Potential Role of Rho-Associated Protein Kinase Inhibitor Y-27632 in Glaucoma Filtration Surgery

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PURPOSE. To investigate the role of Y-27632, a specific inhibitor of Rho-associated protein kinase (ROCK) in regulating human Tenon fibroblast (HTF) activities including proliferation, adhesion, contraction, migratory response, and myofibroblast trans-differentiation. Effects of Y-27632 on prevention of postoperative scar formation were also examined in a rabbit model of glaucoma filtration surgery.

METHODS. After treatment of HTFs with Y-27632, cell toxicity, proliferation, migration, adhesion, and contraction were studied. The cytoskeleton and α-smooth muscle actin (α-SMA) expression were examined via immunohistochemistry. In vivo studies in Japanese white rabbits consisted of a full-thickness sclerectomy followed in the 7-day postoperative period by topical application of Y-27632. Intraocular pressure, morphologic changes in bleb features, and histology of surgical sites were evaluated.

RESULTS. Y-27632 had no direct toxicity or significant effects on cell proliferation of HTF. The cell adhesion assay showed that Y-27632 promoted adhesiveness to both fibronectin and collagen type I. Use of Y-27632 significantly inhibited collagen gel contraction and α-SMA expression in HTFs. Y-27632 also increased HTF motility. In vivo, Y-27632 inhibited wound healing and fibroproliferation after filtration surgery and significantly improved surgical outcome compared with the vehicle. Histologic examination revealed that blebs in the Y-27632-treated group differed from those in the vehicle-treated group in that they lacked significant collagen deposition in the sclerostomy area.

CONCLUSIONS. Y-27632 had profound effects on activities of HTFs and was effective in preventing fibroproliferation and scar formation in a rabbit model of glaucoma surgery. A ROCK inhibitor may be an effective anti-scarring agent after glaucoma filtering surgery. (Invest Ophthalmol Vis Sci. 2007;48: 5549–5557) DOI:10.1167/iovs.07-0878
tion and provided written consent for the procedure. The protocol was approved by the Institutional Review Board at Kyoto University in compliance with tenets of the Declaration of Helsinki. Primary HTFs obtained from expansion cultures of the Tenon explants were propagated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum and antibiotics. Cells were maintained in the logarithmic growth phase, and cells from passages 3 to 6 were used in all experiments, which were performed at least three times with similar results.

Trypan Blue Exclusion Test
The cytotoxicity of Y-27632 was evaluated via a trypan blue exclusion test. Viable cells were counted in vitro according to a previously described method. Briefly, 5 x 10^5 HTFs were plated and grown for 24 hours, then treated with or without Y-27632 (1, 10, or 100 µM) for 24 hours. After trypan blue treatment, stained, and unstained cells were counted by using a hemocytometer. The percentage of cell viability was calculated according to the following formula: % cell viability = (viable cell count/total cell count) x 100. Five independent experiments were performed.

Cell Proliferation Assay
Proliferation of cultured HTFs was measured by use of the commercially available MTT (3-(4,5-dimethyl-2-thiazyl)-2, 5-diphenyl-2-tetrazolium bromide) cell proliferation kit (Nacalai Tesque, Kyoto, Japan), according to the manufacturer’s instructions. Cells were plated at a density of 1 x 10^4 cells per well in 96-well plates and were allowed to adhere for 24 hours. After cultures were washed with phosphate-buffered saline (PBS), they were incubated with or without Y-27632 (1, 10, or 100 µM) for 72 hours and then treated with 5 mg/mL MTT for 4 hours at 37°C. The relative active number of cells was determined by an automated plate reader (Bio-Rad, Hercules, CA).

Cell Adhesion Assay
The cell adhesion assay was performed as previously described. Wells in 96-well plates were coated overnight with 10 µg/mL fibronectin (Sigma-Aldrich-Aldrich, St. Louis, MO) or 0.5 µg/mL collagen type I (Calbiochem, San Diego, CA) at 4°C. Remaining binding sites were blocked by 0.1% bovine serum albumin (BSA) in PBS. HTFs in culture medium containing 2 mg/mL BSA with or without Y-27632 (1, 10, or 100 µM) or 0.5 µg/mL collagen type I (Nitta Gelatin, Osaka, Japan), 10% DMEM, reconstitution buffer (Sigma-Aldrich), HTF cell suspension, and water were mixed in an ice bath at a ratio of 7:1:1:1. Aliquots (0.5 mL) of the resultant mixture were added to each well of 1% BSA-coated 24-well clusters and collagen gel formation was induced. DMEM (0.5 mL), with or without Y-27632 (1, 10, or 100 µM), was then placed on top of the collagen gels. After 1 hour of incubation, gels were freed from the walls of the culture wells, and diameters of the gels were scanned into a computer and measured every 24 hours for 4 days.

Collagen Gel Contraction Assay
This assay was performed as previously described, with minor modifications. Briefly, HTFs were trypsinized and resuspended in culture medium at a density of 2.2 x 10^6 cells/mL, with or without Y-27632 (1, 10, or 100 µM). Collagen type I (Nitta Gelatin, Osaka, Japan), 10% DMEM, reconstitution buffer (Sigma-Aldrich), HTF cell suspension, and water were mixed in an ice bath at a ratio of 7:1:1:1. Aliquots (0.5 mL) of the resultant mixture were added to each well of 1% BSA-coated 24-well clusters and collagen gel formation was induced. DMEM (0.5 mL), with or without Y-27632 (1, 10, or 100 µM), was then placed on top of the collagen gels. After 1 hour of incubation, gels were freed from the walls of the culture wells, and diameters of the gels were scanned into a computer and measured every 24 hours for 4 days.

Immunohistochemistry
HTFs were plated on glass coverslips, cultured overnight, and then serum starved for 24 hours. Then, 10 µM lysophosphatidic acid (LPA; Sigma-Aldrich) was added for 10 minutes, after which it was incubated with Y-27632 (1, 10, or 100 µM) for 30 minutes. After this exposure, the cells were fixed in 2% parafomaldehyde-PBS for 15 minutes and then blocked in 2% BSA for 30 minutes. Coverslips were incubated with anti-vinculin antibody (Sigma-Aldrich) or αSMA antibody (Dako Japan, Kyoto, Japan) for 30 minutes. Rhodamine- phalloidin (Invitrogen-Molecular Probes, Eugene, OR) was used to counterstain the F-actin cytoskeleton. Samples were washed with PBS and incubated with FITC-conjugated secondary antibody (Chemicon, Temecula, CA) for 30 minutes. After they were washed, the cells were mounted in antifade medium and observed via the fluorescence microscope (model IX71; Olympus, Tokyo, Japan). Immunohistochemistry with rabbit specimens used the same routine procedure.

Measurement of Cell Motility Activities
HTFs were grown to confluence in 100-mm tissue culture dishes. Three or four sites in each dish were scraped with a yellow plastic pipette tip to remove the confluent cells and create a linear line. Medium was replaced with fresh medium, with or without Y-27632 (1, 10, or 100 µM). After incubation at 37°C for 9 hours, the movement of cells into the wound area was photographed by a digital microscope camera (Olympus) and analyzed via computer software (Olympus). The shortest distance between the edges of migrated HTFs (including protrusions) from both sides was measured, as previously described.

Animals and Sclerostomy Protocol
Twelve female Japanese White rabbits (Pasteurella free, each 2.0 to 2.4 kg, 12 to 14 weeks old; SRC Japan, Osaka, Japan) were used. Experiments were conducted according to guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Animal Use Committee at Kyoto University. All investigations involving rabbits, including surgery, IOP measurements, bleb scoring, and determination of histologic features, were conducted in a blinded manner.

Rabbits were anesthetized with an intramuscular injection of ketamine hydrochloride (5 mg/kg body weight), and xylazine hydrochloride (5 mg/kg body weight). A limbus-based flap of the conjunctiva and the Tenon capsule was made at a distance of 5 mm from the limbus in the superior nasal quadrant of the right eye. A sclerostomy was performed, and a fistula was constructed toward the anterior chamber. The conjunctiva was closed with three 8-0 polyglactin 910 sutures. After bleb formation was tested, topical Y-27632 or PBS (12 µL of eye drops of 10 mM Y-27632 or the same amount of PBS) was applied to the eye. After surgery, 1 cm of 3% g of ofloxacin ointment was applied to the eye. Topical Y-27632 or PBS was applied for 7 postoperative days.

Clinical Evaluation of Postoperative Y-27632 Effects in a Rabbit Model of Glaucoma Filtration Surgery
Glaucoma Filtration Surgery. Baseline observations were obtained before surgery. The IOP of the rabbit eye was measured during topical instillation of anesthetic before and after surgery by using an application tonometer (Toνονεντ, Tiotlät, Helsinki, Finland). IOP was monitored daily for 7 postoperative days, with values obtained at the same time each day. Blebs were examined via a slit lamp and were graded as previously described by Perkins et al., according to a qualitative scale of 1+ to 4+, reflecting increasing bleb height and size as follows: 1+, minimal height, conjunctiva thickening, no microcysts; 2+, microcysts present; 3+, elevated bleb covering 2 to 3 clock hours of the eye; and 4+, greatly elevated bleb covering more than 4 clock hours. A score of 0 indicated no observable bleb. Bleb failure was defined as the appearance of a flat, vascularized, scarred bleb in the presence of a deep anterior chamber.

Histologic Evaluation. For this study, rabbits were killed humanely 7 days after surgery. An incision was made 90° away from the
surgical site, and the whole globe was fixed in 4% paraformaldehyde in PBS for 48 hours at 4°C and was then embedded in medium (Tissue-Tek; Sakura Finetechical, Tokyo, Japan). Serial sections were cut through the sclerostomy site. Approximately every fifth section was stained with standard hematoxylin-eosin (HE), elastica van Gieson (EVG; for collagen), or α-SMA. The extent of fibroproliferation and scar formation in sections was evaluated in a blinded manner.

**Statistical Analysis**

Data are presented as the mean ± SEM. Statistical comparisons of multiple groups used one-way or two-way repeated-measures analysis of variance (ANOVA) followed by the Bonferroni/Dunn post hoc test. Comparisons of two groups used Student’s t-test with Bonferroni correction. Differences were considered statistically significant at \( P < 0.05 \).

**RESULTS**

**Toxicology of Y-27632: Effects on HTFs**

The result of the trypan blue exclusion test showed that the percentage of living HTFs was 96.4% ± 0.3% in control cultures without Y-27632 treatment. In experimental cultures for 1, 10, and 100 \( \mu \)M Y-27632, percentages of living cells were 96.7% ± 0.4%, 97.3% ± 0.5%, and 96.5 ± 0.4 for 1, 10, and 100 \( \mu \)M Y-27632, respectively. These values were not significantly different from the control value.

Cell proliferation was measured by the MTT assay. The assay is based on measuring the intracellular formazan spectrophotometrically which is facilitated by active cells. The optical absorbance of Y-27632-treated cells were 0.66 ± 0.02, 0.66 ± 0.02, and 0.67 ± 0.01 for 1, 10, and 100 \( \mu \)M Y-27632 respectively, while that of control cells was 0.67 ± 0.03. The MTT assay revealed no significant differences between Y-27632-treated HTFs and control samples. This inhibitor thus displayed little toxicity and had no effect on proliferative activity of HTFs.

**Influence of Y-27632 on Adhesion of HTFs to the ECM**

Compared with controls, greater numbers of Y-27632-treated HTFs adhered to fibronectin and collagen type I, with adhesion to both fibronectin and collagen showing a Y-27632 concentration-dependent effect (Fig. 1). Results were statistically significant for both fibronectin (at 10 \( \mu \)M) and collagen type I (at 100 \( \mu \)M).

**Effects of Y-27632 on the Cytoskeleton and on α-SMA Expression**

In an immunohistochemical study, LPA treatment induced assembly of actin stress fibers and increased the number of focal adhesions in HTFs. Addition of Y-27632 prevented this assembly and focal adhesion expression (Figs. 2A, 2B). Addition of LPA, however, caused redistribution of focal adhesions in the cell periphery. (Fig. 2B).

HTFs were stimulated with LPA in the presence of 10 \( \mu \)M Y-27632 or vehicle control. Although untreated serum-starved cells showed weak α-SMA staining, LPA treatment induced assembly of α-SMA-positive stress fibers in approximately 50% of the cells, and addition of Y-27632 prevented these LPA-induced effects (Fig. 2C).

**Gel Contraction Assay**

Compared with controls, Y-27632 caused significant concentration-dependent inhibition of HTF-mediated collagen gel contraction in the presence of serum (Fig. 3A). At 48 hours after plating and incubation without Y-27632, the original diameter of the gels (16 mm) changed to 5.5 ± 0.2 mm (n = 4). However, with addition of 1, 10, or 100 \( \mu \)M Y-27632, collagen gel diameters at the same time point measured 4.4 ± 0.4, 1.9 ± 0.2, and 0.4 ± 0.2 mm, respectively (Fig. 3B). Experiments at 72 and 96 hours produced similar results. Results were statistically significant, and dose dependency was evident (\( P < 0.001 \)).

**HTF Cell Motility Activities**

At 9 hours after the scraping, the distance between the edges of the exposed regions was 71.7% ± 4.4%, 51.2% ± 3.6%, 24.9% ± 5.0%, 7.6 ± 2.8% respectively, with Y-27632 at 0 (control), 1, 10, and 100 \( \mu \)M (Fig. 4). The increase in Y-27632-induced wound healing was significant and depended on concentration.

**Effects of Y-27632 on Filtration Blebs**

Topical instillation of Y-27632 significantly improved the results of glaucoma filtration surgery in this rabbit model and prolonged bleb survival compared with vehicle-treated control animals. Figure 5A shows the typical appearance of the blebs during the 7 postoperative days. Slit lamp examination showed that the treatment with Y-27632 was associated with elevated, diffuse blebs with mild conjunctival injection rather than the flat, scarred, vascularized blebs in the controls. In the vehicle-treated controls, vascularization in the conjunctiva was significant. Although no significant differences in anterior chamber depth or anterior chamber inflammation were observed between Y-27632-treated and control groups, postoperative IOP was significantly lower in the Y-27632-treated group (Fig. 5B). No significant postoperative changes in pupil dilation or cornea were observed in both groups. Analysis of bleb scores revealed significant differences between the Y-27632-treated and vehicle-treated control groups (Fig. 5C).

**Effects of Y-27632 on Histologic Characteristics of Eyes in a Rabbit Model of Glaucoma Filtration Surgery**

Surgical sites were examined 7 days after the surgery and stained with HE, EVG, or α-SMA. HE stain of vehicle-treated eyes exhibited nearly complete scarring over the sclerostomy site (Fig. 6A), including evidence of new collagen deposition in the scleral gap and bleb area, as shown by EVG stain (Fig. 6C).
In contrast, HE stain showed that postoperative scar formation at day 7 was significantly reduced by Y-27632 treatment (Fig. 6B). EVG stain showed that eyes treated with Y-27632 had bleb cavities of moderate size and evidence of minimal deposition of new collagen in the sclera (Fig. 6D). As judged by EVG with higher magnification, both the conjunctiva and the subconjunctival scar (Fig. 6E) and the area around the failed bleb (Fig. 6G) in the vehicle-treated group consisted of dense collagen fibers and scar tissue. In contrast, the surviving Y-27632-treated bleb showed a much looser architecture with a visible conjunctiva (Fig. 6F) and bleb formation with fewer collagen deposits (Fig. 6H). The sclerostomy area in the vehicle-treated eye consisted of densely packed collagen (Fig. 6I), whereas the site in the Y-27632-treated group differed and showed loose cell infiltration without significant collagen deposition (Fig. 6J).

At the microscopic level, no significant changes in the trabecular meshwork were observed after Y-27632-treatment compared with the vehicle-treated group.

Immunohistochemical staining of blebs on postoperative day 7 demonstrated reduced expression of α-SMA after Y-27632 treatment (Fig. 6K) compared with the control (Fig. 6L).

**DISCUSSION**

Scarring is a major reason for failure of filtration surgery. Several studies showed that subconjunctival scarring of the
filtering bleb site is mainly mediated by HTF proliferation, migration, and contraction. Fibroblasts, including HTFs, are stimulated by growth factors to differentiate into myofibroblasts both in vitro and in vivo. Myofibroblasts are responsible for fibrosis via increased ECM synthesis, for granulation tissue formation, and wound contraction.

The use of antimetabolites has been one of the most important developments in glaucoma surgery. However, antimetabolite treatment can result in several postoperative bleb-related problems. Therefore, alternative anti-scarring agents that do not cause extensive tissue damage are needed. In the present study, a specific ROCK inhibitor, Y-27632, induced profound changes in cultured HTFs without significant toxicity or inhibition of HTF proliferation. In addition, topical instillation of Y-27632 was highly effective in reducing scar tissue formation in a rabbit model of glaucoma filtration surgery.

Our in vitro results revealed that exposure to Y-27632 enhanced adhesiveness of cells to the ECM. This finding correlates well with our previous report on TM cells. The actin cytoskeleton is known to interact with integrins to regulate cell shape and adhesiveness of cells to the ECM. Y-27632 reportedly promotes integrin adhesion in cultured THP-1 monocytes. Our immunocytochemical investigation in the present study documented redistribution of focal adhesions in the cell periphery. This increased adhesiveness of cells to the ECM may be related to alterations in cell shape and redistribution of focal adhesions to the cell periphery.

LPA, a bioactive lipid growth factor that is present in aqueous humor, regulates various cellular events. LPA, associated with activation of the Rho, induces very strong actin fibers and focal adhesions in various kinds of cells. A previous study of myofibroblasts demonstrated that LPA and serum, as well as TGF-β, could activate myofibroblast differentiation, which is supposedly one of the most potent stimulators of HTFs. After glaucoma filtration surgery, HTFs are likely to be exposed to LPA via serum and/or plasma, because the blood–aqueous humor barrier breaks down, and circulating aqueous humor bathes the wound site. Y-27632 has been reported to inhibit LPA-promoted myofibroblast contraction in the collagen lattice model, which suggests that contraction depends on activation of the Rho. In the present study, we investigated LPA-induced α-SMA expression in HTFs to analyze the direct role of the Rho signaling pathway in these cells. Treatment with Y-27632 reduced LPA-induced α-SMA expression in HTFs, which suggests that Y-27632 functions as a potent antiscarring agent via inhibition of transdifferentiation of HTFs into myofibroblasts.

**FIGURE 3.** Effects of Y-27632 on HTF-mediated collagen gel contraction. (A) Collagen gels were incubated without (Control) or with Y-27632 (1, 10, or 100 μM) for up to 96 hours. (B) The extent of contraction of collagen gels mediated by HTFs was expressed as the decrease in gel diameter compared with the initial diameter. Changes in diameters of collagen gels in the absence (control) or presence of Y-27632 at 1, 10, or 100 μM were measured. Data are shown as the mean ± SEM (n = 4). P < 0.001 versus control. Compared with the control, Y-27632 caused statistically significant, concentration-dependent inhibition of HTF-mediated collagen gel contraction (P < 0.001).

**FIGURE 4.** Effects of Y-27632 on cell motility activities of HTFs. (A) Confluent cultures were scraped with a yellow pipette tip to create a cell-free linear wound. Medium was replaced with fresh medium without (control) or with Y-27632 (1, 10, or 100 μM). After 9 hours, migration of cells into the wound area was photographed. Dotted lines: edges of the migrated cells. (B) Distances between edges of migrated cells were measured and are shown as the mean ± SEM (n = 6); the distance before treatment was set at 100%. *P < 0.05, †P < 0.001 versus control.
Gel contraction experiments using three-dimensional cultures of HTFs embedded in collagen type I gels revealed that addition of Y-27632 decreased contraction of gels. This result suggests reduced cell contractility and/or altered interaction between HTFs and collagen type I. Y-27632 treatment increased cell adhesion to collagen type I, and so the change in gel contraction is thought to be due to the relaxation of the HTFs induced by Y-27632.

Our results indicate that administration of Y-27632 enhanced the motility of the HTFs, which is in agreement with the idea that stabilization of actin stress fibers limits cell movement. We have reported that Y-27632 enhances motility of cultured TM cells. In the same study, we demonstrated reduced levels of phosphorylated LIM kinase 2 and cofilin, which is one of the targets downstream of Rho signaling pathways. A previous report from other laboratories showed that constitutively active RhoAV14 significantly inhibited wound closure and that incubation with Y-27632 promotes wound healing. As shown by others and according to findings in the present study, inhibition of formation of stress fibers and focal adhesions via Y-27632 improves cell migration during wound healing. Several candidate antiscarring agents, such as alkylphosphocholines and p38 inhibitors, are also reported to inhibit HTF proliferation and contraction. Although alkylphosphocholines are reported to inhibit migration of HTFs, the main effect of p38 inhibitors is supposed to be an inhibition of transdifferentiation of HTFs into myofibroblasts. In contrast, it is interesting that we found that Y-27632 not only increased migration but also inhibited cell contraction and transdifferentiation of HTFs into myofibroblasts. These findings, taken together, suggest that inhibition of migration may not be necessary for antiscarring effects when associated with decreased myofibroblast transdifferentiation and inhibited cell contraction. However, further studies are needed to elucidate the association of migration and scar formation after Y-27632 treatment.

In an animal model of filtration surgery, obstruction of the sclerostomy site from excessive ECM deposition and contraction.
tion due to transdifferentiation of fibroblasts into myofibroblasts, which is characterized by synthesis of \( \alpha \)-SMA, have been reported.\(^51,52\) In our present study, histologic analysis of rabbit tissues showed that topical instillation of Y-27632 significantly reduced subconjunctival collagen deposition compared with controls. Y-27632 also significantly reduced the population of cells expressing \( \alpha \)-SMA, which indicates inhibition of myofibroblast transdifferentiation in vivo. These observations are in good agreement with the present in vitro data that Y-27632 significantly reduced \( \alpha \)-SMA expression in HTFs, suggesting that beneficial effects of Y-27632 in glaucoma surgery may be mediated by reduced cell contraction and inhibition of transdifferentiation of fibroblasts.

Previously, we have reported the significant IOP-lowering effects of ROCK inhibitors and have shown the possibilities for clinical use.\(^51,52\) In our present study, histologic analysis of rabbit tissues showed that topical instillation of Y-27632 significantly reduced subconjunctival collagen deposition compared with controls. Y-27632 also significantly reduced the population of cells expressing \( \alpha \)-SMA, which indicates inhibition of myofibroblast transdifferentiation in vivo. These observations are in good agreement with the present in vitro data that Y-27632 significantly reduced \( \alpha \)-SMA expression in HTFs, suggesting that beneficial effects of Y-27632 in glaucoma surgery may be mediated by reduced cell contraction and inhibition of transdifferentiation of fibroblasts.

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In conclusion, our results indicate that Y-27632, a selective ROCK inhibitor, effectively reduced subconjunctival scarring after experimental glaucoma filtration surgery. Y-27632 was safe and well tolerated in this model. Further study is needed to determine whether inhibition of the ROCK/ROK family can lead to development of an alternative, more physiological agent to protect against postoperative scarring.

References


**FIGURE 6.** Histologic characteristics of postoperative blebs. Y-27632 treatment reduced scarring at the microscopic level. The images show representative sections from eyes in each group. HE stain revealed that the total amount of scar tissue in the subconjunctival space was significantly greater in vehicle-treated (A) than in Y-27632-treated (B) eyes at day 7. EVG stain revealed that vehicle-treated eyes contained scar tissue with densely packed collagen deposits (C, arrows) and that the size of the collagen deposits was significantly greater than that in Y-27632-treated eyes (D, arrows). EVG also showed that subconjunctival scarring in a vehicle-treated failed bleb consisted of dense collagen fibers and fibroblasts (E, arrows) and that the failed bleb area was densely packed with collagen deposits and fibroblasts (G). In contrast, the surviving Y-27632-treated bleb showed much looser architecture with a visible conjunctiva (F) and bleb formation with less collagen (H). In the area of sclerostomy, the wound site of the failed vehicle-treated bleb consisted of densely packed collagen (I, arrows). The sclerostomy site in the Y-27632-treated group, different from that in the vehicle-treated group, showed loose cell infiltration without significant collagen deposition (J). Immunohistochemical staining for \( \alpha \)-SMA in postoperative blebs on day 7 demonstrated reduced \( \alpha \)-SMA expression after Y-27632 treatment (L) compared with massive \( \alpha \)-SMA expression of the control eye (K, white arrows). Magnification: (A-D) \( \times \) 25; (E, F, K, L) \( \times \) 100; (G-J) \( \times \) 200.
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