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Enhanced anti-tumor activity and alleviated hepatotoxicity of clotrimazole-loaded suppository using poloxamer–propylene glycol gel

Chul Soon Yong [a], Jing Ji Xuan [a], Seung-Hwan Paek [a], Yu-Kyoung Oh [b], Jong-Soo Woo [a], Mann Hyung Lee [c], Jung-Ae Kim [a,**], Han-Gon Choi [a,*]

[a] *College of Pharmacy, Yeungnam University, 214-1, Dae-Dong, Gyongsan 712-749, South Korea*
[b] *School of Life Sciences and Biotechnology, Korea University, Anam-Dong, Seoul 136-713, South Korea*
[c] *College of Pharmacy, Catholic University of Daegu, Gyongsan 712-702, South Korea*

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Abstract

To develop a novel clotrimazole-loaded poloxamer-based suppository with enhanced anti-tumor activity and alleviated hepatotoxicity, the melting point of various formulations composed of P 188 and propylene glycol were investigated. The dissolution and anti-tumor activity of clotrimazole delivered by the poloxamer-based suppository was performed. Furthermore, the hepatotoxicity of clotrimazole was carried out after its rectal administration compared to oral administration in mice. The poloxamer mixtures composed of P 188 and propylene glycol were homogeneous phases. P 188 greatly affected the melting point of poloxamer mixtures. In particular, the poloxamer mixture [P 188/propylene glycol (70%/30%)] with the melting point of about 32 °C was a solid form at room temperature and instantly melted at physiological temperature. The ratio of P 188/propylene glycol greatly affected the dissolution rates of clotrimazole from poloxamer-based suppository. Dissolution mechanism analysis showed the dissolution rate of clotrimazole from poloxamer-based suppositories was independent of the time. The clotrimazole-loaded suppository with P 188 and propylene glycol could not irritate or damage the rectal tissues of rats and gave the improved anti-tumor activity in a dose-dependent manner at mouse. Furthermore, its rectal administration decreased the hepatotoxicity compared to oral administration. Thus, the poloxamer-based solid suppository system with clotrimazole/P 188/propylene glycol was an effective rectal dosage form for the treatment of tumors with alleviated adverse effects.

Keywords: Clotrimazole; Poloxamer 188; Suppository; Melting point; Anti-tumor activity; Hepatotoxicity

1. Introduction

Conventional suppository, a polyethylene glycol (PEG)-based suppository, which may softens or melts lately in the rectum and vagina due to its relatively high melting point, can not be rapidly absorbed in the mucous membranes (Burstein et al., 2000; Eboka et al., 1997). Furthermore, such a PEG-based suppository, which may reach the end of the colon, has a loss of drug at colonic level and may also allow the carried drugs to undergo the first-pass effect (Choi et al., 1998; Kim et al., 1998). To solve the problems of conventional solid suppository, it would be desirable to develop a novel solid suppository, which was a solid phase at room temperature and instantly melted at physiological temperature, and was mucoadhesive to the mucous membranes not to reach the end of the colon. Such a suppository must have the suppository base with the suitable melting points (30–36 °C) and mucoadhesive property.

In this study, as a base of novel poloxamer-based suppository, a mixture of poloxamer 188 (P 188) and propylene glycol with the melting point of about 55 and −10 °C, respectively, has been selected (Yong et al., 2005). In addition, P 188 and propylene glycol are known to have suitable mucoadhesive force (Kim et al., 1998), low toxicity (Yun et al., 1999), less skin irritation (Choi et al., 1998), good drug release characteristics (Miyazaki et al., 1987) and compatibility with other chemicals. Furthermore, clotrimazole was selected here as a model drug, since it

* Corresponding author. Tel.: +82 53 810 2813; fax: +82 53 810 4654.
** Corresponding author. Tel.: +82 53 810 2816; fax: +82 53 810 4654.
E-mail addresses: jakim@yumail.ac.kr (J.-A. Kim), hangon@yumail.ac.kr (H.-G. Choi).

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induced severe hepatotoxicity when orally (Ahmed et al., 1998; Beitner and Penso, 2002; Khalid et al., 2005; Rida et al., 2005).

Thus, in this study, to develop a novel clotrimazole-loaded poloxamer-based suppository with enhanced anti-tumor activity and alleviated hepatotoxicity, the melting point of various formulations composed of P 188 and propylene glycol were investigated. The dissolution and anti-tumor activity of clotrimazole delivered by the poloxamer-based suppository was performed. Furthermore, the hepatotoxicity of clotrimazole was carried out after its rectal administration compared to oral administration in mice.

2. Materials and methods

2.1. Materials

Clotrimazole and propylene glycol were supplied from DC chemical (Seoul, South Korea). Poloxamer 188 (P 188) was supplied from BASF (Mount Olive, NJ, USA). Semipermeable membrane tube (spectra membrane tubing no.1) was from Spectrum Medical Industries Inc. (Los Angeles, CA, USA). All other chemicals were of reagent grade and used without further purification.

2.2. Preparation of poloxamer-based suppository

Various ratios of P 188 and propylene glycol were mixed and heated up to 55 °C. Clotrimazole was then slowly added to the solution with continuous agitation. The resulting solution was moving to the suppository mould and cooled down to 25 °C. The melting point of suppository was determined using DSC (Netzsch, Model 200) at the raising temperature condition of 5 K/min (Burstein et al., 2000; Yong et al., 2005).

2.3. Dissolution test

Various poloxamer-based suppositories [clotrimazole/poloxamer and propylene glycol mixture (5%/95%)] (4 g) were inserted into a semipermeable membrane tube, respectively. The poloxamer and propylene glycol mixture of poloxamer-based suppository were composed of A [P 188/propylene glycol (100%/0%)], B [(80%/20%)], C [(70%/30%)] and D [(50%/50%)], respectively. Both sides of the tube were tied up with a thread to prevent leakage. The semipermeable membrane tube was then placed in a dissolution tester (DST-600, Fine Chemical, Korea). Dissolution test was performed at 36.5 °C using the paddle method at 100 rpm with 400 ml phosphate buffer (pH 4.4) as a dissolution medium. At predetermined interval, 5 ml of the medium was sampled and filtered. The filtrate was analyzed by UV/vis variable wavelength detector (Philips, Model PU8730) at 254 nm (Chang et al., 2002; Choi et al., 2001; Kim et al., 2002).

2.4. Morphology test of rectal tissues in rats

Male Sprague–Dawley rats weighing 250 ± 20 g were fasted for 24–36 h prior to the experiments but allowed free access to water. Poloxamer-based suppository was administered at 1.5 g/kg into the rectum 4 cm above the anus. At 4 h after administration, the rectum was isolated, rinsed with a saline solution, fixed in 10% neutral carbonate-buffered formaldehyde, embedded in paraffin using an embedding center and cut into slices. The slices were stained with hematoxylin–eosin and observed under a light microscope (Leitz; Laborlux 12 Pols, Germany) (Choi et al., 1998).

2.5. In vivo anti-tumor test

2.5.1. Cell culture

CT–26 cells were grown at 37 °C in a humidified incubator under 5% CO_2/95% air in a Dulbeco's modified eagle's medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 200 IU/ml penicillin and 200 μg/ml of streptomycin. Culture medium was replaced every other day. After attaining confluence, the cells were subcultured following trypsinization with 0.25% trypsin–EDTA solution.

2.5.2. Establishment of tumor models in BALB/c Mice

Male BALB/c mice weighing 20–22 g were supplied from Orient Co., Ltd. (Seoul, Korea). Mice were kept in a regulated environment (21 ± 1 °C) with a 12-h light:12-h dark cycle. Mice were given with food pellets and tap water ad libitum, and were kept in the facilities for at least 2 days before the experiments. For the generation of subcutaneous tumors in mice, male BALB/c mice were inoculated with 1×10^6 CT–26 cells in 100 μl PBS into the subcutaneous tissue of the right flank. The tumor volume was first measured two weeks after tumor cell-inoculation and twice a week thereafter, and calculated using the formula $V = 1/2(d_1 \times d_2 \times d_3)$, where d_1, d_2, and d_3 are diameters measured by calipers in different directions (Khalid et al., 2005; Rida et al., 2005). Animal experiments were followed the ethical standards formulated in the institutional guidelines issued by the Korea National Institute of Health for the care and use of laboratory animals.

2.6. Glutamic oxaloacetic transaminase/glutamic pyruvic transaminase (GOT/GPT) assay

GOT/GPT levels were determined by Reitman–Frankel method (1957). The mouse serum (50 μl) was added to a 250 μl of mixture containing L-aspartic acid and α-ketoglutaric acid for GOT determination or to a 250 μl of mixture containing DL–alanine and α–ketoglutaric acid for GPT determination. After the mixture was incubated at 37 °C for 1 h, 250 μl of 2,4-dinitrophenylhydrazine was added. After the mixture was incubated at 25 °C for 20 min, 2.5 ml of 0.4N NaOH was added. After 10 min, the change of absorbance was measured at 505 nm with UV–vis spectrophotometer (Shimadzu, UV-1601, Japan).

2.7. Measurement of lipid peroxidation

Liver tissue homogenate (0.4 ml) was added to 0.1 M potassium phosphate buffer (0.4 ml). After incubation for 4 h at 37 °C,

the mixture was added to 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid solution (pH 3.5) and 1.5 ml of 0.8% thiobarbituric acid. The mixture was heated at 95 °C for 1 h, chilled to room temperature, and extracted with 1 ml of H_2O and 2.5 ml of *n*-butanolpyridine mixture (15:1, v/v). The upper organic layer containing malondialdehyde produced by lipid peroxidation was measured at 532 nm. Synthetic malondialdehyde was used as an external standard, and the level of lipid peroxides was expressed as nmol of malondialdehyde per mg protein (Ohkawa et al., 1979).

3. Results and discussion

The poloxamer mixtures were easily prepared by mixing and heating P 188 and propylene glycol (100%/0%), (80%/20%), (70%/30%), (50%/50%) and (0%/100%), respectively. DSC curve showed that the wide peak at around 15 °C, which was observed for [P 188/propylene glycol (100%/0%)] (A), shifted to high temperature and changed to relatively sharper peak in the poloxamer mixtures B [P 188/propylene glycol (80%/20%)], C [(70%/30%)] and D [(50%/50%)] (Fig. 1). Furthermore, their DSC curves had no peaks of P 188 and propylene glycol, indicating that the poloxamer mixtures composed of P 188 and propylene glycol were not heterogeneous but homogeneous phases (Choi et al., 2001; Fontanella et al., 2000; Yong et al., 2001). The poloxamer mixtures A [P 188/propylene glycol (100%/0%)], B [(80%/20%)], C [(70%/30%)], D [(50%/50%)] and E [(0%/100%)] had the melting point of about 55, 45, 32, 10 and −10 °C, respectively .Our results suggested that propylene glycol affected the melting point of poloxamer mixtures. The poloxamer mixture C [P 188/propylene glycol (70%/30%)] was selected as a suppository base, since it was a solid form at room temperature and instantly melted at physiological temperature (Chang et al., 2002).

To test whether the ratio of P 188 and propylene glycol affected the dissolution rates of clotrimazole from the poloxamer-based suppositories, we performed the dissolution studies on the four formulations composed of 5% clotrimazole and 95% poloxamer mixtures. The poloxamer mixtures were composed of A [P 188/propylene glycol (100%/0%)], B [(80%/20%)], C [(70%/30%)] and D [(50%/50%)], respectively. Among the poloxamer-based suppositories tested, the suppository D [P 188/propylene glycol (50%/50%)] had significantly highest dissolution rates of clotrimazole. The results suggested that it remained in a liquid phase due to its relatively low melting point of 10 °C. Furthermore, the suppository C [P 188/propylene glycol (70%/30%)] gave higher dissolution rates of clotrimazole than did the suppository A and B. However, there were no significant differences among the dissolution rates of clotrimazole from any other poloxamer-based suppositories (Fig. 2A). Our results suggested that the ratio of P 188/propylene glycol greatly affected the dissolution rates of clotrimazole from poloxamer-based suppository. As a possible mechanism by which the dissolution rates of clotrimazole retarded from poloxamer-based suppository A and B, it is speculated that they remained in a solid phase and could not turned into a gel in the dissolution medium due to relatively their high melting point of 55 and 45 °C (Yong et al., 2005). However, poloxamer-based suppository C [P 188/propylene glycol (70%/30%)] might remain in a solid phase and turn into a gel in the dissolution medium due to relatively their optimal melting point of 32 °C (Choi et al., 1998).

To understand the dissolution mechanisms of clotrimazole, we described the dissolution rate using the following equations:

$$\frac{M_t}{M} = kt^n \quad (1)$$

$$\log\left(\frac{M_t}{M}\right) = \log\ k + n\ \log(t) \quad (2)$$

where M_t/M is the fraction of dissolved drug at time t, k a characteristic constant of the poloxamer-based suppository and n is an indicative of dissolution mechanism. As the k value becomes higher, the dissolution occurs faster. The n value of 1 corresponds to zero-order dissolution kinetics, $0.5 < n < 1$ means a non-Fickian dissolution model and $n = 0.5$ indicates Fickian diffusion (Higuchi model) (Peppas, 1985; Choi et al., 2000). From the plot of $\log(M_t/M)$ versus $\log(t)$ (Fig. 2B), kinetic parameters, n and k, were calculated. Table 1 shows that most of n values were close to 1, suggesting that the dissolution rate of clotrimazole from poloxamer-based suppositories was independent of the time (Choi et al., 1998). The relatively parallel slopes of the plots indicated that the ratio of P 188 and propylene glycol might hardly affect the dissolution mechanisms (Choi et al., 2000; Yong et al., 2005). Therefore, the poloxamer-based

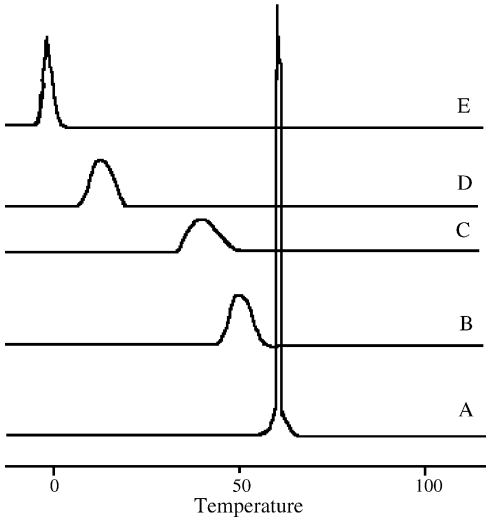

Fig. 1. DSC curves: (A) P 188/propylene glycol (100%/0%); (B) (99%/1%); (C) (98%/2%); (D) (97%/3%); (E) (0%/100%).

Table 1
Dissolution kinetic parameters.

P 188/propylene glycol	Release exponent (n)	Kinetic constant, k (%/h^n)	Correlation coefficient (r)
100%/0%	1.077	1.032	0.943
80%/20%	1.113	1.032	0.921
70%/30%	1.280	0.993	0.911
50%/50%	1.583	0.946	0.917

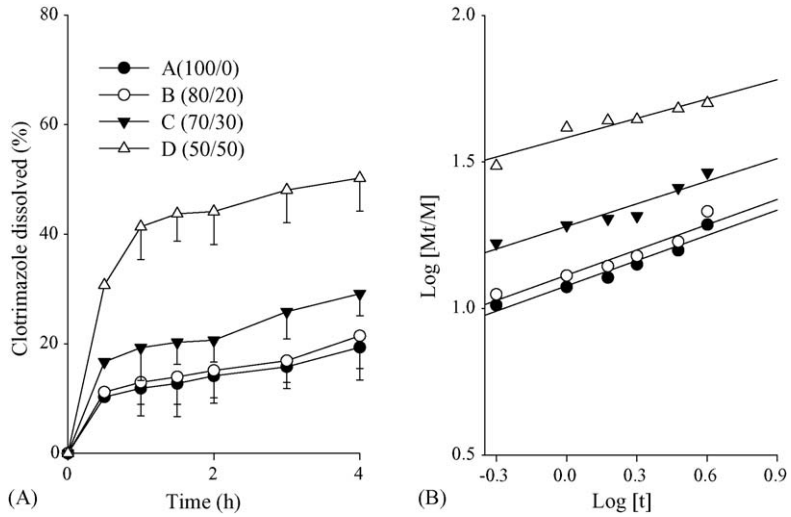

Fig. 2. Effect of poloxamer on the dissolution of clotrimazole from poloxamer-based suppositories (A). Dissolution kinetics of clotrimazole (B). Poloxamer-based suppositories [clotrimazole/poloxamer mixture (5%/95%)] were used as dissolution samples. The poloxamer mixtures of poloxamer-based suppositories were composed of A [P 188/propylene glycol (100%/0%)], B [(80%/20%)], C [(70%/30%)] and D [(50%/50%)], respectively. Each value represents the mean ± S.E. ($n = 6$).

solid suppository composed of 70% P 188 and 30% propylene glycol which was a solid form at room temperature and instantly melted at physiological temperature was selected as a clotrimazole-loaded rectal suppository and carried out in the further study.

The safety test of poloxamer-based suppository composed of 5% clotrimazole and 95% poloxamer mixture [P 124/propylene glycol (90%/30%)] was performed by observing any irritation of poloxamer-based suppository on the rectal tissues of rats (Choi et al., 1998). The morphology of rectal tissues indicated that the poloxamer-based suppository could not irritate or damage the rectal tissues (Fig. 3). Previously, poloxamers, the non-ionic surfactants were reported to be inert, giving no damage to mucous membranes (Choi et al., 1998).

In order to evaluate the effectiveness of the clotrimazole-loaded rectal suppository on tumors, we established a murine skin tumor model and examined the anti-tumor effect of clotrimazole-loaded rectal suppository. When the tumor volume of mouse was reached 200 mm^3, the drug treatment was started. The volume of skin tumors treated with the clotrimazole-loaded suppository in a three intermittent administration regimen was decreased in a dose-dependent manner (Fig. 4). Thus, the clotrimazole-loaded suppository with P 188 and propylene glycol gave the improved anti-tumor activity in a dose-dependent manner.

Since previous other studies have reported that systemic use of CLT as an anti-tumor agent is severely limited by hepatotoxicity associated with the imidazole moiety (Tettenborn, 1974; Cao et al., 2004), we compared the hepatotoxicity of clotrimazole in mice administered through orally and via rectum with the suppository composed of P 188 and propylene glycol. The hepatotoxicity assessed as the activity of glutamate oxaloacetate transaminase and hepatic lipid peroxidation were significantly higher in the mice treated with clotrimazole through oral route than rectal suppository (Fig. 5A and B) (Reitman and Frankel, 1957). Thus, the rectal administration of clotrimazole-loaded suppository with P 188 and propylene glycol decreased the hepatotoxicity compared to oral administration. Our results suggested that the poloxamer-based solid suppository system with clotrimazole/P 188/propylene glycol was an effective rec-

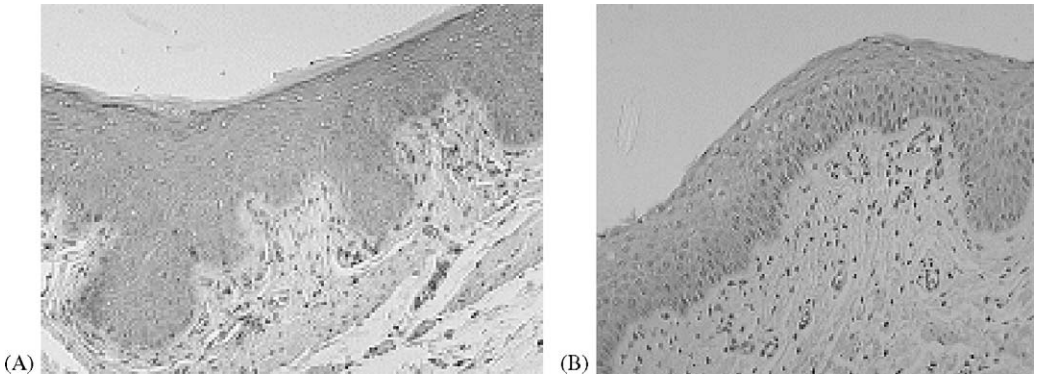

Fig. 3. Morphology of rectal mucosa of rats after rectal administration of poloxamer-based suppository (X 250): (A) before administration and (B) 4 h after administration.

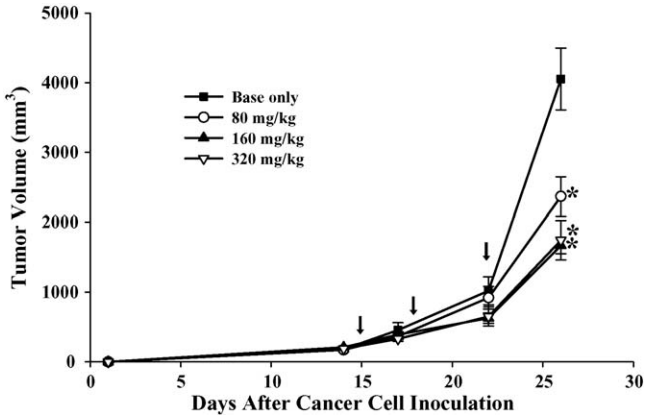

Fig. 4. Anti-tumor effect of clotrimazole rectal suppository on the subcutaneous tumor in mice. The suppository was given in a three intermittent administration regimen starting after the tumor volume of the mice was reached 200 mm^3. Each group of mice was received clotrimazole in a concentration of 80, 160 and 320 mg/kg, respectively. The arrows designate the day of suppository administration. Each value represents the mean ± S.D. ($n = 5$). (*) $P < 0.05$ compared to PEG-based suppository.

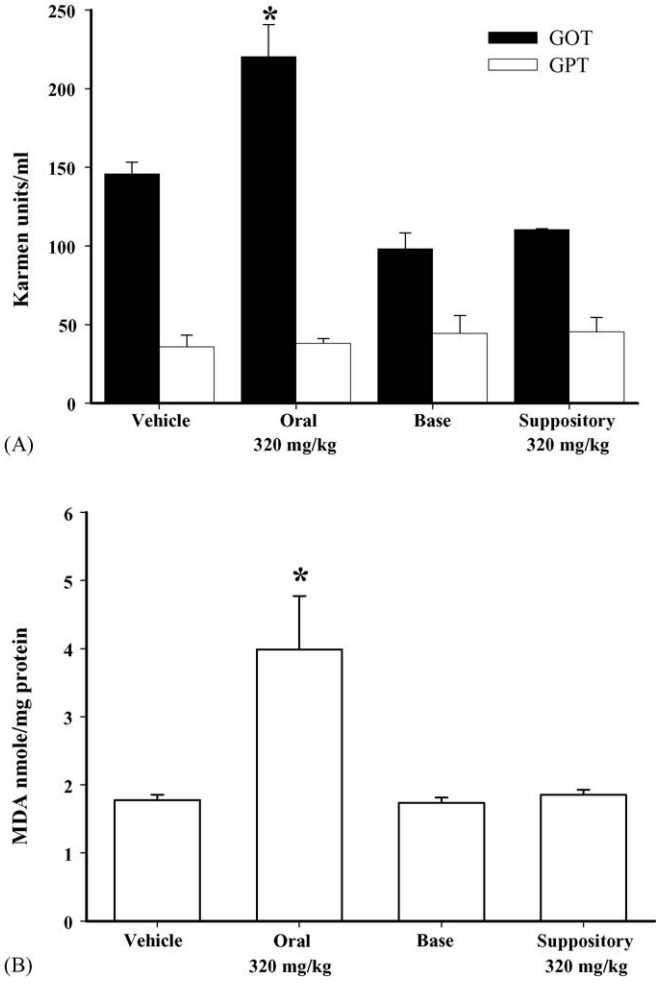

Fig. 5. Effect of clotrimazole on the level of GOT/GPT (A) and hepatic lipid peroxidation (B) in mice. The normal mice were treated with clotrimazole (320 mg/kg) orally or through the rectal suppository. (*) $P < 0.05$ compared to untreated control.

tal dosage form for the treatment of tumors with low adverse effects.

4. Conclusion

Taken together, it is concluded that the poloxamer-based solid suppository composed of 70% P 188 and 30% propylene glycol, which was a solid form at room temperature and instantly melted at physiological temperature, could not irritate or damage the rectal tissues of rats, and gave the improved anti-tumor activity and less hepatoxicity. Thus, the poloxamer-based solid suppository system with clotrimazole/P 188/propylene glycol was an effective rectal dosage form for the treatment of tumors with alleviated adverse effects. The further study on identification test in the rectus and morphology test of rectall tissues will be performed.

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