PROLONGATION OF LIFE SPAN OF DYSTROPHIC HAMSTER BY CYSTEINE PROTEINASE INHIBITOR, LOXISTATIN (EST)

MASAHARU TAMAI, SADAFUMI OMURA, MASAACKI KIMURA, KAZUNORI HANADA* AND HIDEO SUGITA**

*Research Center, Taisho Pharmaceutical Co., Ltd., * Omiya, Saitama, 330 Japan and National Center for Nervous, Mental and Muscular Disorders, ** Kodaira, Tokyo, Japan*

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We studied the effect of loxistatin (EST), an orally applicable cysteine proteinase inhibitor, on dystrophic hamsters, UM-X7.1. E-64-c, an active form of EST, was continuously found in plasma when the feed containing EST was given *ad libitum*. The drug concentration in plasma of dystrophic hamsters receiving EST feed showed distinct dose dependency. A diet containing EST from weaning to death significantly prolonged the survival period of the dystrophic hamsters.

Keywords — loxistatin; ethyl (+)-(2*S*,3*S*)-3-[(*S*)-3-methyl-1-(3-methylbutylcarbamoyl)-butylcarbamoyl]-2-oxiranecarboxylate (EST); (+)-(2*S*,3*S*)-3-[(*S*)-3-methyl-1-(3-methylbutylcarbamoyl)-butylcarbamoyl]-2-oxiranecarboxylic acid (E-64-c); protease inhibitor; cysteine proteinase; oral administration; dystrophic hamster; dystrophy

INTRODUCTION

The most characteristic clinical feature of muscular dystrophy in humans and animals is progressive muscle atrophy and weakness with continuous loss of muscle proteins from muscle cells. The abnormal elevation of cysteine proteinase activities, including calcium activated neutral protease (CANP)^{1,2)} and cathepsins,³⁾ in the muscular dystrophic muscle have suggested that these proteinases contribute to the degradation of muscle proteins. In particular, it has been proposed that CANP removes the Z band to cause fragmentation or disassembly of the myofibrils at the initial stage of muscle fiber degradation.⁴⁾ Therefore, there has been considerable interest in the specific inhibitors against CANP and cathepsins as possible modulators of the atrophy in muscular dystrophy.

E-64-c,⁵⁾ (+)-(2*S*,3*S*)-3-[(*S*)-3-methyl-1-(3-methylbutylcarbamoyl)-butylcarbamoyl]-2-oxiranecarboxylic acid, is a synthetic analog of E-64, (+)-(2*S*,3*S*)-3-[(*S*)-3-methyl-1-(4-guadinobutylcarbamoyl)-butylcarbamoyl]-2-oxiranecarboxylic acid.⁶⁾ It is a specific inhibitor of cysteine proteases such as CANP^{7,8)} and cathepsins.^{9,10)} The inhibitor has been reported to prevent the development of necrotic changes and calcium deposition in hearts of dystrophic hamsters when it was given *via* a subcutaneously implanted osmotic minipump.¹¹⁾ In addition, it has been reported that the intraperitoneal adminis-

tration of E-64-c significantly prolonged the righting ability of genetically dystrophic chickens.¹²⁾ Therefore, E-64-c was expected to be a therapeutic agent of muscular dystrophy. However, E-64-c was not effective when given orally since its absorbability from the intestine was low.

Because an oral drug is desirable for long term use in chronic disease such as muscular dystrophy, we tried to improve the absorbability of E-64-c from the intestine. We found loxistatin (EST), an ethyl ester of E-64-c, to be a prodrug of E-64-c. EST, ethyl (+)-(2*S*,3*S*)-3-[(*S*)-3-methyl-1-(3-methylbutylcarbamoyl)-butylcarbamoyl]-2-oxiranecarboxylate,¹³⁾ showed strong inhibitory activity against cysteine proteinase in tissues when given orally. EST was shown to be converted into E-64-c after permeation through the intestinal membrane.¹⁴⁾

These data encouraged us to extend the period of EST treatment in expectation of some delay in progression of the disease. We report here the effect of feed containing EST on the survival period of dystrophic hamsters.

MATERIALS AND METHODS

Animals and Protocol of the Procedure — Dystrophic hamsters, UM-X7.1, which were developed from BIO 14.6 dystrophic hamsters by Jasmin¹⁵⁾ and healthy hamsters from an unrelated strain (originally from Lakeview Farm, New

Jersey, U.S.A.) were used in this study. These hamsters were kindly provided by Dr. Jasmin and bred in our laboratory.

The animals were maintained under a controlled housing condition and given a diet (CMF, Oriental Yeast Co., Ltd., Tokyo, Japan) containing 0.0375, 0.125 or 0.375% of EST *ad libitum*. From the daily uptakes of these feed, the net uptakes of EST were calculated as approximately 30, 100 and 300 mg/kg body weight/d. Control animals were fed a standard diet (CMF without EST). A litter of animals was divided into three almost equal groups for comparison.

Healthy male hamsters were used in the experiment on the circadian change of the drug concentration in plasma. After feeding with 0.375% feed for a week, animals ($n=4$) were sacrificed and the blood was obtained at the time indicated. Male and female dystrophic hamsters were used to examine dose dependency and the blood was obtained at 10 AM.

In the experiment on survival period of dystrophic hamsters, the control group (18 males and 15 females) received the standard diet, the 30 mg EST group (18 males and 15 females) received 0.0375% EST diet and the 100 mg EST group (17 males and 16 females) received 0.125% EST diet from 21–28 d after birth to death.

Analytical Procedure — E-64-c concentration

FIG. 1. Circadian Change of E-64-c Concentration in Plasma of Normal Hamsters Given Feed Containing EST *ad Libitum*

Vertical bar shows standard deviation.

in plasma was determined by the method previously reported¹⁴⁾ using high performance liquid chromatography (HPLC). In the experiment on dose dependency, each plasma was extracted with ethylacetate and then the residue was obtained by evaporation of the ethylacetate layer. After the methylation of the residue with diazomethane, E-64-c concentration was determined by combined gas chromatography-mass chromatogram method using a Shimadzu LKB-9000 mass spectrometer (Kyoto, Japan).

In the experiment on survival period, the animals were checked clinically every morning at 9–10 AM. All animals found dead were necropsied as early as possible after discovery. For a histopathological examination, the portion of heart, liver and lung in which marked changes were observed macroscopically and the diaphragm as a representative of the respiratory muscles were immediately fixed in 10% neutral formalin solution and embedded in paraffin. The embedded tissues were cut in 4 μm sections which were stained with hematoxylin-eosin or azan.

FIG. 2. E-64-c Concentration in Plasma of Dystrophic Hamsters Treated with Feed Containing EST

The best fitted line is drawn by the method of least squares. Correlation coefficient (r) and regression equation are 0.8792 and $y = 3.464x - 31.094$ ($n = 24$, $p < 0.001$). Vertical bar shows standard deviation.

TABLE I. *Average Survival Period of Dystrophic Hamsters Treated with Feed Containing EST*

	Male	Female	Total
Control	212.2 ± 35.2 (18)	218.2 ± 44.6 (15)	214.9 ± 39.2 (33)
30 mg EST	258.7 ± 43.2 ^{b)} (18)	234.7 ± 57.9 (15)	247.8 ± 51.0 ^{b)} (33)
100 mg EST	258.3 ± 62.7 ^{a)} (17)	254.3 ± 57.9 (16)	256.3 ± 66.6 ^{b)} (33)

a) p < 0.05. b) p < 0.01. Mean ± S.D. (number of animals).

RESULTS

E-64-c Concentration in Plasma

In the plasma of hamsters receiving EST feed, E-64-c was found but the original EST was not detected. The E-64-c concentration in plasma was the highest at 9 AM and the lowest at 9 PM during a given day. The concentration in the daytime was about half that in the nighttime and E-64-c was continuously detected in plasma throughout the 24 h examined (Fig. 1). There was a steady increase in plasma E-64-c concentration in proportion to the EST concentration in the feed (Fig. 2). The relationship was clearly linear with a correlation coefficient (*r*) of 0.8972 (*p* < 0.001). There was no difference between the plasma drug concentrations of male and female.

Survival Period

The average survival period of the untreated dystrophic hamsters was 214.9 d which is approximately one third of the normal life expectancy in this species (Table I). The long term feeding of 30 or 100 mg/kg/d EST feed from weaning to death prolonged their life span with statistical significance (Student's *t*-test, *p* < 0.01). The life span of male hamsters in the control group did not differ from that of the female. Though the life spans of both sexes were prolonged by EST, the effect on the female was not statistically significant. Statistical analysis of the survival curves, using the method of Cox-Mantel, revealed a significant difference between drug-treated and drug-untreated dystrophic hamsters (30, 100 mg EST group, *p* < 0.005). As shown in Fig. 3, all of the control animals died before 300 d after birth, but 4 out of 33 animals in the 30 mg EST group and 10 out of 33 animals in the 100 mg EST group were still alive. The treatment with EST did not influence body weight gain in either sex.

All animals found dead during the survival experiment were necropsied. The gross and his-

topathological findings were similar in treated and non-treated animals. The pathological changes in most of the dead animals were extensive subcutaneous edema, hydrothorax, cardiac enlargement accompanied with mural thrombin and either cavity or congestive liver enlargement, suggesting congestive heart failure.

DISCUSSION

In this study, EST was mixed into the diet and fed to dystrophic hamsters, UM-X7.1. From the fact that E-64-c was constantly observed in the plasma of hamsters receiving EST feed throughout 24 h, it seems likely that hamsters, though they are nocturnal animals, take some food even in daytime. The uptake of EST diet by the animals did not differ from that of the standard diet in a preliminary experiment. Therefore, the effect of EST was considered not to be due to the difference in the feeding condition. These results indicated that feeding a diet containing EST was applicable in an experiment for drug evaluation.

The pathological findings of dead animals

FIG. 3. *Effect of the Feed Containing EST on the Survival Period of Dystrophic Hamsters, UM-X7.1*
—, control; ····, 30 mg EST group; ----, 100 mg EST group.

suggested that the chronic passive venous congestion and the progressive cardiovascular insufficiency were the ultimate causes of death in both treated and non-treated animals. The prolongation of the life span may be attributable to the delay of the progression of heart failure. This was consistent with the results obtained with both EST¹⁶⁾ and E-64-c¹¹⁾ which significantly improved necrotic changes and abnormal deposition of calcium in heart.

The effect on the survival period of female hamsters was not statistically significant, but in the case of male, the effect was significant. However, the life span of female was also prolonged by EST. The survival periods of the female hamsters in control and the 100 mg EST group were nearly identical with the corresponding survival periods of all animals, as shown in Table I. If the number of female hamsters used in the group was increased, it is possible that the effect of EST on the female life span may become statistically significant.

A significant prolongation of the survival period of dystrophic hamsters was the most prominent outcome of EST in view of its application for clinical use. Komatsu *et al.*¹⁷⁾ have also reported that EST treatment significantly improved the survival period and functional ability of hereditary dystrophic mice. This result suggested that EST was effective not only in the heart but also in the skeletal muscle. From these positive effects of the proteinase inhibitor on survival period and functional ability of dystrophic animals, the suppression of muscle protein degradation seems to result in the delay of the progress of muscular dystrophy.

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