ABSTRACT

Despite its potential, use of trans-resveratrol as an anticancer drug is severely constrained because of its tendency to prolong gastric ulceration. We found that in addition to delaying ulcer healing, trans-resveratrol also aggravated acute gastric ulceration induced by the nonsteroidal anti-inflammatory drugs by reducing the synthesis of prostaglandin (PG) E₂ via a specific inhibition of cyclooxygenase (COX)-1 that also hampered angiogenesis. However, for the first time, we showed that the 3′,5′-hydroxylated congener [(E)-HST-1] of resveratrol, synthesized in multigram scale, exerted potential chemotherapeutic property but was nonulcerogenic in nature, rather moderately accelerated healing of indomethacin-induced gastric ulceration. HST-1 did not suppress COX-1, COX-2 expression and synthesis but reduced the level of inflammatory myeloperoxidase (MPO) activity. The healing was augmented primarily through the nitric oxide synthase (NOS)-dependent pathway. HST-1 treatment induced endothelial NOS (eNOS) expression and reduced inducible NOS (iNOS), resulting in increased eNOS/iNOS ratio. The selective NOS inhibitor [L-N^ω-(1-iminoethyl)lysine hydrochloride] and nonselective NOS inhibitor (N^ω-nitro-L-arginine methyl ester) treatment revealed that eNOS could be the probable molecular switch to accelerate the indomethacin-induced ulcer healing in HST-1-treated mice. Furthermore, the anticancer effect of HST-1 on U937 and K562 leukemia cell lines was found to be significantly better than that of trans-resveratrol. Overall, these established HST-1 as a potentially better anticancer compound than trans-resveratrol, considering it is devoid of any ulcerogenic side effects. In conclusion, for the first time, we showed that a novel analog of trans-resveratrol, HST-1, was devoid of ulcerogenic adverse effects of trans-resveratrol but retained potentially better anticancer property.

The naturally occurring dietary compound, trans-resveratrol (trans-3,4′,5′-trihydroxystilbene), a natural phytoalexin present in grape skins, red wines, and grape juices (Celotti et al., 1996; Faustino et al., 2003), is sold over the counter in the United States as a nutritional supplement. At low concentrations, normally occurring in food, it exerts antioxidant property, accounting for its beneficial effects on the cardiovascular system (Pace-Asciak et al., 1995; Pendurthi et al., 1999) and neuroprotection (De Ruvo et al., 2000). However, several in vitro and in vivo studies have shown that at higher doses, it shows anticancer activity by interfering with different cellular events associated with initiation, promotion, and progression of multistage carcinogenesis (Jang et al., 1997; Pozo-Guisado et al., 2002; Kim et al., 2004; Delmas et al., 2006). The chemopreventive activity of trans-resveratrol has been demonstrated notably in breast cancer (Scarlati et al., 2003) and leukemia (Gao et al., 2002). It has also been found that successive treatment of trans-resveratrol delays ulcer healing induced by ischemia reperfusion and acetic acid (Brzozowski et al., 1999, 2001). This kind of ulcerogenic properties of trans-resveratrol demands for the design of its new potential analogs, especially for chemotherapeutic purpose but certainly has to be devoid of ulcerogenic properties, which is a major disadvantage of trans-resveratrol.

Chemically, trans-resveratrol possesses a hydroxystilbeneoid structure that is easy to synthesize and amenable for
subtle modifications to impart different biological activities. As efficient antioxidants and stimulators for prostaglandin (PG) synthesis and angiogenesis, many phenolics are known to show antiulcerogenic activities (Bhattacharya et al., 2007). However, reports on the gastroprotective property of the hydroxystilbenoids are scarce.

Earlier, trans-resveratrol treatment was found to delay healing of gastric ulcer induced by ischemia-reperfusion and acetic acid (Brzozowski et al., 1999, 2001) by reducing PGE$_2$ synthesis via specific inhibition of cyclooxygenase (COX)-1. Later, trans-resveratrol was also found to suppress the phorbol ester-induced COX-2 mRNA expression (Subbaramaiah et al., 1998). It has also been suggested that trans-resveratrol acts as an angiogenesis inhibitor (Subbaramaiah et al., 1998) that may cause the delay of ulcer healing.

Considering the ulcerogenic adverse effect of trans-resveratrol, earlier we synthesized a series of trans-resveratrol congeners as E/Z mixtures and evaluated their anti- and pro-oxidant properties vis-à-vis trans-resveratrol (Subramanian et al., 2004) to develop a novel compound that would retain anticancer properties but devoid of ulcerogenic side effects. True to our expectation, the potency of the compounds was found to be dependent on structural features, like number and positions of the hydroxyl groups, presence of free phenolic and olefinic moieties, and substitution pattern of the olefinic groups (Subramanian et al., 2004). Encouraged by the results, we synthesized the $E$-hydroxystilbene (coded as HST-1) containing a bis-resorcinol moiety. The chemical structures of trans-resveratrol and HST-1 are shown in Fig. 1. In this study, we rationalized the effect of trans-resveratrol and HST-1 on the indomethacin-induced gastrotoxicity in terms of biochemical mechanism and also compared their anticancer activity to explore the potential of HST-1 as a plausible substitute of trans-resveratrol.

**Materials and Methods**

**Chemicals and Reagents.** Indomethacin, 3,4-diaminobenzidine, bovine serum albumin (BSA), misoprostol, trans-resveratrol (RS010, purity > 99% gas chromatography), L-$N^6$-(1-iminoethyl) lysine hydrochloride (L-NIL), N-$N^8$-nitro-$L$-arginine methyl ester (L-NAME), nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, RNAlater solution, hexadecyltrimethylammonium bromide, Gen Elute Mammalian Total RNA miniprep kits, Trizma base, and Tween 20 were procured from Sigma-Aldrich (St. Louis, MO). Other reagents used were hydrogen peroxide (35%; Lancaster, Morecambe, UK); disodium hydrogen phosphate and sodium dihydrogen phosphate (BDH, Poole, Dorset, UK); hematoxylin monohydrate and eosin yellowish (both from Merck, Darmstadt, Germany); von Willebrand Factor (rabbit anti-human; Chemicon, Temecula, CA); dimethyl formamide and tetramethylbenzidine (Acros, Geel, Belgium); polyclonal goat anti-rabbit COX-1, COX-2 antibodies, polyclonal rabbit anti-goat eNOS, and iNOS antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); PGE$_2$ EIA kit (Cayman Chemical, Ann Arbor, MI); Revert Aid H minus first strand cDNA synthesis kit and Dynamo SYBR Green quantitative PCR kit (MBI Fermentas, Hanover, MD); cell proliferation reagent WST-1 nonradioactive cell proliferation colorimetric assay kit (Roche Diagnostics, Mannheim, Germany); cell and tissue staining kits (R&D Systems, Minneapolis, MN); and RPMI 1640, fetal bovine serum, penicillin, streptomycin, Hoechst (Invitrogen, Carlsbad, CA); Ecoline ALAT (GPT), Ecoline alkaline phosphatase, serum albumin, and bilirubin assay kits (Merek); and serum glutamic oxaloacetic transaminase (aspartate aminotransferase) (Bayer AG, Wuppertal, Germany). The synthesis of ($E$)-HST-1 and data for its characterization are presented as supplemental data.

**Animals.** Male Swiss albino mice (6–8 weeks, 25–30 g) bred in-house with free access to food and water were used for all of the experiments. The mice were kept in 12-h light/dark cycles and housed at 25°C. The animal experiments ($n = 15$) were conducted in accordance with the guidelines of the animal ethics committee of the Postgraduate Institute of Basic Medical Sciences, Kolkata, Animal Ethical Committee 507/CPCSEA, Sanction No. IAEC/SB-2/2004/UCM-16, dated June 15, 2004, and were handled following the International Animal Ethics Committee Guidelines, ensuring minimum animal suffering.

**Drug Treatment.** The drugs were prepared using 1% aqueous DMSO solutions of trans-resveratrol and HST-1 and suspending them in 2% gum acacia in distilled water as the vehicle and were administered once daily. Misoprostol (5 mg/kg once daily) in the same vehicle was used as the positive control. In some cases, after ulcer induction, mice were additionally treated intraperitoneally with L-NIL (3 mg/kg twice daily) or L-NAME (15 mg/kg once daily) (Ma and Wallace, 2000).

![Fig. 1. Chemical structures of trans-resveratrol and HST-1 (A) and synthesis of HST-1 (B).](image-url)
Protocol for Gastric Ulceration and Assessment of Healing.

Acute gastric ulceration in mice were induced by oral administration of indomethacin (18 mg/kg, single dose) dissolved in distilled water and suspended in 2% gum acacia as the vehicle (Banerjee et al., 2008). The animals were deprived of food but had free access to tap water 24 h before ulcer induction. The normal and untreated control groups received the vehicle only throughout the course of the experiments. The treatment groups received different doses of trans-resveratrol or HST-1 and misoprostol (5 μg/kg p.o. once daily) as positive control for different periods, starting the first dose 6 h after the indomethacin administration. On the 1st, 2nd, 3rd, 4th, and 7th days, the mice were sacrificed by cervical dislocation under anesthesia (12 mg/kg ketamine). The stomachs from the normal and treated groups were removed rapidly, opened along the greater curvature, and thoroughly rinsed with normal saline. The ulcerated gastric mucosal areas were visualized using a transparent sheet and a dissecting microscope. The damage score (DS) was assessed (Dokmeci et al., 2005) by grading the gastric injury on a scale of 0 to 4, based on the severity of hyperemia and hemorrhagic erosions: 0, almost normal mucosa; 0.5, hyperemia; 1, one or two lesions; 2, severe lesions; 3, very severe lesions; and 4, mucosa full of lesions (lesions, hemorrhagic erosions; hyperemia, vascular congestions). The sum of the total scores divided by the number of animals is expressed as the mean damage score. The experiments were performed by two investigators blinded to the groups and treatment of animals.

Histological Analysis. After scoring the DS, the fundic stomach was sectioned for the histological studies. The tissue samples were fixed in 10% formalin and embedded in paraffin. The sections (5 μm) were cut using microtome, stained with hematoxylin and eosin (Dokmeci et al., 2005), and assessed under an Olympus microscope (BX41; Olympus, Tokyo, Japan).

Myeloperoxidase Assay. The myeloperoxidase (MPO) activity was determined following a reported method (Suzuki et al., 1983), with slight modifications. The whole gastric glandular portions of the stomach taken from all groups (100–150 mg) were homogenized in a 50 mM phosphate buffer, pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide. This was followed by three cycles of freezing and thawing. The homogenate was centrifuged at 12,000g for 20 min at 4°C. To the supernatant (50 μl) was added 80 mM phosphate buffer, pH 5.4, 0.03 M tetramethylbenzidine, and 0.3 M H₂O₂ to make a final reaction volume of 500 μl. After incubating the mixture at 25°C for 25 min, the reaction was terminated by adding 0.5 M H₂SO₄, and the change in the absorbance was measured at 450 nm. The results were expressed as total number of neutrophils by comparing the OD of tissue supernatant with the OD of mice peritoneal neutrophils processed in the same way. A standard curve relating neutrophil numbers and absorbance was obtained by processing purified neutrophils and assaying the MPO activity with 0.0005% hydrogen peroxide as the substrate. The correlation between the number of neutrophils and units of MPO was determined using a reported technique (Bradley et al., 1982). One unit of MPO activity is defined as that converting 1 μmol hydrogen peroxide to water in 1 min at 22°C.

Real-Time PCR. Four days after ulcer induction, the tissue samples of the sham-treated, ulcerated untreated, trans-resveratrol-treated, and HST-1-treated mice were immediately immersed into RNAlater solution. After extracting total RNA and checking its integrity by electrophoresis, the cDNA was synthesized from 5 μg of purified total RNA using a commercial kit. Expressions of COX-1, COX-2, eNOS, and iNOS were detected using suitably designed primers (Primer Express program; Applied Biosystems, Foster City, CA) shown in Table 1. The expressions of the designated enzymes were normalized using the glyceraldehyde-3-phosphate dehydrogenase coding gene as the internal reference. The experiments were performed (Real-Time PCR Systems 7500; Applied Biosystems) in triplicate by using the Dynamo SYBR Green quantitative PCR kit. The samples were quantified for all the above genes using the comparative C₅₀ (ΔΔC₅₀) method, as described in the Assays-on-Demand Users Manual (Applied Biosystems). The fold values (x) were calculated using the equation: x = 2⁻¹⁺ΔΔC₅₀ (eq. 1), where the data for the sample and sham-treated tissues as the calibrator (calibrator being e.g., sham-treated tissue) were first normalized against variations of sample quality and quantity. The ΔΔC₅₀ values were determined using the formula ΔΔC₅₀ = ΔC₅₀ sample − ΔC₅₀ calibrator (eq. 2), where ΔC₅₀ sample = C₅₀ target gene of sample − C₅₀ reference (eq. 3) and ΔC₅₀ calibrator = C₅₀ target gene of calibrator − C₅₀ reference (eq. 4). The expression of the target genes was normalized to the reference gene and relative to the calibrator using the formula 2⁻¹⁺ΔC₅₀ (eq. 1).

PGE₂ Assay. After harvesting of the stomach, the corpus (full thickness) was excised, weighed (100 mg), and suspended in 10 mM sodium phosphate buffer, pH 7.4 (1 ml). The tissues were finely minced and incubated at 37°C for 20 min. After centrifugation (9000g), the PGE₂ levels in the supernatant were measured (Bradford, 1976). The proteins (100 μg) were resolved by electrophoresis, the cDNA was synthesized from 5 μg of total RNA using a commercial kit. Expressions of COX-1, COX-2, eNOS, and iNOS were detected using suitably designed primers (Primer Express program; Applied Biosystems, Foster City, CA) shown in Table 1. The expressions of the designated enzymes were normalized using the glyceraldehyde-3-phosphate dehydrogenase coding gene as the internal reference. The experiments were performed (Real-Time PCR Systems 7500; Applied Biosystems) in triplicate by using the Dynamo SYBR Green quantitative PCR kit. The samples were quantified for all the above genes using the comparative C₅₀ (ΔΔC₅₀) method, as described in the Assays-on-Demand Users Manual (Applied Biosystems). The fold values (x) were calculated using the equation: x = 2⁻¹⁺ΔΔC₅₀ (eq. 1), where the data for the sample and sham-treated tissues as the calibrator (calibrator being e.g., sham-treated tissue) were first normalized against variations of sample quality and quantity. The ΔΔC₅₀ values were determined using the formula ΔΔC₅₀ = ΔC₅₀ sample − ΔC₅₀ calibrator (eq. 2), where ΔC₅₀ sample = C₅₀ target gene of sample − C₅₀ reference (eq. 3) and ΔC₅₀ calibrator = C₅₀ target gene of calibrator − C₅₀ reference (eq. 4). The expression of the target genes was normalized to the reference gene and relative to the calibrator using the formula 2⁻¹⁺ΔC₅₀ (eq. 1).

Western Blot Analysis. The tissue lysates were prepared from the tissue samples using a mammalian cell lysis kit. The tissue (20 mg) was immersed into the lysis buffer (1 ml) containing 200 μl of 5× Tris-EDTA buffer, 200 μl of 5× NaCl, 200 μl of 5× SDS, 200 μl of 5× deoxycholic acid, 200 μl of 5× Igepal, and 10 μl of protease inhibitor cocktail. The tissue samples were homogenized and centrifuged at 12,000g for 10 min, and the protein concentration of the supernatant was measured (Bradford, 1976). The proteins (100 μg) were resolved by 10% nonreducing SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membrane was blocked for 2 h at room temperature in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.02% Tween 20 (Tris-buffered saline/Tween 20) containing 3% BSA followed by overnight incubation at 4°C in 1:500 dilution of the respective antibodies for COX-1, COX-2, eNOS, and iNOS in 3% BSA. The membrane was washed three times with Tris-buffered saline/Tween 20 and incubated with alkaline phosphatase-conjugated secondary antibody, and the bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazo-
Cell Culture. The U937 (human leukemic monocyte lymphoma), K562 (human myelogenous leukemia) cells (National Centre for Cell Science, Pune, India) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified incubator containing 5% CO₂ and 95% air.

WST-1 Assay. The antiproliferative assay was carried out using the commercially available colorimetric assay kit WST-1. The cells (5 × 10⁴ cells in 100 μl medium/well) were plated in 0.07% DMSO in media as control in 96-well plates. The cells as such or in the presence of trans-resveratrol or HST-1 (dissolved in 0.07% DMSO in media) were incubated for 48 h. At the end of the treatments, each well was treated with WST-1 solution (10 μl), and after incubation for 2 h, the absorption at 450 nm was read with a microplate reader (Bio-Rad model 680; Bio-Rad, Hercules, CA). The inhibition of cell proliferation by HST-1 and trans-resveratrol was evaluated by calculating the IC₅₀ value.

Cellular and Nuclear Morphology Analysis. TCS-SP-2 Leica confocal microscope (Wetzlar, Germany) was used for all microscopic imaging with either phase contrast for cellular morphology or fluorescence for nuclear morphology with Hoechst staining, as described previously (Kristin et al., 2007).

Toxicity Tests. Toxicity tests were carried out for both HST-1 and trans-resveratrol, which were treated to separate groups of mice (22–25 g, n = 20) at the dose of (10 mg/kg) for a period of 1 month. The serum bilirubin, albumin, aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen, and creatine kinase were measured using commercially available assay kits.

Statistical Analyses. Data were expressed as mean ± S.D. unless mentioned. Comparisons were made between different treatments (analysis of variance) using the software GraphPad InStat (GraphPad Software Inc., San Diego, CA), where an error protecting the multiple comparison procedure, namely Tukey-Kramer multiple comparison tests, was applied for the analysis of significance of all the data.

Results

Synthesis of (E)-HST-1. The compound (E)-HST-1 was synthesized by McMurry olefination of the aldehyde 3 using the low-valent titanium (LVT) reagent, prepared from titanium tetrachloride/Zn/tetrahydrofuran. The McMurry reaction is known to produce the olefins as cis/trans mixtures (McMurry, 1989). However, the stereochemical course of the reaction can be controlled using a suitable LVT reagent, as shown by our group earlier (Rele et al., 2001). The present protocol was designed after screening several LVT reagents, prepared using different titanium salts, reducing agents, and solvents (data not shown). The optimized protocol, shown in (Fig. 1), provided (E)-HST-1 as the sole isomer, which was confirmed by NMR spectra (supplemental data).

Ulcer Index. The mice receiving vehicle only showed no lesions in the gastric mucosa. Indomethacin (18 mg/kg) administration produced typical time-dependent acute mucosal lesions in mice, as assessed by histology (Fig. 2), and quantified in terms of DS (Fig. 3A). Maximum ulcerative damage was observed on the 4th day, when the DS increased by 60.0% (P < 0.001) compared with that on the 1st day. Thereafter, natural recovery reduced it gradually, and on the 7th day, the DS value was less than that of the day of ulcer induction. We have chosen the dose of trans-resveratrol (10 mg/kg), based on a previous report (Brzozowski et al., 1999), which was also supported by our dose-dependent study (Fig. 3B). Treatment with trans-resveratrol progressively increased the ulcerative damage up to the 4th day, followed by a gradual decline. However, mice receiving trans-resveratrol for 4 and 7 days showed higher (25.4%, P < 0.001; 108.3%, P < 0.001) DS values compared with the respective ulcerated untreated group mice, which signified that trans-resveratrol treatment not only aggravated ulcerated condition, but also delayed the healing process. A dose-dependent study with HST-1 showed the best result with a dose of (10 mg/kg) (Fig. 3C), which was chosen for the present studies. It is interesting that on day 4, treatment with HST-1 significantly reduced DS by 60.0% (P < 0.001) and 68.1% (P < 0.001), and on day 7, it reduced to 40.0% (P < 0.001) and 71.2% (P < 0.001), respectively, compared with ulcerated untreated and trans-resveratrol-treated mice. Although mice treated with misoprostol (5 μg/kg once daily) exerted profound healing effect from day 4, DS value was 82.1% (P < 0.001) less and reduced 75.0% (P < 0.001) on day 7, respectively, compared with the ulcerated untreated mice. It is notable that neither trans-resveratrol nor HST-1 alone showed any ulcerogenic effect (data not shown).

trans-Resveratrol Induced MPO Activity, but HST-1 Reduced It. Compared with the normal mice, the MPO status in the ulcerated untreated mice increased immediately, reaching maximum on the 4th day, then gradually

![Image](https://image-url.com)
declined, and on the 7th day, the value was close to that of the 1st day ulcerated control. The MPO levels in the trans-resveratrol-treated mice were significantly higher \((P < 0.001)\) on both days 4 and 7, compared with the ulcerated untreated mice. In contrast, both HST-1 and misoprostol reduced it from the initial days (Fig. 4), restoring it to the normal value on the 7th day. However, after 4-day treatment, HST-1 showed a pronounced effect reducing the MPO activity by 53.4\% \((P < 0.001)\) and 59.2\% \((P < 0.001)\), compared with the ulcerated untreated and trans-resveratrol-treated mice. Neither trans-resveratrol nor HST-1 on their own had any effect on the MPO level in normal mice.

Effects of trans-Resveratrol and HST-1 on the Biochemical Parameters. Overall, maximum ulceration was evident in both ulcerated untreated and trans-resveratrol-treated mice on the 4th day of indomethacin administration. It is interesting that HST-1 \((10 \text{ mg/kg})\) showed a pronounced healing effect on the same day. Although an extended treatment regime produced better healing, a large part of it was due to natural recovery. Hence, the biochemical parameters of the mice receiving trans-resveratrol and HST-1 \((10 \text{ mg/kg})\) for 4 days were only selected and compared with those of the ulcerated untreated mice.

Effects of the Drugs on COX Expressions and PGE_2 Synthesis. The real-time PCR technique revealed that both COX-1 and COX-2 mRNA were down-regulated in the mucosa of the ulcerated untreated mice. trans-Resveratrol suppressed COX-1 expression \((P < 0.01)\) further, without affecting COX-2 expression significantly. In contrast, HST-1 did not suppress the expressions of both COX-1 and COX-2, and the fold value remained by 2.1- and 1.3-fold, respectively. These values were with respect to the globally expressed glyceraldehyde-3-phosphate dehydrogenase gene levels.

\[ \text{Fig. 3. Ulcer healing by trans-resveratrol and HST-1. Stomach ulceration in mice was induced by oral administration of indomethacin (18 mg/kg). Treatment was carried out with trans-resveratrol or HST-1 (each 10 mg/kg) or misoprostol (5 \text{ mg/kg}), and the ulcer indices (in terms of damage scores) were scored as stated under Materials and Methods. A, time-dependent healing capacities of trans-resveratrol and HST-1 against indomethacin-induced stomach ulceration in mice.} \]

\[ a, b, c, d, \text{ and } e, P < 0.001. \]

\[ \text{B and C, dose-dependent effects of trans-resveratrol and HST-1 on indomethacin-induced ulcer in mice.} \]

\[ \$\$, \text{ and } \#\#\#\$, P < 0.001 \text{ with respect to trans-resveratrol dose of 20 mg/kg; } \|, P < 0.05; \|\|\|\|, P < 0.001 \text{ with respect to 20 mg/kg HST-1, respectively. ns, nonsignificant. The values are mean } \pm \text{ S.E.M. (} n = 15). \]
These data (Figs. 5, A and C) were also supported by the Western blot study (Fig. 5, B and D).

Colorimetric study revealed that on the 4th day of ulceration, PGE$_2$ synthesis in the sham-treated, ulcerated untreated, trans-resveratrol-treated, and HST-1-treated mice were 45 ± 3.20, 24.2 ± 2.59, 18.69 ± 2.1, and 32 ± 3.52 pg/mg protein, respectively. Hence, mucosal PGE$_2$ was markedly suppressed because of ulceration (46.2%, $P < 0.001$) and trans-resveratrol treatment (58.5%, $P < 0.001$), compared with the normal value. However, PGE$_2$ suppression was not evident in HST-1-treated mice compared with ulcerated untreated group.

**Effects of the Drugs on Nitric Oxide Synthase Expression.** Ulceration led to increased expressions of eNOS (1.5-fold) and iNOS (2.5-fold) in the ulcerated untreated mice, whereas in the trans-resveratrol-treated mice, the increases for the respective parameters were 2.5- and 1.9-fold. In contrast, HST-1 induced eNOS gene expression much higher (4.8 fold), while suppressing the iNOS expression (Fig. 6, A and C) significantly ($P < 0.001$) compared with the trans-resveratrol-treated and ulcerated untreated mice. The Western blot results (Fig. 6, B and D) further supported the above data.

**HST-1 Increased vWF VIII More Than trans-Resveratrol.** Angiogenesis plays a major role in healing of ulcer, which was studied microscopically by immunostaining the vWF VIII. The microscopic results revealed the presence of ~15 ± 3.01 microvessels/mm$^2$ in normal submucosa. The immunohistochemical studies confirmed substantial formation of the microvessels on the 4th day of ulceration, when the number of microvessels in the ulcerated untreated and trans-resveratrol- and HST-1-treated mice were 23.6 ± 4.24, 19.3 ± 3.16, and 35.2 ± 3.98 mm$^{-2}$, respectively. Therefore, HST-1 increased the microvessel number by 49.1% ($P < 0.001$) and 82.3% ($P < 0.001$) compared with the ulcerated untreated and trans-resveratrol-treated mice.

**HST-1 Promotes Ulcer Healing in an Nitric Oxide Synthase-Dependent Way.** The gene expression study revealed profound effect of HST-1 on the expressions of both eNOS and iNOS. Hence, we also studied the effects of L-NAME, a nonselective nitric oxide synthase (NOS) inhibitor, and L-NIL, a selective iNOS inhibitor, to assess the relative contributions of different isoforms of NOS to gastric ulcer healing in response to the HST-1 treatment. Treatment with L-NAME significantly increased the ulcer index (87.1%, $P < 0.001$) (Fig. 7A) and MPO activity (78.6%, $P < 0.001$) (Fig. 7B), while reducing the numbers of microvessels (47.1%, $P < 0.001$) (Fig. 7C) compared with the HST-1-treated ulcerated mice. L-NIL did not have significant impact on any of these parameters.

**Chemopreventive Role of trans-Resveratrol and HST-1.** HST-1 was devoid of ulcerogenic contrainductive effects and moderately accelerated the healing process but still retained significant chemopreventive potential. The dose response effects of trans-resveratrol and HST-1 on the growth of U937 and K562 leukemia cell types were compared by the WST-1 assay method. trans-Resveratrol and HST-1 exhibited a differential growth-inhibitory effect after 48 h of incubation. WST-1 assay revealed that trans-resveratrol showed its IC$_{50}$ value at the dose of 50 μM for the U937 cell and 100 μM for the K562 cell, whereas HST-1 showed its IC$_{50}$ value comparatively at low dose, 25 and 50 μM, respectively, for U937 and K562 cells (Table 2). Furthermore, the control and treated cells were stained with Hoechst, and confocal imaging clearly exhibited morphologic changes with cell membranes that appeared irregular and jagged compared with the control (Fig. 8), consistent with the appearance of punc-
tuated and brightly stained nuclei, which signified chromosomal condensation and cell death.

Evaluation of Toxicity of HST-1 and trans-Resveratrol on Mice. HST-1 and trans-resveratrol treatment at the dose of 10 mg/kg to a group of mice for the period of 1 month did not show any toxic side effects, as confirmed by comparing the values with sham-treated (Table 3).

Discussion

trans-Resveratrol is currently being evaluated in preclinical studies as a potential cancer chemoprevention agent. Brozowski et al. (1999) recently reported that trans-resveratrol treatment delayed the gastric ulcer-healing process in the acetic acid-induced ulcerated rat. This ulcerogenic adverse effect severely constrains its use despite the potential chemotherapeutic property. Hence, we tried to develop a congener of trans-resveratrol, which would exert at least similar chemotherapeutic potential as trans-resveratrol but would be devoid of ulcerogenic contraindication property of trans-resveratrol. Of a series of synthetic trans-resveratrol analogs, synthesized in our laboratory (data not shown), only the compound (E)-HST-1 did not aggravate ulcerated condition or delay the healing process; rather, it fairly accelerated the healing despite having strong anticancer potential. Hence, for the first time, we showed that at the similar dose (10 mg/kg), unlike trans-resveratrol, HST-1 accelerated the healing process in indomethacin-induced ulcerated mice but simultaneously showed anticancer potential in the cell culture system.

Our histological examinations revealed that administration of indomethacin caused maximum ulcerative damage on the 4th day (Fig. 3A). However, even after 7 days, the authealing was partial. The MPO activity, a marker of neutrophil aggregation at the site of inflammation frequently increases in ulcerated condition and reduces with the healing process (Souza et al., 2004). In consonance with this, we also found that ulceration increased the MPO activity up to the 4th day, followed by its gradual reduction on the 7th day (Fig. 4). Between the two test hydroxystilbenes, trans-resveratrol dose-dependently aggravated the ulceration (Fig. 3C) and also delayed the healing process as observed earlier (Brzozowski et al., 1999). The DS results were well supported by the data of MPO activity that was found appreciable, even on the 7th day in the trans-resveratrol-treated mice compared with those ulcerated untreated (Fig. 4). This established a strong contraindication of trans-resveratrol regarding gastric ulceration, induced by indomethacin in mice. In contrast, its synthetic congener, HST-1, reduced the ulcerative damage significantly with concomitant decrease in the MPO activity (Fig. 4), confirming its ulcer-healing potency. However, trans-resveratrol on its own did not cause ulcer.

The WST-1 assay also revealed a better antiproliferative activity of HST-1 compared with trans-resveratrol against the human leukemic monocyte lymphoma U937 and human myelogenous leukemia K562 cell lines (Table 2), which were further supported by confocal imaging of the cells (Fig. 8). This suggested that unlike trans-resveratrol, HST-1 could provide efficient healing to the indomethacin-induced gastric
ulceration, without compromising its anticancer property as trans-resveratrol.

The nonsteroidal anti-inflammatory drugs exert both their therapeutic and toxic effects, mainly through inhibiting cyclooxygenase and decreasing the levels of circulating PGE$_2$ at the gastric mucosa. There has been accumulating evidence that PGE$_2$ contributes to ulcer healing by inducing proangiogenic factors (Miller, 1983), bicarbonate secretion, and maintaining mucosal integrity. Our gene and protein expression studies revealed reduced expression of both COX-1 and COX-2 because of indomethacin administration. However, trans-resveratrol selectively suppressed the expression of COX-1, but not of COX-2 (Fig. 5). It is believed that gastric injury develops only when both COX-1 and COX-2 are inhibited. Given that trans-resveratrol did not alter the COX-2 status, it is expected that trans-resveratrol is nonulcerogenic when treated to nonulcerated healthy mice, as observed in the studies. Our results on the depletion of the mucosal PGE$_2$ level because of ulceration itself and more by trans-resveratrol also correlated well with the COX-1 results. In comparison, the mRNA expression and Western blot results revealed that HST-1 did not show any inhibitory effects on expression of COX isozymes and PGE$_2$ synthesis.

Another inducible enzyme that is actively involved in inflammation and healing process is NOS. Earlier, it was reported (Ma and Wallace, 2000) that eNOS expression played a major role in gastric ulcer healing. A number of in vitro and in vivo studies have demonstrated that inhibition of eNOS prevented angiogenesis and profoundly delayed gastric ulcer healing (Konturek et al., 1993). On the other hand, iNOS largely induced under certain pathological conditions (Anggård et al., 1994). We found low eNOS expression associated with significant enhancement of iNOS on indomethacin treatment (Fig. 6). This might contribute to the stimulation of the inflammatory situation, explaining the ulcerogenic property of indomethacin. Likewise, trans-resveratrol also induced the iNOS expression substantially, whereas its augmenting power for the eNOS was much less than that of HST-1. In contrast, HST-1 treatment significantly increased the eNOS expression and simultaneously reduced the iNOS expression. Immunohistochemical study of vWF VIII revealed that trans-resveratrol treatment suppressed angiogenesis, but HST-1 treatment induced it. trans-Resveratrol induced less eNOS expression and strongly inhibited expression of COX-1, which played a major role in endothelial cell proliferation. All these factors would lead to impaired angiogenesis, accounting for the delayed ulcer healing by trans-resveratrol. However, HST-1 significantly stimulated eNOS expression without any further suppression of COX isozyme, explaining its ulcer-healing property.

To further reconfirm the fact that up-regulation of eNOS by HST-1 played the predominant role in its healing action, the effects of the NOS inhibitors, L-NAME and L-NIL, were examined in the HST-1-treated mice (Fig. 7). In the present study, L-NAME, but not L-NIL, reduced and even delayed the healing of gastric ulcer by HST-1 depicted by DS, which was also associated with reduced microvessel numbers and increased MPO activity. These implied that HST-1 accelerated the ulcer-healing process in an eNOS-dependent manner.

A structure-activity correlation of the observed results would, at best, be speculative. However, it can be easily suggested that the resorcinol moiety possibly holds the key with regard to the gastrotoxicity of the hydroxystilbenes. We have also found that the hydroxystilbenes, devoid of this unit, were toxic to the gastrointestinal tract (data not shown). However, trans-resveratrol containing one such unit was nonulcerogenic to healthy mice but delayed ulcer healing. In contrast, the biresorcinol derivative, HST-1, could even accelerate healing of the indomethacin-induced gastric ulceration. Given that we also examined the possible toxic effects of HST-1 on mice (Table 3) and found them to be...
nontoxic at this dose (10 mg/kg). These findings suggested that the test samples at the dose for ulcer healing did not have any potential side effect in mice.

In conclusion, this study suggested that HST-1, a novel analog of trans-resveratrol, did not aggravate ulcerated condition, like trans-resveratrol; rather, it moderately accelerated indomethacin induced ulcer healing in an NOS-dependent way. Furthermore, it had been found that HST-1 showed significantly better antiproliferative activity on leukemia cell lines compared with trans-resveratrol, suggesting its better chemopreventive potential also. Therefore, further vivid study of HST-1 on different cancer cells can establish it as a potential candidate of chemotherapy that has overcome the ulcerogenic side effects of trans-resveratrol; currently, we are examining this aspect.

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