ANTICANCER RESEARCH *39*: 201-206 (2019)
doi:10.21873/anticanres.13098

Therapeutic Effect of GGsTop, Selective Gamma-glutamyl Transpeptidase Inhibitor, on a Mouse Model of 5-Fluorouracil-induced Oral Mucositis

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Abstract. *Background: Oral mucositis (OM) induced by cancer chemotherapy has a high incidence and serious symptoms, which often force chemotherapy to be stopped. GGsTop is a newly-discovered gamma-glutamyl transpeptidase (GGT) inhibitor. Previous research suggested that inhibition of GGT suppressed reactive oxygen species and induced the production of collagen and elastin. We hypothesized that GGsTop could safely treat OM. Materials and Methods: A mouse model of OM was treated with GGsTop and ulcer area, weight, and white blood cell count were determined. The treatment effect was also evaluated by hematoxylin-eosin and collagen staining. Results: The therapeutic effect of GGsTop was better than that of an existing drug and may be safely used in combination with chemotherapy. Furthermore, GGsTop promoted collagen production in oral mucosa. Conclusion: GGsTop treated OM quickly and safely. GGsTop is highly valuable for use as a treatment for OM.*

Oral mucositis (OM), one of the side-effects induced by chemotherapy, has reported incidence of 40%, increasing to approximately 100% for chemotherapy in combination with radiotherapy (1, 2). OM caused by anticancer drug treatment is shown as erythema, edema or ulceration that can be accompanied by alterations ranging from mild burning sensation to large and painful ulcers and has a wider range of presentations than general stomatitis (3, 4). Symptoms include eating disorders, communication obstacles, and sleep disorders as well as the sharp pain, which reduce the patients' quality of life (QOL) (5). Loss of consciousness was also reported to occur as a result of deterioration of OM which forced the treatment to stop (6). Preventing OM or treating it quickly brings improvement of QOL, and reduces the need for treatment interruption. At present, various treatments (oral cryotherapy, brushing, administration of steroidal anti-inflammatory drug, disinfectant mouthwash, *etc.*) are performed (5, 7). However, they do not seem to bring enough relief nor preventive effect (8). A novel drug for OM is needed.

Anticancer drugs cause stomatitis mainly by two methods. One is by direct mucosal injury and the other is indirectly by causing a bacterial infection in the oral mucosa (9). Previous research reported that since the cell cycle of oral mucosal cells is as short as that of cancer cells, oral mucosal cells were damaged by anticancer drugs. In addition, reactive oxygen species (ROS) generated by anticancer drugs cause mucosal damage (10). It is also reported that bacterial infection is due to immunosuppression by anticancer agents, reduced mucus secretion, a decrease in the number of mucosal cells, and reduced barrier function (10). Because of these characteristics, it is impossible to use existing medicine for stomatitis, which mainly comprises steroidal anti-inflammatory drugs (11). This fact is a major cause for stomatitis during cancer chemotherapy and is regarded as a problem.

Gamma-glutamyl transpeptidase (GGT) is a highly glycosylated heterodimeric enzyme that is widely found in organisms from bacteria to mammals (12-15). GGT catalyzes the early stages of glutathione (GSH) degradation and produces cysteinyl-glycine (16). Cysteinyl-glycine is a thiol compound with very high reactivity and produces ROS which subsequently facilitates oxidative reaction (17, 18). GGT plays a central role in mediating the redox balance of cells, and the detoxification of xenobiotics and ROS through glutathione metabolism (19-21). Inhibition of GGT has been reported to suppress ROS, induce the production of collagen and elastin (22).

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Key Words: Oral mucositis, chemotherapy, GGT inhibitor, wound healing, mouse model.

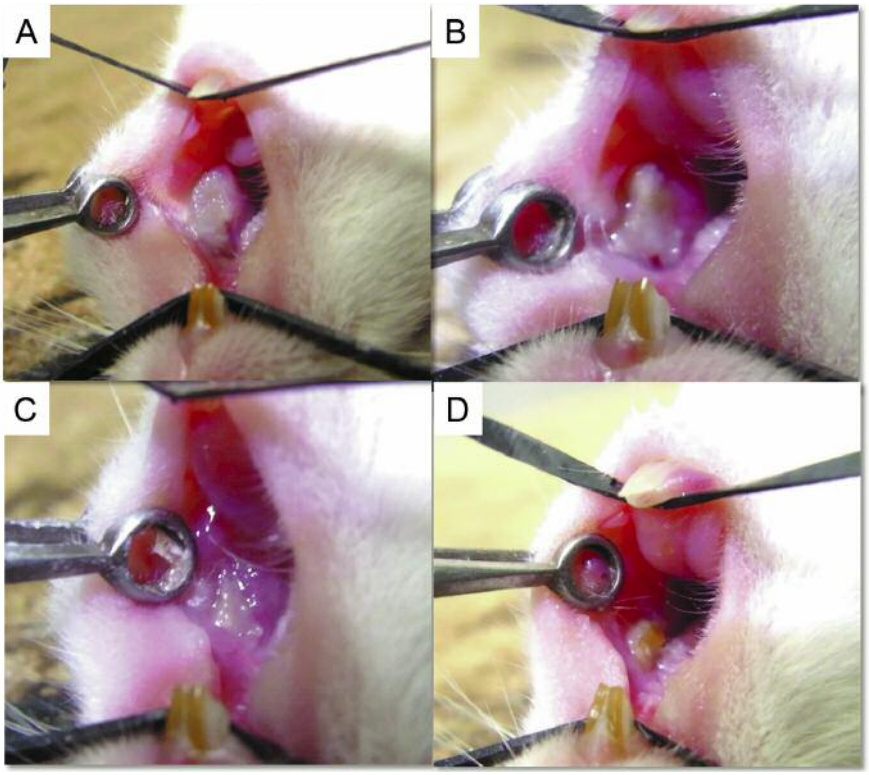

Figure 1. *Oral mucositis of model mice on days 1 (A), 6 (B), 11 (C), and 14 (D).*

Acivicin, L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (AT-125; produced by *Streptomyces sviceus*), has been used as an inhibitor of GGT (23). However, acivicin irreversibly inhibits various glutamine amidotransferases, including imidazole glycerol phosphate synthase and guanine monophosphate synthetase, and inactivates a number of biosynthetic enzymes for purine and pyrimidine, amino acids, and amino sugars (23-25), resulting in potent cytotoxicity (26, 27).

GGsTop™, 2-amino-4-{[3-(carboxymethyl)phenoxy](methyl)phosphoryl} butanoic acid, is a newly discovered GGT inhibitor. GGsTop is chemically stable and nontoxic (28, 29). Furthermore, GGsTop exhibits activity toward human GGT of more than 100-fold that of acivicin, inhibits only GGT, and does not inhibit glutamine amidotransferases (28). In addition, GGsTop induces expression of collagen, elastin synthesis, heat-shock protein 47, and keratinocyte induction of human skin fibroblasts (30, 31).

In our previous studies, 5-fluorouracil (5-FU) and acetic acid were used to prepare model mice that reproduce stomatitis developing during cancer chemotherapy (32). We hypothesized that GGsTop might safely treat stomatitis. The purpose of this study was to evaluate whether GGsTop has any therapeutic effect using model mice.

Materials and Methods

Chemicals and drug preparation. Acetic acid (purity ≥99.7%) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was diluted in distilled water at 20%. 5-FU (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was diluted in saline at 8 mg/mI. Turk reagent (Nacalai Tesque, Inc., Kyoto, Japan) was used for white blood cell (WBC) count. Isoflurane (Intervet Inc., Millsboro, DE, USA) was used for mouse anesthesia. Collagen stain kit (Collagen Research Center, Co., Ltd., Tokyo, Japan) was used to stain collagen in oral mucosal tissue. GGsTop was kindly provided by NAHLS. Co., Ltd (Kyoto, Japan). All other chemicals were of reagent grade.

Animals. Nine-week-old male ICR mice, weighing 30-40 g, were purchased from Japan SLC, Inc. (Shizuoka, Japan). The animals were housed in groups of five per cage in a room maintained under standardized light (12:12 h light-dark cycle) at an ambient temperature of 23±2˚C, humidity of 60±10˚C with free access to food pellets and drinking water, and were acclimated for 7 days prior to use in experiments. The experimental protocol used in this study was in accordance with the guidelines of Tokyo University of Science (approval number: Y18041).

OM induction. The protocol for the induction of OM was modified on the basis of a previously published protocol (32). To replicate immunosuppression induced by anticancer drug, mice were

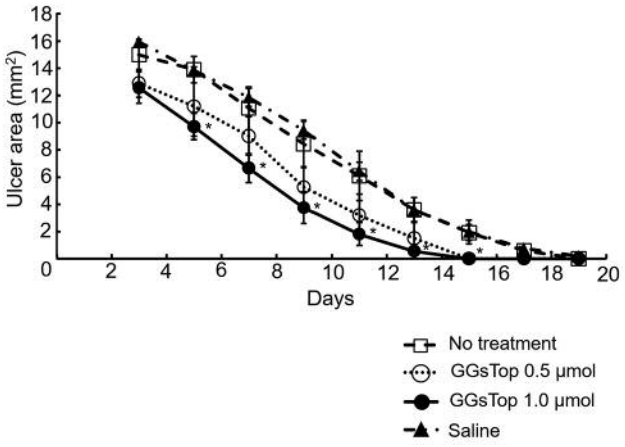

Figure 2. *Changes in ulcerous area of mucosa of the left cheek of mice with oral mucositis. Data are the mean±S.D., n=5. *Significantly different at p<0.05 compared ti the group without treatment (Dunnett's test).*

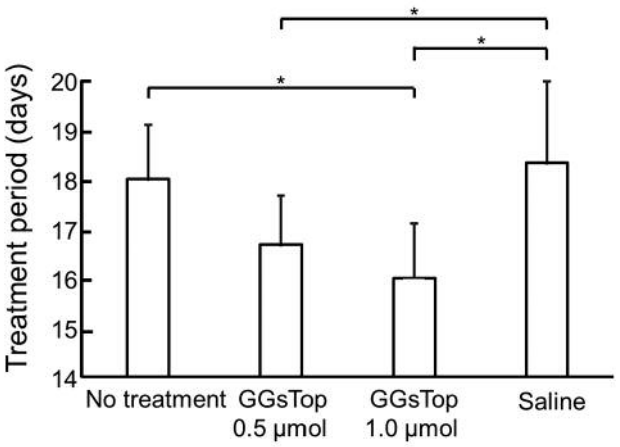

Figure 3. *Treatment period of oral mucositis in modeI mice. Data are the mean±S.D., n=5. *Significantly different at p<0.05 compared to the group without treatment (Dunnett's test).*

administered an intraperitoneal injection of 5-FU on days −5, −3, and −1 of the experiment at a dose of 50 mg/kg body weight. On day 0, under isoflurane anesthesia, the left cheek of each mouse was extended outside the oral cavity. Subsequently, mucosal ulcers were induced by injection of 20% acetic acid using a microsyringe with a 31-G needle. Figure 1 shows the state of the oral cavity of the model mouse at intervals during the experiment.

OM treatment. From the day following the administration of acetic acid, GGsTop solution was administered to the ulcer site once with a p100 micropipette once a day. The amount of GGsTop was adjusted to be 0.5 or 1.0 μmol mouse (in 33 μl). This operation was carried out until the end of complete healing, defined as the treatment period. All mice survived during the experimental period.

Assessments. From day 3 to the end of treatment, once every two or three days, the mice were anesthetized with isoflurane and the left cheek was inverted and the area of mucositis measured (as mm^2) using image analysis software (Image J; National Institutes of Health, Bethesda, MD, USA). Furthermore, the tail venous blood was collected and diluted in Turk's reagent, and WBC count was performed with a hemacytometer.

Histological evaluation. On days 3 and 15, oral mucosal tissue sections were produced using control mice, oral mucositis mice with OM treated with saline, and mice with OM treated with 1.0 μmol GGsTop. Preparation of frozen tissue sections was performed based on the method of Kawamoto (33). The obtained tissue sections were examined for changes of mucosal tissues by hematoxylin-eosin staining and collagen staining. In collagen staining, red/pink color shows collagenous proteins and green color shows non-collagenous proteins.

Statistical analysis. Analysis of variance and multiple comparison tests using Dunnet's method was applied to determine differences in measures. Data are expressed as the mean±SD and differences were considered significant at $p<0.05$ (n=5).

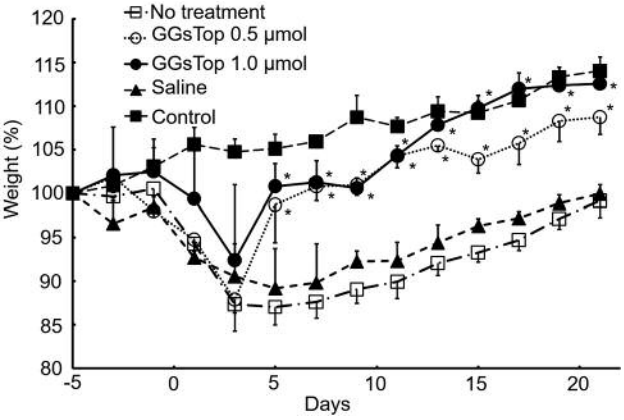

Figure 4. *Change in body weight of mice with oral mucositis. Data are the mean±S.D., n=5. *Significantly different at p<0.05 compared to the group without treatment (Dunnett's test).*

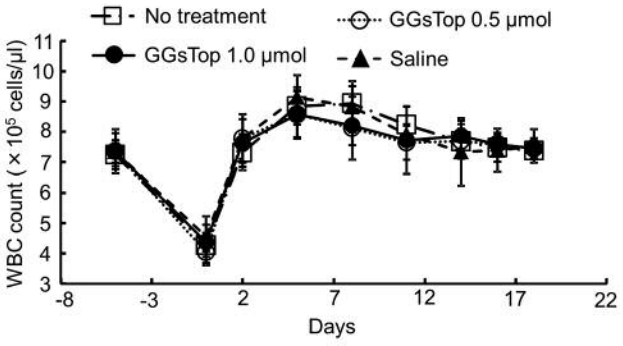

Figure 5. *Changes in white blood cell (WBC) count in mice with oral mucositis. Data are the mean±S.D., n=5. *Significantly different at p<0.05 compared to the group without treatment (Dunnett's test).*

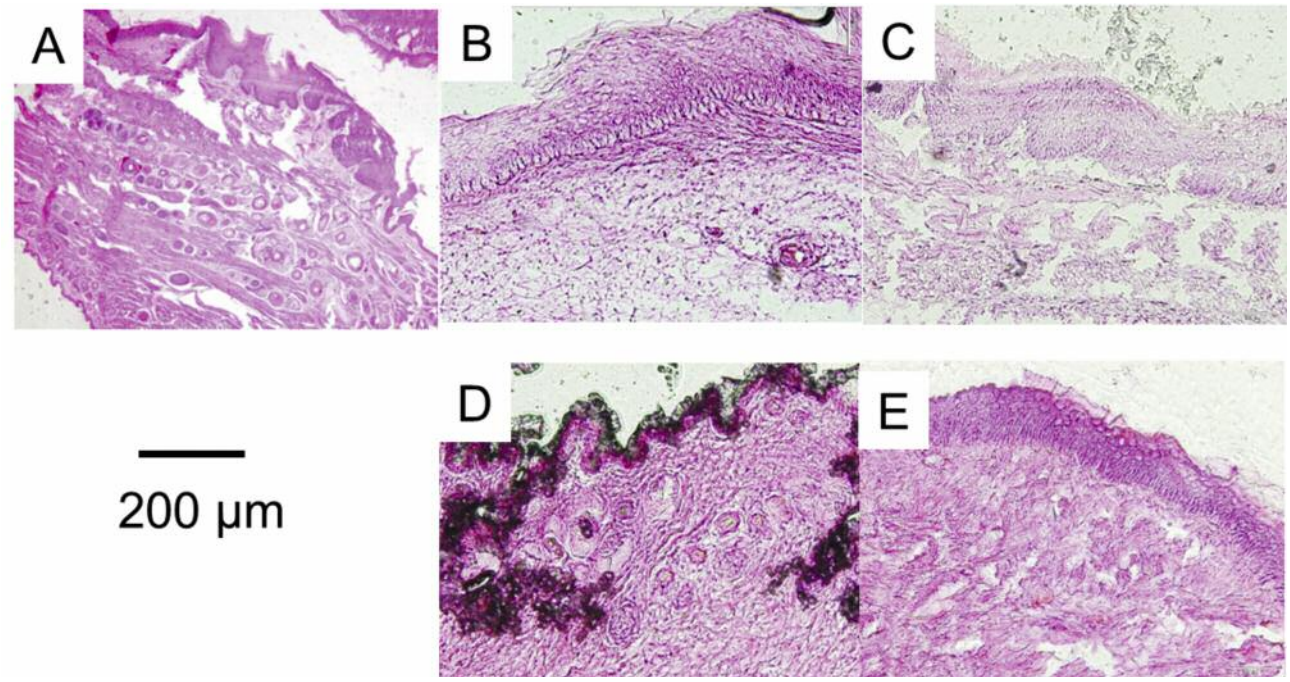

Figure 6. *Hematoxylin-eosin-stained oral mucosal tissue section of normal healthy mouse (A) compared to that on day 3 and 15 in mice with oral mucositis without treatment (B and C, respectively) and those treated with 1 μmol GGsTop (D and E, respectively).*

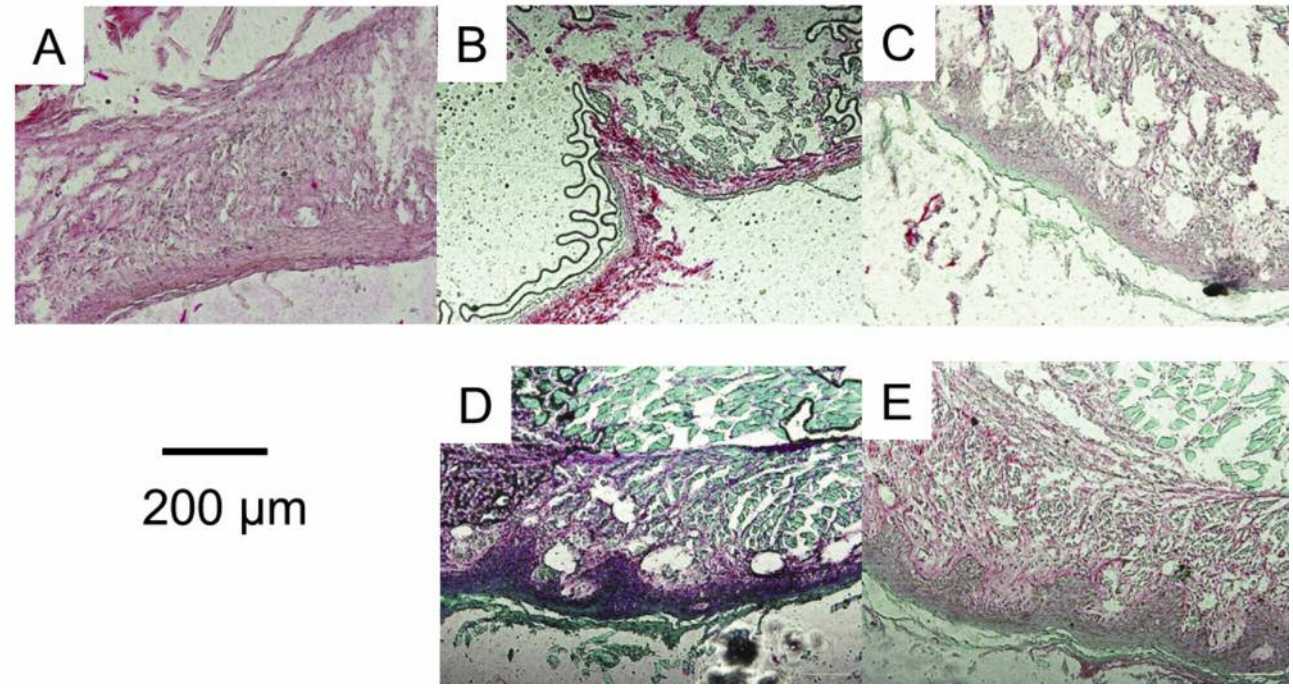

Figure 7. *Collagen-stained oral mucosal tissue section of normal healthy mouse (A) compared to that on days 3 and 15 in mice with oral mucositis without treatment (B and C, respectively) and those treated with 1 μmol GGsTop (D and E, respectively).*

Results

Therapeutic effect of GGsTop on OM. Administration of GGsTop reduced the maximum ulcerated area and shortened the treatment period in a dose-dependent manner (Figures 2 and 3). Body weight was also improved by administration of GGsTop (Figure 4). The WBC count remained normal throughout the experiment in all groups (Figure 5).

Histological evaluation. HE-stained oral mucous showed GGsTop administration promoted early recovery as compared with no treatment of OM (Figure 6). Administration of GGsTop promoted collagen production in the oral mucosa (Figure 7).

Discussion

Figures 1-6 show that GGsTop not only has a therapeutic effect on stomatitis, but can also be used under immunosuppression due to pre-administration of anticancer drug. Anticancer drugs such as 5-FU are often immunosuppressive, and anti-inflammatory drugs for treating stomatitis cannot be used in combination with such chemotherapy. However, since GGsTop does not have an immunosuppressive effect, it is an effective novel therapeutic drug against OM as a side-effect of anticancer therapy.

GGsTop is a selective GGT inhibitor. In addition, GGsTop is chemically stable and nontoxic. GGsTop has been reported to promote the production of collagen, elastin, heat-shock protein 47, and glutathione in the skin through GGT inhibition. GGsTop promotes the production of antioxidant substance, migration of epidermal keratinocytes, improvement of skin barrier function, moisturizing.

In this study, collagen production, which is most closely related to mucous membrane repair (34), was examined. Collagen is one of the main components constituting the mucosa. As the production of collagen was promoted by treatment of OM with GGsTop, mucosa damaged by inflammation was repaired quickly.

Much concerning the antioxidant action of GGsTop has been reported. GGsTop reduced oxidative stress by increasing the amount of glutathione only during inflammation and suppressed the onset of asthma (35). GGsTop prevented ischemia/reperfusion-induced renal injury by inhibiting GGT activity and ROS production (22, 36). From these reports, it is presumed that mucosal protection from inflammation due to antioxidant activity also applied to the stomatitis model in this study.

The result of the therapeutic effect of GGsTop was equivalent to that using Kenalog™ in the previous study (32). However, excessive reduction of WBC count occurred using Kenalog due to steroidal anti-inflammatory effect; in contrast, using GGsTop maintained a normal leukocyte number. These findings suggest that GGsTop may be a novel remedy for stomatitis.

Conclusion

In this study, GGsTop showed that it was non-toxic and had a therapeutic effect equal to or better than that of previous stomatitis remedy. Therefore, GGsTop is highly valuable for use as a treatment for OM, and further study of the treatment mechanism in detail is needed.

Acknowledgements

The Authors are grateful for GGsTop kindly provided by NAHLS. Co., Ltd (Kyoto, Japan).

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Received November 15, 2018
Revised December 2, 2018
Accepted December 4, 2018