Pharmacological preconditioning with resveratrol: role of nitric oxide

REIJI HATTORI,1 HAJIME OTANI,2 NILANJANA MAULIK,1 AND DIPAK K. DAS1
1Cardiovascular Research Center, University of Connecticut School of Medicine, Farmington, Connecticut 06030-1110; and 2Kansai Medical University, Moriguchi, Osaka, Japan

Received 20 November 2001; accepted in final form 21 January 2002

Hattori, Reiji, Hajime Otani, Nilanjana Maulik, and Dipak K. Das. Pharmacological preconditioning with resveratrol: role of nitric oxide. Am J Physiol Heart Circ Physiol 282: H1988–H1995, 2002; 10.1152/ajpheart.01012.2001.—Resveratrol (trans-3,4',5-trihydroxystilbene), a recently described grape-derived polyphenolic antioxidant, has been found to protect the heart from ischemic-reperfusion injury. The present study sought to determine the mechanisms of cardioprotection by investigating the ability of resveratrol to precondition the heart. Isolated perfused rat hearts were randomly divided into six groups: group I was perfused for 15 min with Kreb-Henseleit buffer (KHB) only; group II was perfused with 10 μM resveratrol; group III was perfused with 10 μM resveratrol plus 100 μM Nω-nitro-L-arginine methyl ester (L-NAME), a nonselective nitric oxide (NO) synthase (NOS) inhibitor; group IV was perfused with 10 μM resveratrol plus 100 μM aminoguanidine (AG), an inducible NOS (iNOS) blocker; and groups V and VI consisted of hearts perfused with L-NAME and AG, respectively. The perfusion was then switched to working mode, and all hearts were made globally ischemic for 30 min followed by 2 h of reperfusion. Preconditioning of the hearts with resveratrol provided cardioprotection as evidenced by improved postischemic ventricular functional recovery (developed pressure and aortic flow) and reduced myocardial infarct size and cardiomyocyte apoptosis. Resveratrol-mediated cardioprotection was completely abolished by both L-NAME and AG. In a separate study, hearts were examined for iNOS mRNA beginning at 30 min after reperfusion, increasing steadily up to 60 min of reperfusion, and then decreasing progressively up to 2 h after reperfusion. Preperfusion of the hearts with AG almost completely blocked the induction of iNOS. The results of our study demonstrate that resveratrol can pharmacologically precondition the heart in a NO-dependent manner.

inducible nitric oxide synthase; grapes; wine; antioxidants; polyphenols; heart; ischemia-reperfusion

There is a continuing effort to develop ideal cardioprotective compounds to cure ischemic heart disease. Apart from pharmacological cardioprotective agents, alternative strategies are also developed among which ischemic preconditioning has received significant attention. Ischemic preconditioning mediated by one or more brief periods of ischemia and reperfusion is considered to be the most powerful cardioprotective technique of recent years (9). Unlike pharmacological interventions that protect the ischemic myocardium by neutralizing toxic components, ischemic preconditioning exerts cardioprotection by upregulating endogenous protective mechanisms, which include several cardioprotective enzymes and proteins (25, 36). Unfortunately, ischemic preconditioning-mediated cardioprotection is realized through two windows, first, early, or classical ischemic preconditioning lasting up to 3–4 h and delayed ischemic preconditioning lasting from 1 to 3–4 days (8). Attempts to increase these windows have met with limited success. There is a definite need for developing a pharmacological preconditioning approach that can significantly increase these windows of cardioprotection.

Recently, resveratrol (trans-3,4',5-trihydroxystilbene), a naturally occurring phenolic compound abundantly found in grape skins and in wines, has been found to protect the heart from ischemic-reperfusion injury (10, 30). Resveratrol is a polyphenol phytoalexin (trans-3,5,4'-trihydroxystilbene) that possesses diverse biochemical and physiological actions, which include estrogenic, antiplatelet, and anti-inflammatory properties (2, 13). Recently, resveratrol was found to protect the kidney, heart, and brains from ischemic-reperfusion injury (1, 10, 16, 30). In kidney cells, resveratrol was found to exert its protective action through upregulation of nitric oxide (NO) (16). A growing body of evidence supports the cardioprotective role of constitutive expression of NO (14, 24). For example, a NO donor or a precursor for NO synthesis like L-arginine has been found to ameliorate myocardial ischemic-reperfusion injury (7, 12). Recently, NO has been found to play a crucial role in ischemic preconditioning in which NO functions as the mediator of ischemic preconditioning (17).

Because resveratrol can protect a variety of tissues against ischemic injury and this polyphenol can enhance the constitutive NO in some cells, including the kidney (5, 20), we hypothesized that resveratrol may function as a pharmacological preconditioning agent...
acting through NO. To test the hypothesis, a group of rat hearts were pretreated with resveratrol for 15 min. The resveratrol-treated rat hearts were found to be resistant against ischemic-reperfusion injury compared with those from nontreated hearts. A NO inhibitor, $N^G$-nitro-l-arginine methyl ester (l-NAME), as well as an inducible NO synthase (iNOS) blocker, aminoguanine (AG), abolished the resveratrol-mediated cardioprotection, suggesting a role of NO in resveratrol preconditioning.

**MATERIALS AND METHODS**

*Isolated perfused heart preparation.* Sprague-Dawley rats weighing 275–300 g were anesthetized with pentobarbital sodium (80 mg/kg ip injection, Abbott; North Chicago, IL). After intravenous administration of heparin (500 IU/kg, Elkins-Sinn; Cherry Hill, NJ), the chests were opened, and the heart from each rat was rapidly excised and mounted on a nonrecirculating Langendorff perfusion apparatus (19). The perfusion buffer used in this study consisted of a modified Krebs-Henseleit bicarbonate buffer (KHB) (in mM: 116 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, 10 glucose, and 1.7 CaCl2), pH 7.4, gassed with 95% O2-5% CO2, and filtered through a 5-μm filter to remove any particulate contaminants. The buffer was maintained at a constant temperature of 37°C and was gassed continuously for the entire duration of the experiment. Left atrial cannulation was then carried out, and after allowing for stabilization of 10 min in the retrograde perfusion mode, the circuit was switched to the antegrade working mode, which allowed for the measurement of myocardial contractility as well as aortic and coronary flows, as described in detail in a previous paper (11). Essentially, it is a left heart preparation in which the heart is perfused with a constant preload of 17 cmH2O (being maintained by means of a Masterflex variable speed modular pump, Cole Parmer Instrument; Vernon Hills, IL) and pumps against an afterload of 100 cmH2O. At the end of 10 min after the attainment of steady-state cardiac function, baseline functional parameters were recorded as usual. The circuit was then switched back to the retrograde mode. The hearts were divided into six groups, with six to eight hearts in each group. In *group I*, isolated hearts were perfused for 15 min with KHB buffer after 10 min of KHB buffer perfusion for stabilization. In *group II*, isolated hearts were preperfused with 10 μM resveratrol for 15 min after 10 min of stabilization. In *group III*, isolated hearts were preperfused with 100 μM l-NAME, a NO inhibitor, and 10 μM resveratrol for 15 min after 10 min of stabilization. In *group IV*, isolated hearts were preperfused with 100 μM l-NAME, an iNOS blocker, and 10 μM resveratrol for 15 min after 10 min of stabilization. In *group V* and *group VI*, isolated hearts were preperfused with 100 μM l-NAME and 100 μM AG, respectively, for 15 min after 10 min of stabilization. The hearts were then perfused with fresh KHB buffer for 10 min to allow washout of the drugs. All groups were subjected to 30 min of global ischemia followed by 120 min of reperfusion with the same KHB buffer. The protocol of the experiment is shown in Fig. 1. The first 10 min of reperfusion was in the retrograde mode to allow for postischemic stabilization and thereafter in the antegrade working mode to allow for assessment of functional parameters, which were recorded at 15, 30, 60, 90, and 120 min into reperfusion.

*Measurement of cardiac function.* Aortic pressure was measured using a Gould P23 XL pressure transducer (Gould Instrument Systems; Valley View, OH) connected to sidearm of the aortic cannula. The signal was amplified using a Gould 6600 series signal conditioner and monitored on a CORDAT II real-time data acquisition and analysis system (Triton Technologies, San Diego, CA). Heart rate, developed pressure (defined as the difference of the maximum systolic and diastolic pressures), and the maximal first derivative of developed pressure (dP/dtmax) were all derived or calculated from the continuously obtained pressure signal. Aortic flow was measured using a calibrated flowmeter (Gilmore Instruments; Barrington, IL), and coronary flow was measured by timed collection of the coronary effluent dripping from the heart.

*Evaluation of infarct size.* Hearts to be used for infarct size calculations were taken at the end of the experiment, and 1% triphenyltetrazolium solution in phosphate buffer (88 mM Na2HPO4, 33 mM NaH2PO4) was perfused into the coronary artery directly for 15 min at 37°C and then stored at −70°C for subsequent processing (32). Frozen hearts (including only ventricular tissue) were sliced transversely in a plane perpendicular to the apex-basal axis into ~0.5-mm-thick sections, blotted dry, placed in between microscope slides, and scanned on a Hewlett-Packard Scanjet 5p single pass, flat bed scanner (Hewlett-Packard; Palo Alto, CA). Using the National Institutes of Health 1.61 Image processing software, each digitized image was subjected to equivalent degrees of background subtraction, brightness, and contrast enhancement for improved clarity and distinctness. Risk (equivalent to total left ventricular muscle mass) as well as infarct zone of each slice were traced, and the respective areas were calculated in terms of pixels. The weight of each slice was then recorded to facilitate the expression of total and infarct masses of each slice in grams. The risk and infarct volumes (in cm³) of each slice were then calculated on the basis of slice weight to remove the introduction of any errors due to nonuniformity of heart slice thickness. The risk volumes and infarct volumes of each slice were summed to obtain the risk and infarct volumes for the whole heart. Infarct size was taken to be the percent infarct per risk area for any one heart.

*Cardiomyocyte apoptosis.* Immunohistochemical detection of apoptotic cells was performed using an Apop Tag Plus in situ apoptosis detection kit (Oncon; Gaithersburg, MD) according to the following principles: residues of digoxigenin-labeled dUTP are catalytically incorporated into the DNA by terminal deoxynucleotidyl transferase (transferase-mediated dUTP nick-end labeling, TUNEL), an enzyme that catalyses a template-independent addition of nucleotide triphosphate to the 3’-OH ends of double- or single-stranded DNA. The incorporated nucleotide was stained immunohistochemically.
with a peroxidase-conjugated sheep polyclonal anti-digoxigenin antibody and diaminobenzidine as described by the manufacturer. Counterstain was performed with methyl green. Positive control samples were prepared by incubating sections with 10 U/ml DNase I for 20 min at 37°C before treatment with terminal deoxynucleotidyl transferase. Negative control slides were processed in the absence of terminal deoxynucleotidyl transferase. The number of TUNEL-positive cardiomyocytes was counted in 60 random high-power fields on the left ventricular free wall at the mid left ventricular level from the endocardial to the epicardial portion. The percentage of TUNEL-positive cardiomyocytes was calculated by dividing the number of TUNEL-positive cardiomyocytes by the total number of cardiomyocytes in 60 microscopic fields.

**Estimation of malonaldehyde formation.** Malonaldehyde (MDA) was assayed as described previously to monitor the development of oxidative stress (6). In short, coronary effluents were collected in 2 ml of a solution containing 20% trichloroacetic acid and 5.3 mM sodium bisulfite, kept on ice for 10 min, and centrifuged at 3,000 g for 10 min; supernatants were then collected, derivatized with 2,4-dinitrophenylhydrazine (DNPH), and extracted with pentane. Aliquots of 25 μl in acetonitrile were injected onto a Beckman Ultrasphere C18 (3 μm) column. The products were eluted isocratically with a mobile phase containing acetonitrile-water-acetic acid (40:60:0.1 vol/vol/vol) and measured at three different wavelengths (307, 325, and 356 nm) using a Waters M-490 multichannel ultraviolet detector. The peak for MDA was identified by cochromatography with DNPH derivative of the authentic standard, peak addition, ultraviolet pattern of absorption at the three wavelengths, and by gas chromatography-mass spectrometry.

**Northern blot analysis for iNOS induction.** For RNA extraction, hearts were obtained at baseline, after 15 min perfusion with or without resveratrol, and at different periods of reperfusion. Hearts were excised, instantly frozen in liquid N2, and stored at −70°C for subsequent RNA preparation. At a later date, total RNA was extracted from the heart by the acid-guanidinium-thiocyanate-phenol-chloroform method as described previously (26). For Northern blot analysis, total RNA was electrophoresed in 1% agarose-formaldehyde-formamide gel and transferred to GeneScreen Plus. After prehybridization, membranes were hybridized with a 1.8-kb fragment of mouse macrophage iNOS cDNA obtained from Cayman Chemical (Ann Arbor, MI). Each hybridization was repeated at least three times with different membranes. After each hybridization, the iNOS cDNA was removed and rehybridized with a GAPDH cDNA probe, the result of which served as loading controls.

The autoradiographs were evaluated quantitatively by a computerized densitometric scanner. The results of densitometric scanning were normalized relative to signal obtained for the GAPDH-cDNA probe.

**Statistical analysis.** A two-way ANOVA followed by Scheffé’s test was first carried out using the Primer Computer Program (McGraw-Hill, 1988) to test for differences between groups. If differences were established, the values were compared using Student’s t-test for paired data. The values were expressed as means ± SE. The results were considered significant if P was <0.05.

**RESULTS**

**Functional recovery.** There were no differences in baseline function among the groups. As was expected, upon reperfusion, the absolute value of developed pressure, dP/dt max, and aortic flow were decreased in all groups compared with the baseline values, whereas coronary flow and heart rate (not shown) did not exhibit significant change. Resveratrol (10 μM)-treated rat hearts displayed significant recovery of postischemic myocardial function. We also tried two higher doses (25 and 100 μM) of resveratrol and found 10 μM to be an optimal dose for preconditioning. Aortic flow was significantly higher than control values in the resveratrol-treated group (Fig. 2). The cardioprotective role of resveratrol was also evidenced by significantly higher developed pressure and dP/dt max readings throughout the reperfusion period (Fig. 3). In contrast, L-NAME- and AG-treated rat hearts displayed significantly lower postischemic recovery in developed pressure, dP/dt max, and aortic flow compared with those for the control group. L-NAME and AG when combined
Inhibition of cardiomyocyte apoptosis with resveratrol. We performed double antibody staining using the antibody in Apop Tag kit and the monoclonal antibody recognizing cardiac myosin heavy chain to specifically identify cardiomyocyte apoptosis. A significant number of apoptotic myocytes as well as nonmyocyte cells were visible in the normal hearts subjected to 30 min of ischemia and 2 h of reperfusion (Fig. 5). The number of apoptotic cells expressed as a percentage of total cardiomyocyte population was higher in the hearts that were pretreated with either L-NAME or AG compared with resveratrol-treated hearts. In contrast, only a few apoptotic cells were visible in the resveratrol-treated hearts. This anti-apoptotic property of resveratrol was completely abolished by L-NAME and AG, because the number of apoptotic cells returned to control levels when the hearts were simultaneously treated with resveratrol and L-NAME or AG.

Effects of resveratrol on MDA formation. The production of MDA is an indicator for lipid peroxidation and development of oxidative stress. As shown in Fig. 6, after 3 min of reperfusion, MDA levels in control hearts (8.0 ± 1.3 pmol/ml) were markedly elevated compared with hearts treated with resveratrol (5.2 ± 1.6 pmol/ml). This trend was also evident after 5 min into reperfusion, at which time the control hearts still exhibited a significantly higher level of MDA production (15.6 ± 5.5 pmol/ml) than hearts treated with resveratrol (8.1 ± 3.1 pmol/ml). The amount of MDA formation was significantly increased (compared with resveratrol) when hearts were simultaneously treated with resveratrol and L-NAME or AG.

Effects of resveratrol on iNOS mRNA induction. In both control (without resveratrol) resveratrol-preconditioned hearts, induction of the expression of iNOS mRNA was detected in the hearts reperfused for 30 min after 30 min of ischemia (Fig. 7). None to a very small amount of iNOS mRNA was detected in the hearts reperfused for 30 min after 30 min of ischemia (Fig. 7). None to a very small amount of iNOS mRNA was detected in the hearts reperfused for 30 min after 30 min of ischemia (Fig. 7). None to a very small amount of iNOS mRNA was detected in the hearts reperfused for 30 min after 30 min of ischemia (Fig. 7).

DISCUSSION

There are several salient findings of the present study which indicate that resveratrol may function as a pharmacological preconditioning agent. One, and the most important one, is that resveratrol in a low dose could precondition the heart as evidenced by its ability...
Fig. 4. Effects of resveratrol on myocardial infarct size. A group of isolated perfused rat hearts was treated with resveratrol in the absence or presence of NO and iNOS blockers for 15 min followed by 30 min of ischemia and 2 h of reperfusion. Infarct size was determined by scanning the images of the rat heart ventricular sections with triphenyltetrazolium. Bottom: representative infarct size of six groups of hearts. Infarct size is expressed as percent infarct relative to area at risk. Results are expressed as means ± SE of at least 6–8 rats per group (top). *P < 0.05 vs. control; †P < 0.05 vs. resveratrol.

Fig. 5. Effects of resveratrol on cardiomyocyte apoptosis. A group of isolated perfused rat hearts was treated with resveratrol in the absence or presence of NO and iNOS blockers for 15 min followed by 30 min of ischemia and 2 h of reperfusion. Double immunofluorescent staining was performed with TdT-mediated dUTP nick-end labeling staining and antibody recognizing cardiac myosin heavy chain to detect apoptotic nuclei (bottom) Results are expressed as means ± SE of 6–8 rats per group (top). *P < 0.05 vs. control; †P < 0.05 vs. resveratrol.
Our study demonstrated that resveratrol reduced the amount of MDA formation in the heart, suggesting that resveratrol functioned through the induction of iNOS expression and reduced apoptosis and to improve postischemic functional recovery. We tried three different doses of resveratrol to lower the infarct size by reducing both necrosis and apoptosis and to improve postischemic functional recovery. Among the three different doses, 10 μM resveratrol was found to be optimal for preconditioning purpose. Both L-NAME and AG abrogated the cardioprotective abilities of resveratrol. Resveratrol enhances the induction of the iNOS expression and AG inhibited the expression of iNOS, suggesting that resveratrol functioned through the induction of iNOS mRNA.

Resveratrol is a naturally occurring polyphenol that possesses some antioxidant property. Resveratrol, which can scavenge peroxyl radicals, is not a very potent antioxidant in vitro (24). However, it functions as a potent antioxidant in vivo. Indeed, the results of our study demonstrated that resveratrol reduced the amount of MDA formation in the heart, suggesting reduction of oxidative stress due to ischemia-reperfusion. Previous studies also showed that resveratrol prevented lipid peroxidation (15) and lipid peroxidation-induced cell death (33). A recent study demonstrated reduction of MDA formation in the ischemic reperfused myocardium of resveratrol-treated rat (2).

The in vivo antioxidant property of resveratrol may be explained by its ability to enhance the induction of the expression of iNOS mRNA and stimulate NO formation. NO, which itself is a free radical because 1 atom of nitrogen combines with 1 atom of oxygen to produce NO with an unpaired electron, behaves as a potent antioxidant in vivo. NO can rapidly react at or near the diffusion-limited rate [6.7 × 10^9 (mol/l)^-1.s^-1] with the superoxide anion (O_2^-), which is presumably formed in the ischemic reperfused myocardium, to form highly reactive peroxynitrite radical (ONOO^-) (28). Although peroxynitrite can be subsequently protonated to form ·OH in vitro (31), there is no evidence that such reactions can occur in biological systems. In physiological systems such as the heart where adequate amounts of thiols and ascorbate are present, the ONOO^- radical preferentially reacts with the -SH group of the thiols and ascorbate. Most importantly, the affinity of NO for O_2^- is far greater than of superoxide dismutase for O_2^- (35). During reperfusion, local levels of O_2^- exceed the capacity of superoxide dismutase, creating a situation where O_2^- competes for NO and thereby reduces its bioavailability. Furthermore, NO can protect against oxidative damage produced by oxoferryl-myoglobin (Mb), which contributes to myocardial ischemia-reperfusion injury of the heart, in which high concentrations of Mb and lipid hydroperoxides set the stage for the enhancement of oxidative damage through the formation of oxoferryl-Mb (19).

Interestingly, resveratrol shares many common physiological functions with NO. For example, both resveratrol and NO possess anti-inflammatory and antiplatelet activities (21, 23) and can exert vasodilatory effects on blood vessels (29). Similar to NO, resveratrol...
is a potent scavenger for peroxyl radicals (10, 30). NO exists as a free radical, and resveratrol is a weak free radical scavenger in vitro, but both of them possess potent antioxidant capacity in vivo and can attenuate lipid peroxidation. The fact that resveratrol augments NO availability and both of them share common physio-

logical function strongly suggests that resveratrol exerts its cardioprotective effects through NO.

Recently, NO has been found to act as the mediator of ischemic preconditioning mediated by four or more brief cycles of ischemia and reperfusion (34, 37). The role of NO in ischemic preconditioning was confirmed from the observation that an NO blocker or iNOS inhibitor could abrogate the cardioprotection afforded by ischemic preconditioning. Increased iNOS mRNA levels were observed 30 min after the onset of reperfusion compared with baseline values or with the values after ischemia, suggesting that reperfusion induces enhanced expression of iNOS. Resveratrol further up-

regulated the expression of iNOS, indicating the ability of resveratrol to induce iNOS. These results are consistent with these previous reports, which showed the ability of resveratrol to induce NO synthesis (16, 29).

A large number of studies support the concept that the adapting mechanism for cardioprotection is trig-

gered by enhanced availability of endogenous NO during ischemia and reperfusion. A recent study demonstrated a distinctive role of NOS isoforms in late preconditioning, with endothelial NOS (eNOS) serving as the trigger on day 1 and iNOS as the mediator on day 2 (4). In this study, the authors documented that, in conscious rabbits, ischemic preconditioning elicits a biphase response in cardiac NOS activity, i.e., an immediate activation of NOS (most likely eNOS) followed 24 h later by a delayed upregulation of iNOS. The results of our study do not disagree with these reports because AG used in the present study may also have blocked eNOS activity in addition to iNOS expression, because the IC50 for iNOS (250 μM) is only half that for eNOS (526 μM). Nevertheless, our results suggest an enhanced induction of iNOS expression with resveratrol. In the naive or nonpreconditioned heart, NO plays a crucial role in the reduction of ischemic-reperfusion injury, where both NOS (or eNOS) and iNOS may be involved. It appears that resveratrol contributes to the early induction of iNOS, whereas ischemia-reperfusion serves to activate eNOS early and to induce iNOS at later time points postreperfusion. These results are also in consistent with the reports that resveratrol is a poor antioxidant in vitro, but it functions as a potent antioxidant in vivo through its ability to up-

regulate NO (16). The contribution of iNOS versus eNOS in ischemic preconditioning has been reviewed recently (37).

Ischemic preconditioning, which occurs by a mecha-
nism involving endogenous mediators leading to the upregulation of endogenous defense system, is consid-
ered as the state-of-the-art technique for myocardial protection. However, as mentioned earlier, ischemic preconditioning-mediated cardioprotection is only short lived; it lasts for a few hours during the earlier ischemic preconditioning or classical preconditioning and for a few days for delayed preconditioning. To prolong this preconditioning window, pharmacological approaches have been undertaken with limited success. For example, monophosphoryl lipid A was found to precondition a heart after 24 h, and such preconditioning was blocked with either L-NAME or AG, suggesting a role of NO in monophosphoryl lipid A medi-
ated cardioprotection (22, 27). Unlike monophosphoryl lipid A, resveratrol can protect the heart from ischemic-reperfused injury when applied only 15 min before the induction of ischemia. Also, unlike lipid A, resveratrol is a naturally occurring substance present in grapes and wines. Thus use of resveratrol as a possible pharmacological preconditioning agent is highly war-

anted.

In summary, resveratrol appears to function as a phar-

camological preconditioning agent. Resveratrol-treated rat hearts were resistant against ischemic-reperfusion injury as evidenced by greater postischemic functional recovery and reduced myocardial infarction and cardiomyocyte apoptosis. Cardioprotective effects of resveratrol were abrogated by L-NAME or AG, suggesting that NO plays a crucial role in resveratrol preconditioning. Resveratrol reduced ischemia-reperfusion-mediated increase in oxidative stress. Resveratrol resulted in an induction of iNOS mRNA, which was completely blocked with AG, suggesting that resveratrol exerts its physiological func-

tion, at least partially, through the induction of NOS, a property shared with ischemic preconditioning.

This study was supported in part by National Heart, Lung, and Blood Institute Grants HL-25359, HL-33889, HL-56803, and HL-56322.

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