrum, *in vivo* inhibitory activity and absorbability will be described and the usefulness of EST as an oral drug will be also discussed.

MATERIALS AND METHODS

1. Materials — EST and E-64-c were synthesized in our laboratory and the structures are shown in Fig. 1. Purified cathepsins B and H were kindly donated by Dr. N. Katunuma (Tokushima university, Tokushima). Elastase, amino acid arylamidase (microsomal aminopeptidase), chymotrypsin (type A_4), glyceraldehyde-3-phosphate dehydrogenase and hexokinase were purchased from Boehringer Mannheim GmbH (W. Germany). Trypsin, papain, cathepsin D and collagenase (type VII) were obtained from Sigma Chemical Co. (USA).

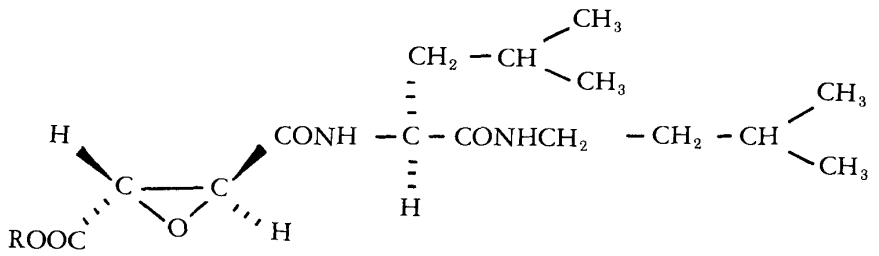
2. Determination of in Vitro Inhibitory Activity — Inhibitory activities of EST and E-64-c against cathepsins B and H, papain and collagenase were determined by the methods of Barrett *et al.*[6] Inhibitory activities against elastase, chymotrypsin, trypsin, cathepsin D, microsomal amino peptidase, hexokinase and glyceraldehyde-3-phosphate dehydrogenase were assayed according to the methods of Travis and Johnson,[13] Travis and Morii,[14] Cechova,[15] Mycek,[16] Pfleiderer,[17] Grossbard and Schinke[18] and Duggleby,[19] respectively.

A solution of EST in dimethylsulfoxide was used in these assays. E-64-c was neutralized with sodium bicarbonate to dissolve and diluted with distilled water containing dimethylsulfoxide before use.

3. Assay of Enzyme Activity in Animal Tissues — Male Syrian hamsters (7 weeks old) were orally given 100 mg/kg body weight of EST or E-64-c. The inhibitors were given to animals as a suspension in 0.5% carboxymethylcellulose solution containing 0.1% Tween 80. Blood for determination of drug concentration was collected into heparinized test tubes from the femoral artery and vein at the time indicated. After elimination of the remaining blood by perfusion of saline, liver, heart and quadriceps femoris were removed and separately homogenized in phosphate buffer-saline (pH 7.4) containing 0.1% of Triton X-100 using Ultra Tarrax (Janke & Kunkel GmbH, W. Germany). Each homogenate was then measured of cathepsin B&L, cathepsin D and acid phosphatase activity.

Suc-L-Tyr-L-Met-naphthylamide[20] was used for the assay of whole activity of cathepsin B and L (shown as cathepsin B&L activity hereafter). Each assay tube (1 ml) contained 0.1 M acetate buffer, pH 5.0, 2 mM cysteine, 1 mM ethylenediamine tetraacetic acid, 7.5% dimethylsulfoxide, 0.3% Triton X-100 and homogenate. After 10 min preincubation at 37 °C, 0.025 ml of 80 mM substrate in dimethylsulfoxide was introduced to start the reation. Precisely 60 min later, released 2-naphthylamine was determined by the method of Barrett.[21]

Assay of cathepsin D activity was carried out in 0.2 ml of reaction mixture containing 0.1 M acetate buffer, pH 3.8, 0.3% Triton X-100, 0.84 mg of [^{14}C]methylhemoglobin (0.2 μCi/mg)[22] and homogenate at 30 °C. After 30 min incubation, the reaction was stopped with 0.2 ml of



EST: R = C_2H_5 E-64-c: R = H

FIG. 1. *Structures of Inhibitors*

10% trichloroacetic acid and the radioactivity of the supernatant fluid was determined.

Acid phosphatase activity was assayed in 3 ml of reaction mixture containing 6.0 mM sodium *p*-nitrophenylphosphate, 33 mM acetate buffer, pH 5.0, 0.3% Triton X-100 and homogenate. After 10 min incubation at 37 °C, the reaction was stopped with 1 ml of 1 N sodium hydroxide and A_{405} of the solution was determined.

4. Determination of Absorbability —The absoption from the intestine of rats was examined by an *in situ* loop method. Fasted male Wistar rats weighing about 150 g were anesthetized with urethane. The pylorus, the intestine at 5 cm from pylorus and bile duct were ligated. Each drug suspension (1 mg/0.1 ml 5% gum arabic solution) was injected into the ligated intestine. After a given period of time, the intestinal loop and blood were removed for determination of drug content.

5. Determination of EST and E-64-c by HPLC —The plasma (0.5 ml) was mixed with 0.5 ml of 5% phosphoric acid solution and then extracted with 7 ml of ethylacetate. Five ml of the upper layer were evaporated and the residue was dissolved in 1 ml of the solution for mobile phase (methanol : water : phosphoric acid = 54.5 : 45.0 : 0.5) and applied to a Nucleosil C-18 column (4×150 mm, packed with 5 μm particles) which was run at 1.0 ml/min at 40 °C and monitored at 210 nm using a high performance liquid chromatography (HPLC).

The intestinal loop which was taken from a rat was homogenized in 5 ml of 5% phosphoric acid solution and 30 ml of ethylacetate. After centrifugation, 10 ml of organic layer was obtained. The concentrations of EST and E-64-c were determined by the use of HPLC as described above for plasma.

RESULTS AND DISCUSSION

The effects of EST and E-64-c on various purified enzymes were investigated. Although both compounds inhibited cysteine proteinases such as cathepsins B and H and papain, as shown in Fig. 2, E-64-c was 100 to 1000 times stronger than EST.

E-64-c and EST showed no inhibitory activities even at the concentration of 1 mM, against non-cysteine proteinases such as trypsin, chymotryspin, elastase, aminopeptidase (microsomal), collagenase (bacterial) and cathepsin D

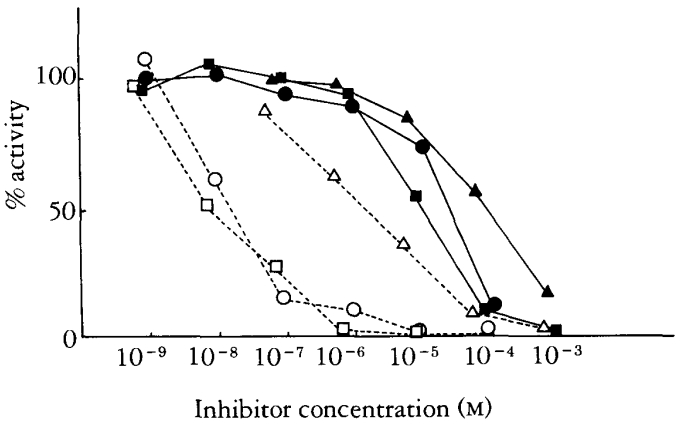


FIG. 2. *Inhibitory Activities of EST and E-64-c against Cysteine Proteinases in Vitro*
The remaining activity of cathepsin B (●, ○), cathepsin H (▲, △) or papain (■, □) was measured as described in the "Materials and Methods" after preincubation for 5 min with EST (filled symbols) or E-64-c (open symbols) in the same condition used for measurement of the activity.

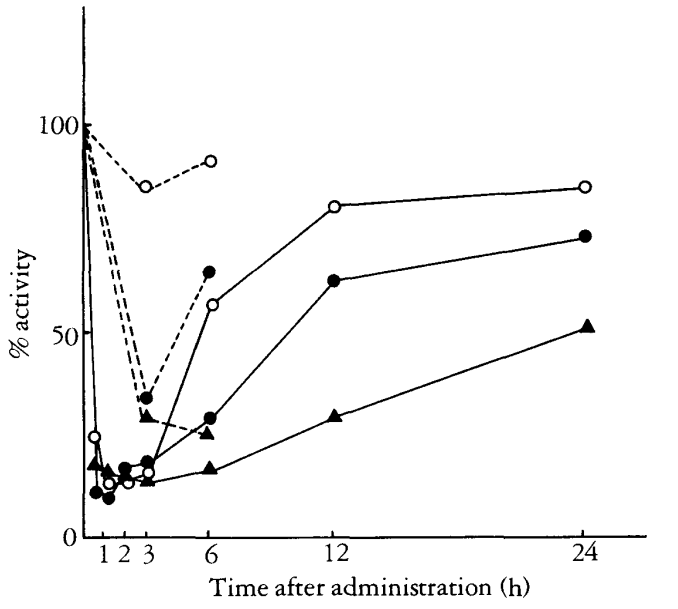


FIG. 3. *Change in Cathepsin B&L Activity in Hamster after Oral Administration of EST or E-64-c*
Syrian hamsters were orally given 100 mg/kg body weight of EST (——) or E-64-c (-----). At the time indicated, hamsters (n=4) were killed. The activity of cathepsin B&L in heart (○), muscle (●) or liver (▲) was measured as described in the "Materials and Methods."

and non-proteolytic thiolenzymes, such as glyceraldehyde-3-phosphate dehydrogenase and hexokinase. These results indicate that both E-64-c and EST are strictly specific cysteine proteinase inhibitors in analogy with E-64.

The effects of the orally administered EST and E-64-c on the cathespsin B&L activities (whole activity of cathepsins B and L) in skeletal muscle, heart and liver were examined using normal hamsters. Figure 3 shows the time courses of change in cathepsin B&L activity after an oral treatment with 100 mg/kg body weight of each compound. Most of the cathepsin B&L activity was inhibited within 0.5 h after the administration of EST in every tissue. The inhibition continued for at least 3 h and then disappeared gradually within 24 h. It seems likely that the recovery of enzyme activity is due to the regeneration but not to the reversible restoration of the activity, since E-64-c irreversibly reacts with cathepsin B.[6,7] The inhibition was the highest in liver and the lowest in heart, showing the distribution amount of E-64-c into the corresponding tissue cells. When administered orally, E-64-c was far less effective than EST in inhibition. Both EST and E-64-c had no effect on cathepsin D and acid phosphatase activity as shown in Figs. 4 and 5. These results suggest that these inhibitors affected only systeine proteinases *in vivo* as well as *in vitro*.

Figures 6 shows the time dependent profile of E-64-c concentration in plasma of the same hamsters described above. E-64-c was found in the plasma of hamster treated with EST, but unchanged EST was not detected (<0.1 μg/ml). E-64-c appeared within 0.5 h after the administration and completely disappeared within 6 h. The concentration of E-64-c in plasma of hamsters treated with E-64-c was far less than that of the group treated with EST. This difference may be attributed to the difference in the absorbability of these compounds from the intestine.

The excellent absorbability of EST and its conversion to E-64-c was confirmed by the absorption experiment using an *in situ* loop method. As shown in Fig. 7, about 40% of EST

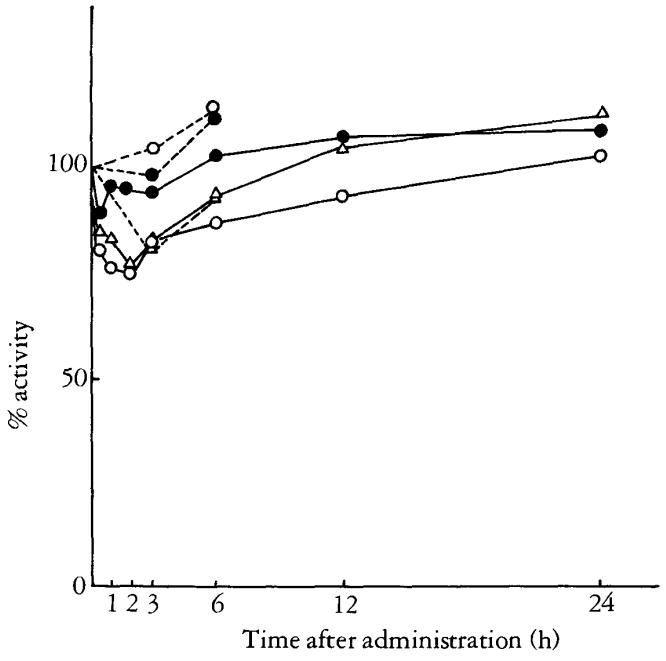


FIG. 4. *Change in Cathepsin D Activity in Hamster after Oral Administration of EST or E-64-c*

Syrian hamsters were orally given 100 mg/kg body weight of EST (——) or E-64-c (-----). At the time indicated, hamsters (n=4) were killed. The activity of cathepsin D in heart (○), muscle (●) or liver (△) was measured as described in the "Materials and Methods."

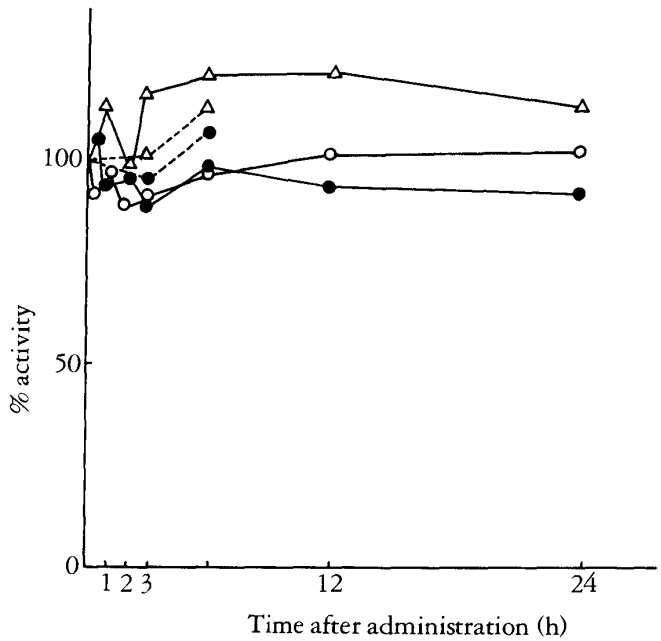


FIG. 5. *Change in Acid Phosphatase Activity in Hamster after Oral Administration of EST or E-64-c*

Syrian hamsters were given orally 100 mg/kg body weight of EST (——) or E-64-c (-----). At the time indicated, hamsters (n=4) were killed. The activity of acid phosphatase in heart (○), muscle (●) or liver (△) was measured as described in the "Materials and Methods."

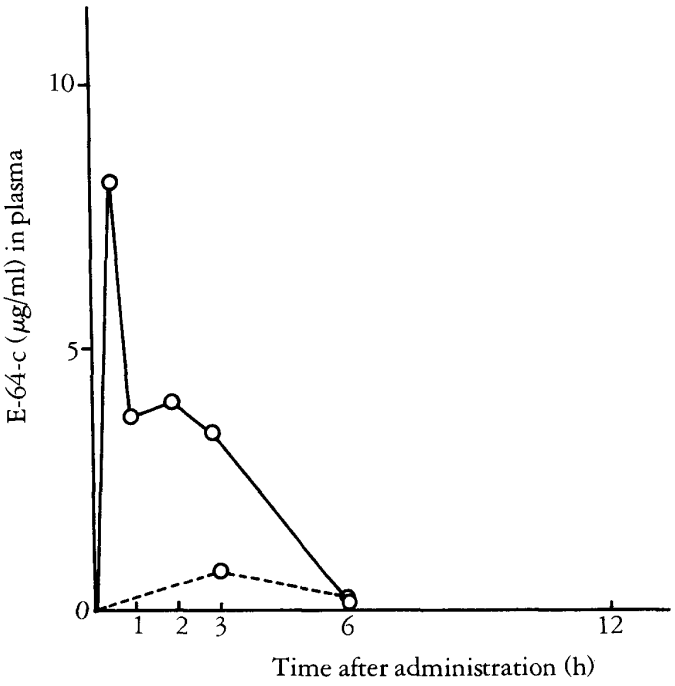


FIG. 6. *Time Dependent Profiles of Changes in the Concentration of E-64-c in Plasma of Hamsters Treated with EST or E-64-c*

The concentrations of E-64-c in plasma of hamsters (n=4) treated with EST (○——○) or E-64-c (○-----○) were determined as described in the "Materials and Methods."

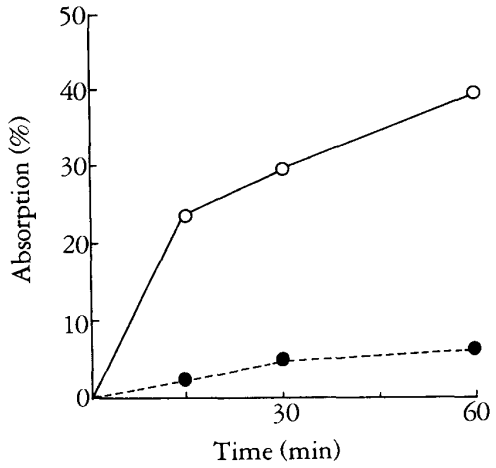


FIG. 7. *Time Dependent Profiles of Changes in the Absorption from Intestine of Rats Treated with EST and E-64-c*

EST (○——○) or E-64-c (●-----●) was injected into the intestinal loop of rats (n=3) as described in the "Materials and Methods." The absorption was calculated from the remaining amount of the drug in the intestinal loop.

given was adsorbed from rat intestinal loop within 60 min after injection, while only about 5% was absorbed in case of E-64-c. In plasma of the rats treated with EST, E-64-c was found but the original EST was not found as it was in the plasma of hamsters. E-64-c was not observed in the plasma of rats treated with E-64-c. In addition, the study using ^{14}C labeled compound showed that EST was converted to E-64-c during the permeation through intestinal membrane.[23]

As a general rule, the absorbability of a compound that dissociates into the ionic form in intestine is not good. E-64-c is presumed to be in an ionic state in neutral intestinal fluid since it is strongly acidic owing to the carboxyl group in its structure. Therefore, it seems likely that the prevention of dissociation into the ionic form by esterification of the carboxyl group should result in an improvement in absorbability.

The cathepsin inhibitions with orally administered EST in the tissue of hamster were more potent than that expected from the *in vitro* activity. This was anticipated from the excellent absorbability from intestine, followed by the conversion to E-64-c with a stronger inhibitory activity. These results indicate that EST is useful as an oral drug.

There are many reports concerning the marked elevation in proteinase activity, such as cathepsins and CANP, accompanying the progressive loss in muscle protein associated with the development of some diseases including muscular dystrophy.[1,2,24,25] The inhibition of EST against CANP, which was presumed to cause the fragmentation of myofibrils at the initial stage of muscle degradation, was not determined because the selective assay system for the activated CANP *in vivo* was not established. Suzuki, however, has reported that E-64-c irrversibly inactivated CANP only in the presence of calcium.[10] EST was naturally expected to inhibit not only cathepsins but the action of CANP *in vivo* from the fact that orally administered EST was converted to E-64-c soon after the permeation through intestinal membrane. Therefore, EST was expected to delay the progress of muscle breakdown when it was given orally.

We have done some therapeutic investigation with dystrophic hamsters by feeding food containing EST and found it helpful. This work will

be described elsewhere.

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