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Suppression of Metastasis of Human Breast Cancer Cells by Chitosan Oligosaccharides

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The present study investigated the antimetastatic property of chitosan oligosaccharides (COS) by evaluating motility, invasion, and the amount and activity of MMP-9 in MDA-MB-231 human breast carcinoma cells. Treatment of MDA-MB-231 cells with increasing concentrations of COS led to a concentration-dependent decrease in cell migration. COS significantly inhibited the invasion of MDA-MB-231 cells through a Matrigel-coated membrane. The treatment of MDA-MB-231 cells with COS reduced the amounts of secreted MMP-9. The activity and amount of MMP-9 protein in MDA-MB-231 cells were decreased by treatment with COS and occurred in a concentration-dependent manner. Our data indicated that COS can serve as a potential novel therapeutic candidate for the treatment of metastatic breast cancer.

Keywords: Breast cancer, chitosan oligosaccharides, invasion, metastasis, matrix metalloproteinase

Metastasis is the primary cause of breast cancer mortality in women globally. Despite successful treatment of the primary malignancy, relapse and subsequent metastatic spread can still occur at distant sites [5]. Development of effective chemopreventive and therapeutic strategies for metastatic disease are urgently needed.

Metastasis of cancer cells occurs by a complex multistep process that involves cell adhesion, invasion, proliferation, and vessel formation. Proteolytic enzyme degradation of the extracellular matrix (ECM) is a crucial step of tumor invasion and metastasis [19]. Matrix metalloproteinases (MMPs) comprise a family of zinc-dependent endopeptidases that have been associated with tumor cell invasion and metastasis, owing to their capacity to degrade extracellular matrix components [10]. MMP production appears to be a marker for cancer cells with a higher metastatic potential [16]. It has been suggested that MMPs were involved in breast cancer invasion and metastasis [6]. Recent clinical studies also demonstrated the prognostic importance of MMPs [7].

The water-soluble chitosan oligosaccharides (COS) possess various biological activities, including antiadiopogenesis [4], cancer chemopreventive and antimetastatic activities [12, 13], antitumor activity and immuno-enhancing effects [14, 18], and have been shown to be particularly useful in many fields [8, 15].

In the present article, we report the effects of COS on motility, invasion, and the activity and expression of MMP-9 in MDA-MB-231 human breast carcinoma cells.

Materials and Methods

Chemicals and Cell Line

COS (1 kDa<MW<3 kDa) were kindly obtained from Kitto Life (Kyunggi, Korea). Transwell chambers were purchased from Corning Inc. (Corning, NY, U.S.A.). Matrigel matrix was obtained from BD Biosciences (Bedford, MA, U.S.A.). Quantikine MMP-9 kit was purchased from R&D Systems (Minneapolis, MN, U.S.A.). Human anti-MMP-9 antibody was purchased from Neomarker (Fermont, CA, U.S.A.). Biotin-rabbit anti-mouse IgGAM (H+L) was obtained from Zymed (San Francisco, CA, U.S.A.). 4-Nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate substrate was purchased from Promega (Madison, WI, U.S.A.). All other necessary reagents of analytical grade were bought from Sigma-Aldrich Chemical Company (St. Louis, MO, U.S.A.). The MDA-MB-231 human breast carcinoma cell line was purchased from the Korean Cell Line Bank and cultured at 37°C in 5% CO_2 in RPMI medium 1640 supplemented with 10% (v/v) fetal bovine serum (FBS).

Cell Growth Assay

MDA-MB-231 cells were plated at an initial cell concentration of 0.5×10^4 cells per well of a 96-well plate for 24 h and treated with

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COS for 24, 48, 72, and 120 h. Measurement of cell growth was determined using 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay.

Wound Healing Migration Assay

MDA-MB-231 cells were seeded on a 6-well plate precoated with collagen (20 μl/ml). After reaching confluency, the cell monolayer was scratched with a pipette tip to obtain a "wounded" monolayer culture. The media and dislodged cells were aspirated, and then replaced by serum-free medium with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) plus COS at 0.1, 0.5, 1, 3, and 5 mg/ml, respectively. After incubation for 48 h, cell migration was observed and photos were taken under a phase-contrast inverted microscope. The experiment was repeated three times separately.

Invasion Assay

Cell migration through Matrigel-coated filters was measured by using Transwell chambers with 8-μm-pore polycarbonate filters coated with Matrigel matrix. MDA-MB-231 cells were seeded in the upper compartment of each invasion chamber and incubated in the presence of TPA plus COS or EGCG for 48 h. Nonmigrating cells on the upper surface of the membrane were gently scrubbed with a cotton swab, and the invading cells on the lower surface were fixed with 100% methanol and stained with Hematoxylin and Eosin Y solution. The number of cells was counted under a microscope at 100× magnification.

Measurement of MMP-9 Production

The cells were treated with COS as described previously. The levels of secreted MMP-9 in the culture supernatant were measured using an MMP-9 Quantikine enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol.

Gelatin Zymographic Assay

MDA-MB-231 cells were plated in a 12-well plate and grown to subconfluency. Growth-arrested cultures were treated with the TPA plus COS or EGCG in fresh medium without FBS for 48 h. Conditioned medium was collected, normalized to cell number, mixed with 10× nonreducing sample buffer, and subjected to electrophoresis in a 10% SDS-polyacrylamide gel containing 0.1% (w/v) gelatin. The resulting gel was rinsed, and then enzyme degradation was performed at 37°C for 18 h in 50 mM Tris-HCl (pH 7.5), 5 mM $CaCl_2$, and 0.04% NaN_3. Gel was subsequently stained with 0.05% Coomassie Blue and destained in 10% (v/v) acetic acid/25% (v/v) methanol.

Western Blot Analysis

Protein lysates (30 μg) of COS-treated cells were loaded onto 7% SDS-polyacrylamide gels to determine the expression of MMP-9. Proteins were electrophoresed and electrotransferred to a polyvinylidene difluoride membrane. Membranes were incubated with primary antibodies to MMP-9. Primary antibodies were detected using biotin-rabbit anti-mouse IgGAM (H+L) and alkaline phosphate-conjugated streptavidin and visualized by 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate substrate.

Statistical Analysis

The data were analyzed for statistical significance using Student's *t*-test. *p* Values less than 0.05 were considered to be significant.

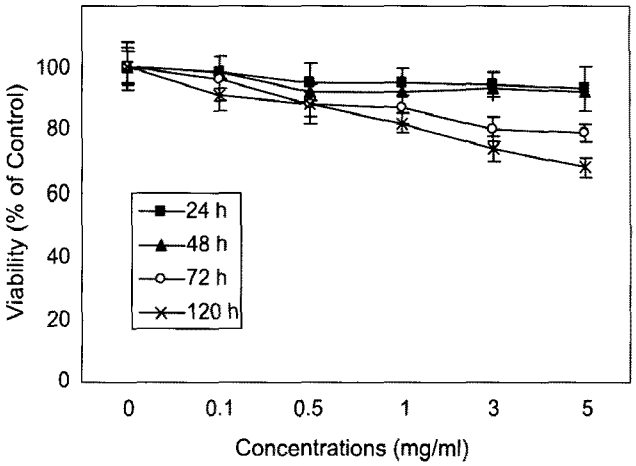

Fig. 1. Effect of chitosan oligosaccharides on growth of MDA-MB-231 human breast cancer cells.
Data shown are mean values with bars indicating the SD of the mean (n=3).

Results

COS Inhibit Growth of MDA-MB-231 Cells

We investigated the effect of COS on the growth of highly invasive and dedifferentiated MDA-MB-231 human breast carcinoma cells to determine whether COS were toxic or inhibitory to the breast cancer cells. As shown in Fig. 1, COS inhibition of MDA-MB-231 cell growth was dose- and time-dependent; however, the effect became significant only after 72 or more hours of treatment.

COS Influence the Motility and Invasion of Human Breast Carcinoma Cells

The motility and invasion of the MDA-MB-231 human breast carcinoma cell line were examined. The treatment of MDA-MB-231 cells with increasing concentration of COS led to a concentration-dependent decrease in wound healing cell migration (Fig. 2A). COS also caused a concentration-dependent decrease in the invasion of MDA-MB-231 cells through the Matrigel chamber (Fig. 2B). These effects on cell migration and invasion were not the result of cell toxicity, as these cells do not exhibit significant growth inhibition under the conditions used (Fig. 1).

COS Suppress the Secretion of MMP-9 by MDA-MB-231 Cells

MMP-9 is produced by breast cancer cells, and its increased expression is associated with disease progression [2]. We examined whether the reduced secretion and activity of MMP correlated with reduced invasion of MDA-MB-231 cells after COS treatment. The total amounts of secreted MMP-9 proteins in conditioned media were quantified using ELISA kits. COS suppressed the secretion of MMP-

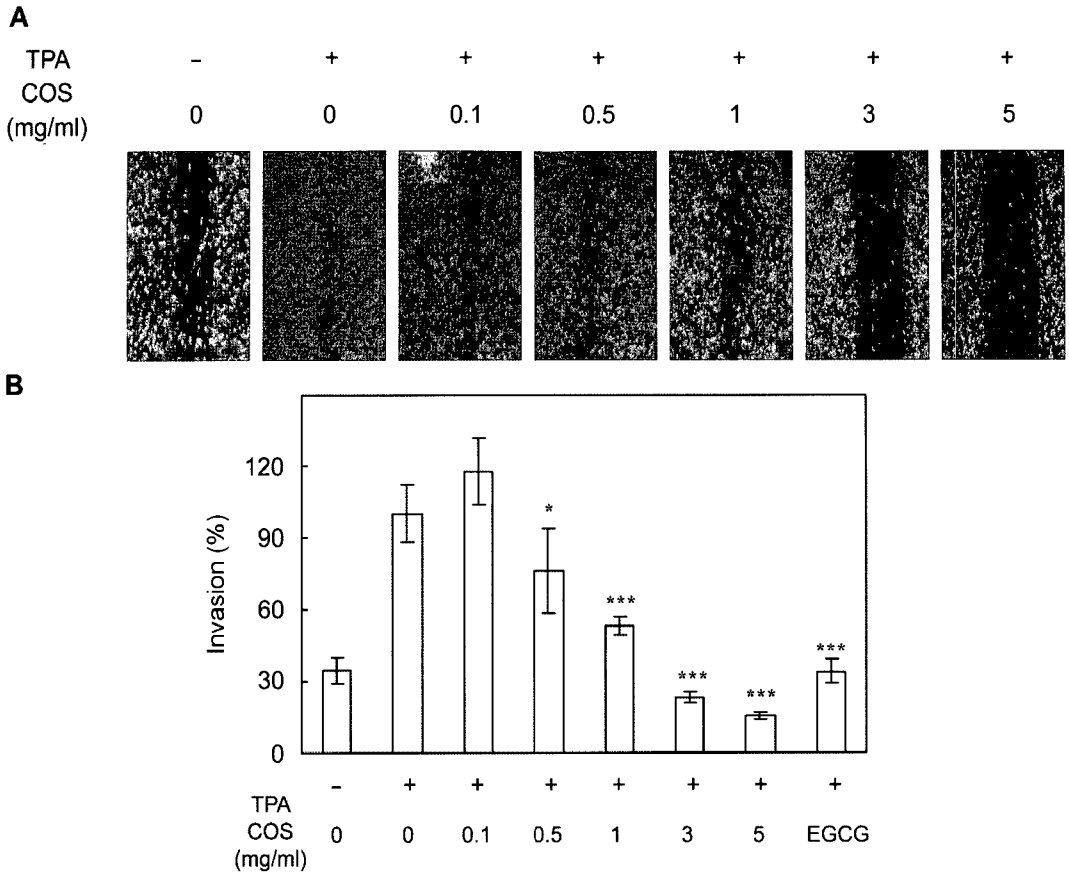

Fig. 2. Effect of chitosan oligosaccharides (COS) on TPA-induced migration (**A**) and invasiveness (**B**) of MDA-MB-231 cells.
EGCG, 20 μM epigallocatechin gallate from green tea. Data shown are mean values with bars indicating the SD of the mean (n=3). *$p<0.05$, ***$p<0.001$ compared with the control.

9 in a concentration-dependent manner (Fig. 3). Gelatin zymography was also carried out to assess the activity of MMP-9 in cells treated with COS. As shown by gelatinolytic activity data, COS inhibited the activity of MMP-9 in a concentration-dependent manner (Fig. 4). Quantification analysis indicated that the MMP-9 activity reduced by 27.4%, 36.7%, and 56.1% when cells were treated with 1, 3, and 5 mg/ml of COS, respectively (Fig. 4).

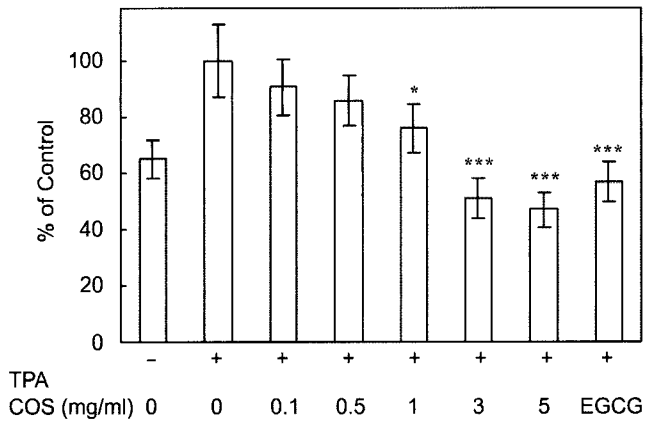

Fig. 3. Effect of chitosan oligosaccharides (COS) on levels of secreted MMP-9 by MDA-MB-231 cells.
EGCG, 20 μM epigallocatechin gallate from green tea. Data shown are mean values with bars indicating the SD of the mean (n=3). *$p<0.05$, ***$p<0.001$ compared with the control.

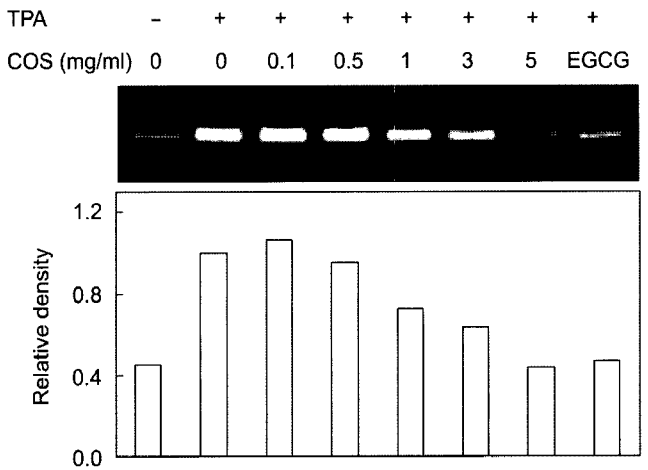

Fig. 4. Effect of chitosan oligosaccharides (COS) on activity of MMP-9 in MDA-MB-231 cells.
EGCG, 20 μM epigallocatechin gallate from green tea.

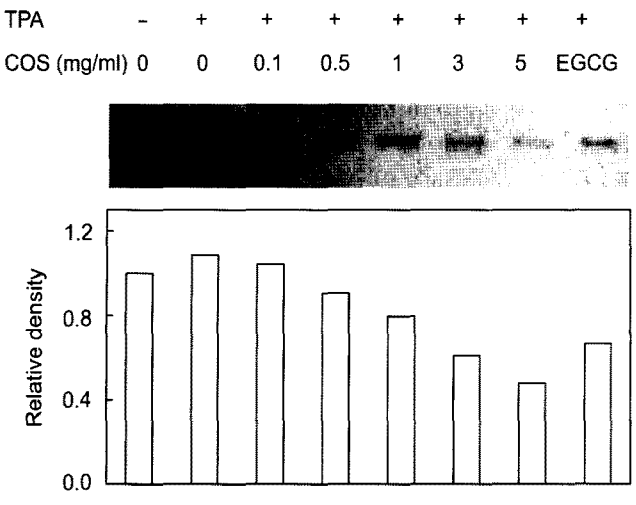

Fig. 5. Effect of chitosan oligosaccharides (COS) on protein expression levels of MMP-9 in MDA-MB-231 cells.
EGCG, 20 μM epigallocatechin gallate from green tea.

COS Suppress the Protein Expressions of MMP-9 in MDA-MB-231 Cells

Western blot analysis was performed to determine the effect of COS on the protein expression of MMP-9. Pretreatment of MDA-MB-231 cells with COS decreased the protein expression levels of MMP-9 in a concentration-dependent manner (Fig. 5).

Discussion

Metastasis of cancer cells is a complex multistep process involving cell adhesion, invasion, and motility. Hence, interruption of one or more of these steps is one approach for antimetastatic therapy. The present study demonstrated that COS can inhibit the metastatic process of MDA-MB-231 human breast cancer cells.

The process of tumor cell invasion and metastasis requires the degradation of connective tissue associated with the vascular basement membrane (BM; i.e., Matrigel) and interstitial connective tissue [17, 20]. The BM is the largest barrier between a free malignant cell and the bloodstream, and it must be traversed before malignant cells can enter circulating blood [11]. Therefore, invasion through a BM is a critical step in metastasis [3]. Breast cancer cell invasiveness was investigated in this study using a Matrigel chamber invasion assay. The present study showed that COS displayed obvious inhibition on the invasive ability of MDA-MB-231 breast cancer cells in a concentration-dependent manner (Fig. 2B). At 1, 3, and 5 mg/ml, COS resulted in 47.1%, 76.9%, and 84.6% inhibition of TPA-induced invasiveness of MDA-MB-231 cells, respectively. A comparable effect was observed with EGCG (Fig. 2B).

Tumor metastasis from the original site to another distant organ needs migration. Any alteration of the cell migration would interrupt the metastatic cascade [3]. The present study showed that treatment with various concentrations of COS could reduce the motility of MDA-MB-231 cells. Taken together, these results demonstrate that COS inhibits processes that lead to metastasis of breast cancer cells.

For cancer cells, in order to invade and migrate through the basement membrane, proteolysis of the extracellular matrix must occur. This is accomplished by the secretion and activation of MMPs that degrade all extracellular matrix components. In particular, MMP-9 (gelatinase-B) degrades components of the basement membrane and has been associated with high potential of metastasis in several human carcinomas including breast cancer [1, 9]. To further explore the exact mechanism of COS-induced inhibition on the migration and invasion, we performed a set of experiments, including ELISA assay, gelatin zymography, and Western blot to detect the secretion, activity, and protein levels of MMP-9, respectively. The results showed that COS notably downregulated the secretion, activity and protein levels of MMP-9. At 5 mg/ml, COS was a more potent inhibitor of the secretion, activity, and protein expression of MMP-9 than EGCG (Figs. 3, 4, and 5).

The data presented herein indicate that COS attenuates processes involved in breast cancer metastasis by inhibiting the migration, invasion, and secretion, activity, and protein expression of MMP-9 in human breast cancer cells.

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References

1. Aye, M. M., C. Ma, H. Lin, K. A. Bower, R. C. Wiggins, and J. Luo. 2004. Ethanol-induced *in vitro* invasion of breast cancer cells: The contribution of MMP-2 by fibroblasts. *Int. J. Cancer* **112:** 738–746.
2. Bachmeier, B. E., A. G. Nerlich, R. Lichtinghagen, and C. P. Sommerhoff. 2001. Matrix metalloproteinases (MMPs) in breast cancer cell lines of different tumorigenicity. *Anticancer Res.* **21:** 3821–3828.
3. Chen, J. and L. U. Thompson. 2003. Lignans and tamoxifen, alone or in combination, reduce human breast cancer cell adhesion, invasion and migration *in vitro*. *Breast Cancer Res. Treat.* **80:** 163–170.
4. Cho, E. J., Md. A. Rahman, S. W. Kim, Y. M. Baek, H. J. Hwang, J. Y. Oh, H. S. Hwang, S. H. Lee, and J. W. Yun. 2008.

Chitosan oligosaccharides inhibit adipogenesis in 3T3-L1 adipocytes. *J. Microbiol. Biotechnol.* **18:** 80–87.

5. Friedel, G., U. Pastorino, R. J. Ginsberg, P. Goldstraw, M. Johnston, H. Pass, J. B. Putnam, and H. Toomes. 2002. Results of lung metastasectomy from breast cancer: Prognostic criteria on the basis of 467 cases of the International Registry of Lung Metastases. *Eur. J. Cardiothorac. Surg.* **22:** 335–344.
6. Garibyan, L., C. Lenahan, and S. Pories. 2004. Metalloproteinases: Promising tumor markers for breast cancer management. *Curr. Surg.* **61:** 255–259.
7. Gonzalez, L. O., I. Pidal, S. Junquera, M. D. Corte, J. Vazquez, J. C. Rodriguez, *et al.* 2007. Overexpression of matrix metalloproteinases and their inhibitors in mononuclear inflammatory cells in breast cancer correlates with metastasis-relapse. *Br. J. Cancer* **97:** 957–963.
8. Jeon, C. 2005. Mercury ion removal using a packed-bed column with granular aminated chitosan. *J. Microbiol. Biotechnol.* **15:** 497–501.
9. Jones, J. L., J. A. Shaw, J. H. Pringle, and R. A. Walker. 2003. Primary breast myoepithelial cells exert an invasion-suppressor effect on breast cancer cells *via* paracrine down-regulation of MMP expression in fibroblasts and tumour cells. *J. Pathol.* **201:** 562–572.
10. Khasigov, P. Z., O. V. Podobed, T. S. Gracheva, K. D. Salbiev, S. V. Grachev, and T. T. Berezov. 2003. Role of matrix metalloproteinases and their inhibitors in tumor invasion and metastasis. *Biochemistry (Mosc.)* **68:** 711–717.
11. Liotta, L. A., K. Tryggvason, S. Garbisa, I. Hart, C. M. Foltz, and S. Shafie. 1980. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature* **284:** 67–68.
12. Nam, K. S., M. K. Kim, and Y. H. Shon. 2007. Chemopreventive effect of chitosan oligosaccharide against colon carcinogenesis. *J. Microbiol. Biotechnol.* **17:** 1546–1549.
13. Nam, K. S., M. K. Kim, and Y. H. Shon. 2007. Inhibition of proinflammatory cytokine-induced invasiveness of HT-29 cells by chitosan oligosaccharide. *J. Microbiol. Biotechnol.* **17:** 2042–2045.
14. Nishimura, K., S. Nishimura, N. Nishi, I. Saiki, S. Tokura, and I. Azuma. 1984. Immunological activity of chitin and its derivatives. *Vaccine* **2:** 93–99.
15. Shin, W. S., J. C. Kil, and G. M. Park. 2006. Antiprotozoal activity of deacetylated chitosan oligosaccharide (dp 2-8) on *Trichomonas vaginalis*. *J. Microbiol. Biotechnol.* **16:** 1984–1989.
16. Sreenath, T., L. M. Matrisian, W. Stetler-Stevenson, S. Gattoni-Celli, and R. O. Pozzatti. 1992. Expression of matrix metalloproteinases genes in transformed rat cell lines of high and low metastatic potential. *Cancer Res.* **52:** 4942–4947.
17. Stetler-Stevenson, W. G., S. Aznavoorian, and L. Liotta. 1993. Tumor cell interactions with the extracellular matrix during invasion and metastasis. *Annu. Rev. Cell Biol.* **9:** 541–573.
18. Suzuki, K., T. Mikami, Y. Okawa, A. Tokoro, S. Suzuki, and M. Suzuki. 1986. Antitumor effect of hexa-N-acetylchitohexaose and chitohexaose. *Carbohydr. Res.* **151:** 403–408.
19. Yilmaz, M., G. Christofori, and F. Lehembre. 2007. Distinct mechanisms of tumor invasion and metastasis. *Trends Mol. Med.* **13:** 535–541.
20. Yu, A. E., R. E. Hewitt, D. E. Kleiner, and W. G. Stetler-Stevenson. 1996. Molecular regulation of cellular invasion – role of gelatinase A and TIMP-2. *Biochem. Cell Biol.* **74:** 823–831.