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A Novel Clotrimazole-loaded Suppository with Effective Anti-tumor Activity

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ABSTRACT – To develop a poloxamer-based solid suppository with poloxamer and polyethylene glycol mixtures, 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 were performed. 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. Furthermore, the ratio of P 188/propylene glycol greatly affected the dissolution rates of clotrimazole from poloxamer-based suppository. It gave the more effective anti-tumor activity than conventional PEG-based suppository due to fast dissolution. Thus, the clotrimazole-loaded poloxamer-based solid suppository was an effective rectal dosage form with anti-tumor activity.

Key words – Clotrimazole, Suppository, Anti-tumor activity

Clotrimazole is an antifungal imidazole derivative that has been in clinical use for more than 20 years. Recent studies have shown that clotrimazole inhibits proliferation of human cancer cells[1] and vascular endothelial cells, leading to inhibition of growth factor-stimulated angiogenesis,[2] and thus, tumor growth. However, it has not been reported whether clotrimazole modulates inflammatory angiogenesis or not. In this study, we investigated that clotrimazole suppresses intestinal inflammation via down-regulating pro-inflammatory cytokine IL-8 expression and blocking the epithelial and endothelial responses to IL-8.[3]

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.[4,5] 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.[6-8] To solve these 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.

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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.[9] Furthermore, P 188 are known to have suitable mucoadhesive force, low toxicity, less skin irritation, good drug release characteristics and compatibility with other chemicals.[6,8,10]

Thus, in this study, the melting point of various formulations composed of P 188 and propylene glycol were investigated, and the dissolution and anti-tumor activity of clotrimazole delivered by the poloxamer-based suppository were performed. Furthermore, the oral administration of clotrimazole might induce the severe hepatic toxicity.[1,2] Thus, to investigate the induction of hepatic toxicity via rectal administration of clotrimazole, its glutamic oxaloacetic transaminase/glutamic pyruvic transaminase (GOT/GPT) assay were checked.

Materials and methods

Materials

Clotrimazole and propylene glycol were supplied from DC chemical (Seoul, South Korea). Poloxamers (P 188) were supplied from BASF Aktiengesellschaft (Ludwigshafen,Germany). Semipermeable membrane tube (Spectra membrane tubing No.1) was from Spectrum Medical Industries Inc. (Los Angeles, California, USA). Acetonitrile and methanol were from Aldrich Chemical Co. (Milwaukee, WI, USA). All other chemicals were of reagent grade and used without further puri-

fication.

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.[3,9]

Dissolution test

Various poloxamer-based suppositories [clotrimazole/ poloxamer and propylene glycol mixture (5/95%)] (4 g) and conventional PEG-based suppository [clotrimazole/PEG 4000 (5/95%)] (4 g) containing 200 mg clotrimazole 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/visible variable wavelength detector (Philips, Model PU8730) at 254 nm.[11,12]

***In vivo* anti-tumor test**

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.

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-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 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 tumor volume was first measured two weeks after cancer cell-inoculation and twice a week thereafter, and calculated using the formula $V=1/2$ ($d1\times d2\times d3$), where d1, d2, and d3 are diameters measured by calipers in different directions.[13,14] 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.

Glutamic oxaloacetic transaminase/Glutamic pyruvic transaminase (GOT/GPT) assay

GOT/GPT levels were determined by Reitman-Frankel method.[13,14] The normal mice were treated with clotrimazole (320 mg/kg, dispersed in 1% povidone solution) orally or through the rectal suppository. After antitumor experiments, 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 mL 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 0.4 N NaOH was added. After 10 min, the change of absorbance was measured at 505 nm with UV-VIS spectrophotometer (Shimadzu, UV-1601, Japan).

Measurement of lipid peroxidation

The normal mice were treated with clotrimazole (320 mg/kg) orally or through the rectal suppository. After antitumor experiments, liver was taken, weighed, suspended in 0.5 mL of ice-cold sample buffer per 50 mg of tissue, and homogenized 30 s using a tissue homogenizer at 4°C (Biospec Products Inc., Switzerland). 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.[15]

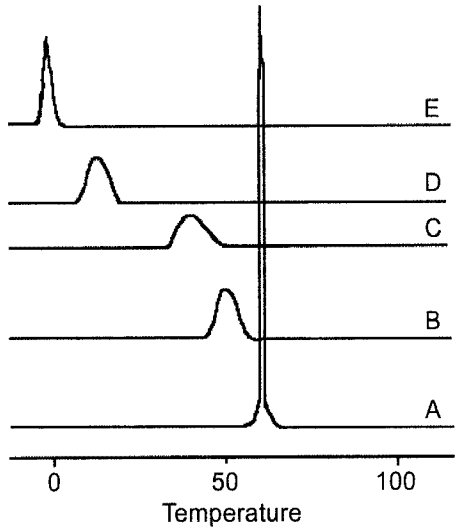

Figure 1–DSC curves: (A) P 188/propylene glycol (100/0%); (B) (99/1%); (C) (98/2%); (D) (97/3%); (E) (0/100%).

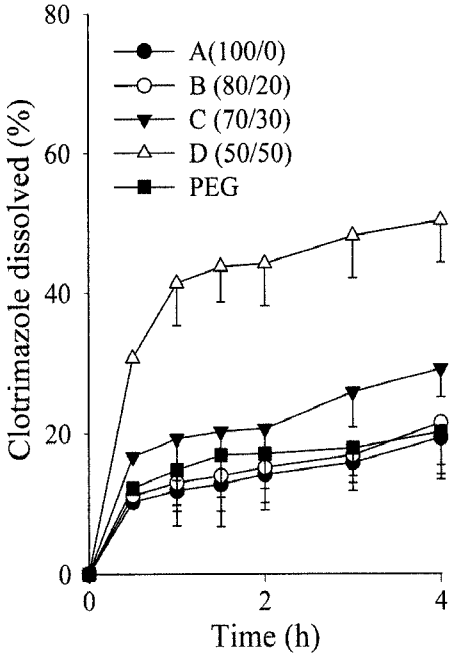

Figure 2–Effect of poloxamer on the dissolution of clotrimazole from poloxamer-based suppositories. Poloxamer-based suppositories [clotrimazole/poloxamer mixture (5/95%)] (4 g) and PEG-based suppository [clotrimazole/PEG 4000 (5/95%)] (4 g) containing 200 mg of clotrimazole were used as dissolution samples. 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).

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 55°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.[9,10] 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.[11]

To test whether the ratio of P 188/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 was composed of A [P 188/propylene glycol (100/0%)], B [(80/20%)], C [(70/30%)] and D [(50/50%)], respectively. Additionally, the dissolution test of PEG-based suppository [clotrimazole/PEG 4000 (5/95%)] was carried out. 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. 2). 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.[9] 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. On the other hand, the PEG-based susspository had relatively lower dissolution rates of clo-

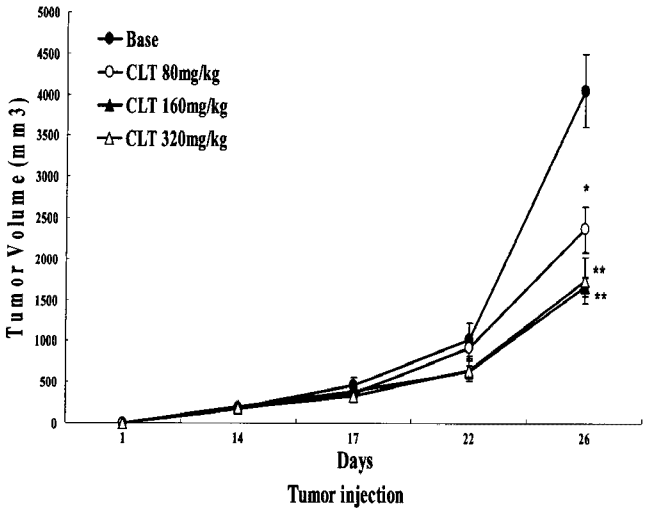

Figure 3–Anticancer effect of clotrimazole rectal suppository on the subcutaneous tumor in mice. The suppository was given in a three intermittent administration regimen starting after the tumor volume of the mice was reached 200 mm^3. Each group of mice was received clotrimazole in a concentration of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

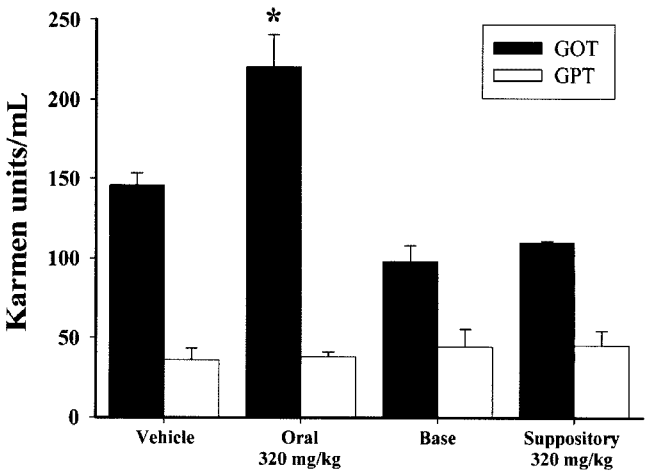

Figure 4–The effects of clotrimazole on the level of GOT/GPT in mice. The normal mice were treated with clotrimazole (320 mg/kg) orally or through the rectal suppository. *$P<0.05$, compared to untreated control.

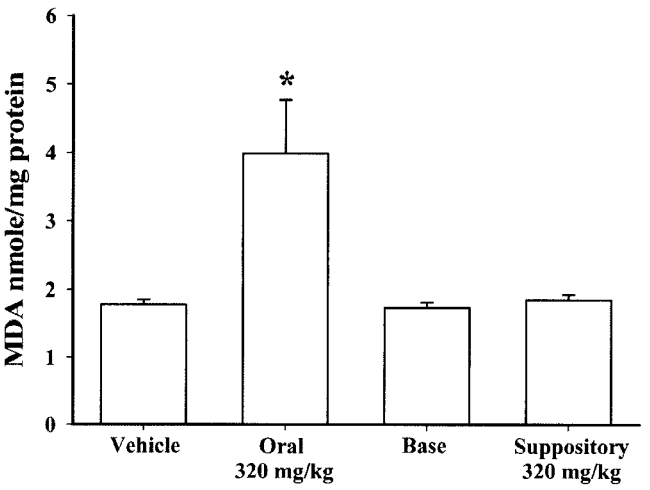

Figure 5–The effects of clotrimazole on the level of hepatic lipid peroxidation in mice. The normal mice were treated with clotrimazole (320 mg/kg) orally or through the rectal suppository. *$P<0.05$, compared to untreated control.

trimazole than poloxamer-based suppository C [P 188/propylene glycol (70/30%)]. These results suggested that PEG was remained initially in a solid phase and slowly soluble in the dissolution medium.[6]

In order to evaluate the effectiveness of the clotrimazole rectal suppository on cancers, we established a murine skin tumor model and examined the anticancer effect of clotrimazole rectal suppository. When the tumor volume of mouse was reached 200 mm^3, the drug treatment was started. The volume of skin cancers treated with the clotrimazole suppository in a three intermittent administration regimen was decreased in a dose-dependent manner (Fig. 3). Since previous other studies have reported that systemic use of CLT as an anticancer agent is severely limited by hepatotoxicity associated with the imidazole moiety,[16-18] 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 (Figs. 4 and 5). Our results suggest that a rectal poloxamer gel system with clotrimazole/P 188/propylene glycol is an effective rectal dosage form for the treatment of cancers with low adverse effects.

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, gave the more effective anti-tumor activity. Thus, the clotrimazole-loaded poloxamer-based solid suppository was an effective rectal dosage form with anti-tumor activity.

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