Tryptamine-Gallic Acid Hybrid Prevents Non-steroidal Anti-inflammatory Drug-induced Gastropathy

CORRECTION OF MITOCHONDRIAL DYSFUNCTION AND INHIBITION OF APOPTOSIS IN GASTRIC MUCOSAL CELLS

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Background: Non-steroidal anti-inflammatory drugs (NSAIDs) induce gastropathy by promoting mitochondrial pathology, oxidative stress, and apoptosis in gastric mucosal cells.

Results: We have synthesized SEGA (3a), a tryptamine-gallic acid hybrid, which prevents NSAID-induced gastropathy by preventing mitochondrial oxidative stress, dysfunction, and apoptosis.

Conclusion: SEGA (3a) bears an immense therapeutic potential against NSAID-induced gastropathy.

Significance: This novel molecule is a significant addition in the discovery of gastroprotective drugs.

This article has been withdrawn by the authors. In Fig. 4A, a portion of the indomethacin images overlaps with the indomethacin + SEGA (3a) images. The images corresponding to the indomethacin panel in Fig. 4B are an inappropriate representation of the original experimental data. In Fig. 6C, the Control and indomethacin + SEGA (3a) images overlap. In Fig. 7, the 20-h indomethacin image was reused in the 4-h indomethacin + SEGA (3a) image. Additionally, the 0- and 8-h indomethacin images were reused from Table 1 of Maity, P., et al. (2008) J. Biol. Chem. 283, 14391-14401. The authors regretfully state that the mistakes occurred while preparing the figures; the authors stand by the overall conclusions of the study.
Tryptamine-GA Hybrid against NSAID-induced Gastropathy

offers toxic insult by oxidatively damaging and inactivating mitochondrial aconitate, resulting in the release of iron from its Fe-S cluster (6, 17). Again, the released iron in presence of H$_2$O$_2$ generates OH through the Fenton reaction (17). Heme oxygenase 1 may also generate free iron by catalyzing excess free heme. Heme oxygenase 1 translocates to mitochondria and decreases intramitochondrial free heme accumulated during gastric injury by NSAID. Excess free heme and overactivity of heme oxygenase 1 inside mitochondria may favor the accumulation of free iron in excess to ferritin sequestration (18). Free iron overload in cells has been shown to be associated with the development and progression of several pathological conditions (19–21). Intramitochondrial free iron and ROS lead to MOS and consequent dysfunction (19–25). MOS disrupts cellular integrity and promotes cell death (5, 26, 27), which ultimately leads to organ damage.

The overproduction of ROS develops mitochondrial pathology (22, 24, 28, 29), as indicated by the defect in electron transport chain and ATP synthesis, opening of mitochondrial permeability transition pore (MPTP), fall in transmembrane potential ($\Delta V_m$), oxidative damage of mitochondrial DNA, proteins, and phospholipids (30), and finally the activation of the mitochondrial pathway of apoptosis (6, 31). Thus, mitochondrial dysfunction triggers the mitochondrial pathway of apoptosis (6, 32–38). Mitochondrial dysfunction and current apoptosis play an important role in NSAID-induced gastropathy (4, 6–7, 12). Hence, it is clear that the molecule that will prevent MOS and consequent mitochondrial dysfunction will be effective against NSAID-induced gastropathy.

The aim of the present study is to design a small molecule that will correct NSAID-induced apoptosis, and gastropathy by blocking MOS, chelating intramitochondrial free iron, and correcting the mitochondrial pathology entering into mitochondria.

**EXPERIMENTAL PROCEDURES**

Indomethacin, thiobarbituric acid, 5,5’-dithiobis(nitrobenzoic acid), 2,2-diphenyl-1-picrylhydrazyl (DPPH), DMSO, albumin, collagenase type I, hyaluronidase, paraformaldehyde, the caspase-3 assay kit, and 4-hydroxycinnamic acid were obtained from Sigma. Serotonin was purchased from Alfa Aesar. 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) was obtained from Acros Organics (Geel, Belgium). Gallic acid and indole-3-acetic acid were procured from SRL (New Delhi, India). Fetal bovine serum was obtained from Invitrogen. 5,5’,6,6’-Tetrachloro-1,1’,3,3’-tetraethylbenzimidazolocarbocyanine iodide (JC-1) was procured from Molecular Probes (Eugene, OR), and the caspase-9 assay kit was bought from Biovision (Mountain View, CA). MitoSOX, MitoTracker Red, and Phen Green SK were purchased from Invitrogen. The mitochondria isolation kit was purchased from the Biochain Institute (Hayward, CA). The Dead-End colorimetric TUNEL assay kit was purchased from Promega, and the APO-BrdU™ TUNEL assay kit was purchased from Invitrogen. Anti-active caspase-3 antibody was purchased from Cell Signaling Technology. All other reagents were of analytical grade purity.

**General EDC Coupling Procedure for Formation of Esters or Amide**—To a solution of 3,4,5-tris(benzyloxy)benzoic acid/4-hydroxycinnamic acid/indole-3-acetic acid (1 eq, 12 mmol), amines (hydrochloride)/alcohol (1.2 eq, 14.4 mmol), 1-hydroxybenzotriazole (1 eq, 12 mmol, for amines), and Et$_3$N (6 eq, 72 mmol, for amines)/DMAP (1 eq, 12 mmol, for alcohols) in N,N-dimethylformamide, EDC hydrochloride (1.2 eq, 14.4 mmol) was added at 0 °C. Then the reaction mixture was stirred at room temperature overnight until completion of the reaction, monitored by thin layer chromatography (TLC). After that reaction, the mixture was quenched by the addition of ice-cold H$_2$O and extracted with ethyl acetate. The combined organic phase was washed with brine and dried over Na$_2$SO$_4$. The organic phase was then reduced in vacuo; the concentrated ethyl acetate extracts were chromatographed over a silica gel column.

**General Procedure—Hydrolysis (Hydrogenolysis)**—A mixture of compound 1 and palladium on carbon (catalytic) in a methanol and water mixture (5:1) (10 ml) was hydrogenated at room temperature overnight under pressure. The mixture was evaporated in vacuo to remove the methanol from the materials and the concentrate was kept in a drybox for further use. The detailed methods for synthesis are described in the supplemental material.

**Determination of in Vitro Antioxidant Property by Following Ferric Reducing Antioxidant Power (FRAP)**—The assay was performed in a 96-well microplate as described earlier (4). The FRAP solution was prepared by mixing of 10 ml of acetate buffer (50 mM, pH 3.6), 1 ml of TPTZ solution (10 mM in 40 mM HCl), and 1 ml of ferric chloride solution (20 mM) in distilled water. The solution was kept for 1 h in a water bath at 37 °C. In a 96-well microplate, 25 μl of the compounds under investigation (in methanol or water) at different concentrations in the range 1–100 μM were placed in triplicate, and freshly prepared FRAP solution (175 μl) was added to this sample. Absorbance was monitored at 595 nm at different time intervals up to 150 min in a microplate reader. Absorbance of 175 μl of FRAP solution and 25 μl of methanol or water mixture was taken, which was subtracted from the absorbance of the samples at each time interval to calculate the absorbance change (ΔA). The FRAP value at time interval t (FRAP value) was calculated according to the formula,

$$\text{FRAP value (m)} = (\Delta a/T \times \Delta a/Fe^{2+}) \times 10^{-5}$$  

(Eq. 1)

where $\Delta a/T$ is the absorbance change after the time interval t (6 min) relative to the tested tryptamine derivatives at a concentration of 10 μM, and $\Delta a/Fe^{2+}$ is the absorbance change at the same time interval relative to ferrous sulfate at the same concentration (4).

**Free Radical-scavenging Activity by Following DPPH Radical Assay**—DPPH is a stable free radical and shows absorbance at 517 nm. Antioxidant molecules scavenge the DPPH radical by donating hydrogen, as visualized by discoloration of the DPPH radical from purple to yellow (4, 39). The assay system contained 1 ml of compounds under investigation dissolved (in

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methanol or water) at different concentrations in the range 10–100 μM and 4 ml of DPPH (0.15 mM) in methanol (80% (v/v) in water) and mixed well. It was allowed to stand for 30 min at room temperature away from light. Ascorbic acid and gallic acid were used as positive control. The absorbance of the solution was measured at 517 nm.

Iron Chelating Activity in Vitro—The assay system has a total volume of 1 ml containing Fe(II) (10 μM) in 20 mM phosphate buffer, pH 7.4. SEGA (3a) at different concentrations (500 nm to 100 μM) and TPTZ (20 μM solution) were added to the Fe(II) solutions in small volumes to the sample cuvette with the concomitant addition of the same volume of DMSO to the reference cuvette (SEGA (3a) was dissolved in DMSO). For the control group, the assay system is the same without SEGA (3a). Desferrioxamine, a well known iron chelator was used as a positive control. Iron chelating ability of SEGA (3a) at different concentrations was monitored by recording the absorbance of the Fe(II)-TPTZ complex immediately after each addition in the quartz cells of the 1-cm light path in a PerkinElmer Life Sciences Lamda 15 UV-visible spectrophotometer at 25 ± 1 °C. The contents were mixed well before the spectrum was recorded.

Animals and Indomethacin (NSAID)-induced Gastric Damage (Gastropathy)—All of the in vivo studies were done in accordance with the institutional animal ethical committee guidelines. Sprague-Dawley rats (180–220 g) were used in this study. Each group (control or experimental) of animals was maintained at 24 ± 2 °C with a 12-h light-dark cycle. The animals were fasted for 24 h before the study to avoid food-induced increased acid secretion and its effect on gastric lesions. The rats were randomly divided into gastric mucosal lesion induction groups and control, indomethacin-treated, and indomethacin-treated groups. Oral administration of indomethacin (30 mg/kg b.w.) was given to all the animals to induce gastric injury. In the drug-pretreated indomethacin-treated groups, the animals were given SEGA (3a) (50, 20, 10, 5, 3, and 1 mg/kg b.w.), intraperitoneally 30 min prior to indomethacin treatment. The control group received vehicle only. After 4 h of indomethacin treatment, the animals were sacrificed under proper euthanasia, and stomachs were collected. The severity of mucosal lesions was scored as the injury index (40) according to the following scale: 0, no pathology; 1, a small injury (1–2 mm); 2, a medium injury (3–4 mm); 4, a large injury (5–6 mm); 8, a larger injury (>6 mm). The sum of the total scores in each group of rats divided by the number of the animals was expressed as the mean injury index (4–6).

For the healing study, gastric mucosal injury was first induced with indomethacin treatment at a dose of 48 mg/kg–1 b.w. 4 h after the induction of mucosal injury, some of the animals were divided into two different groups (n = 6) (i.e. autohealing and SEGA (3a)-induced healing). This time point was referred as 0 h of healing. At this point of time, in the SEGA (3a)-induced healing group, SEGA (3a) was administered (intraperitoneally) at a dose of 50 mg/kg–1 b.w. (this dose was selected from the dose-response curve). The animals, which were not treated with SEGA (3a) and received only indomethacin, served as the autohealing group. Starting from 0 h, the stomach was dissected out from all groups at intervals of 2, 4, 8, and 24 h, respectively, for measuring injury index as described (6, 12) and histological studies.

Histological Study—Stomach tissue from control and experimental groups was washed a number of times with phosphate-buffered saline (PBS, pH 7.4) and fixed in 10% buffered formalin for 12 h at 25 °C. The fixed tissues were then dehydrated and embedded in paraffin for preparing semithin sections (4). A microtome was used to prepare the semithin sections, which were then taken over poly-l-lysine-coated glass slides for hematoxylin-eosin staining. The stained sections were observed under a microscope (Leica DM-2500, Leica Microsystems GmbH, Wetzlar, Germany) and were documented by a high resolution digital camera.

Soret Spectroscopy to Detect Hemoglobin Released in Stomach during Mucosal Injury—After opening the stomach, gastric mucosal tissues from control and experimental groups were washed with PBS (pH 7.4). A PBS solution was collected and centrifuged at 12,000 × g for 20 min. The clarified PBS solutions were used to detect hemoglobin by recording Soret absorbance immediately in quartz cells of 1-cm light path in a PerkinElmer Lamda 15 UV-visible spectrophotometer for 12 h at 25 °C. The fixed tissues were then dehydrated and suspended in HBSS (pH 7.4), containing 100 units/ml penicillin, 100 units/ml streptomycin, and 10 μg/ml gentamycin. The mucosa was minced and suspended in HBSS pH 7.4, containing 100 units/ml penicillin, 100 units/ml streptomycin, and 0.1% collagenase type I. The suspension was incubated for 30 min at 37 °C in a 5% CO2 environment with shaking and then filtered through a sterile nylon mesh. The filtrate was centrifuged at 600 × g for 5 min, and the cell pellet was washed with HBSS (pH 7.4) and further centrifuged. The pellet was incubated in 5 ml of Ham’s F-12 medium in a T25 flask supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin, 100 units/ml streptomycin, and 10 μg/ml gentamycin. Cells were cultured at 37 °C with 5% CO2 and grown to ~90% confluence before treatment. 90% of the cells obtained following this protocol possessed epithelial characteristics. For all in vitro experiments, cultured cells were first divided into three groups: control, indomethacin-treated, and SEGA (3a)-pretreated indomethacin-treated in 12-well plates with 106 cells/well. Each well of the “indomethacin group” was treated with indomethacin, each well of the “indomethacin plus SEGA (3a) group” was treated with SEGA (3a) 30 min prior to indomethacin treatment, and the control was treated with only vehicle.

Measurement of Intramitochondrial Superoxide Anion (O2−) in Gastric Mucosal Cells—Mitochondrial O2− was detected by fluorescence microscopy using the specific dye MitoSOX. Equal numbers of gastric mucosal primary cultured cells from control and experimental groups were used for the detection of intramitochondrial O2− using MitoSOX, a superoxide-sensitive fluorescence probe, following the protocol as described in the product catalogue (6, 43). Cells were stained with the fluorescent probe in HBSS (pH 7.4) and incubated for 15 min at 37 °C.
Tryptamine-GA Hybrid against NSAID-induced Gastropathy

in the dark. After the incubation, cells were washed with HBSS three times and used for fluorescence microscopy (Leica DM-2500). Staining of MitoSOX was visualized using a red filter.

**Measurement of Intramitochondrial Free Iron in Gastric Mucosal Cells**—Equal numbers of gastric mucosal primary cultured cells from control and experimental groups were used for free iron localization using Phen Green SK, an iron-sensitive fluorescence probe, following the protocol as described in the product catalogue. Cells were first incubated with Phen Green SK (20 μM) for 15 min at 37 °C in the dark. After the incubation, cells were washed with HBSS and used for fluorescence microscopy (Leica DM-2500). The fluorescence of Phen Green SK was visualized using a green filter (6).

**Isolation of Mitochondria**—Mitochondria were isolated and purified by following the protocol reported earlier (18, 44). In brief, the scraped gastric mucosa from the control, indomethacin-treated (48 mg·kg⁻¹·b.w.), and SEGA (3a)-pretreated (50 mg·kg⁻¹·b.w.) indomethacin-treated groups were minced and homogenized in isolation buffer, followed by centrifugation at 600 × g for 10 min to remove the cell debris and nuclear pellet. This was further centrifuged at 12,000 × g for 15 min to obtain the crude mitochondrial pellet. A 25–50% Percoll density gradient was prepared. Over the 25% Percoll, the crude mitochondrial pellet (resuspended in cold 15% Percoll solution) was layered. It was further centrifuged at 30,000 × g at 4 °C for 45 min to obtain pure mitochondria at the interface between Percoll (25–50%) layers. The mitochondria were washed three times and used for fluorescence microscopy (Leica DM-2500).

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**Measurement of MOS**—Isolated mitochondria from stomach tissue of control, indomethacin-treated (48 mg·kg⁻¹·b.w.), and SEGA (3a)-pretreated (50 mg·kg⁻¹·b.w.) stomach tissue of rats were used to detect MOS. MOS was measured as described earlier through the quantification of total thiol, lipid peroxidation products, and protein carbonyl in mitochondria. In brief, thiol content was measured by its reaction with 5,5′-dithiobis(nitrobenzoic acid) to yield the yellow chromophore of thionitrobenzoic acid, which was measured at 412 nm. Mitochondrial lipid peroxidation was assayed by adding 1 ml of the mitochondrial fraction in 0.9% normal saline to 2 ml of thiobarbituric acid/TCA mixture (0.375% (w/v) and 15% (w/v), respectively) in 0.25 N HCl and was mixed and boiled for 15 min. The samples were then cooled, and after centrifugation, the absorbance of the supernatant was read at 535 nm. Tetraethoxypropane was used as a standard. Protein carbonyl was measured by following the standard colorimetric method that measures the binding of dinitrophenylhydrazine to the carbonyl group and was quantified by taking the absorbance at 280 nm.

**Assessment of Mitochondrial Respiratory Function by Following Mitochondrial Oxygen Consumption**—Mitochondrial oxygen consumption was measured using a Clark-type electrode in a liquid phase oxygen measurement system (Oxygraph, Hansatech, Norfolk, UK) (46). Complex I (state 3)-mediated oxygen consumption was initiated by the incorporation of glutamate and malate (5 mM each) to 1 ml of respiratory medium (250 mM sucrose, 5 mM KH₂PO₄, 5 mM MgCl₂, 0.1 mM EDTA, and 0.1% BSA in 20 mM HEPES, pH 7.2). Basal respiration (state 2) was measured following the addition of mitochondrial suspension. The addition of ADP (1 mM) marks the initiation of state 3 respiration. State 4 respiration was recorded in the absence of ADP. The respiratory control ratio (RCR) was obtained from the ratio of state 3 respiration (nmol of O₂ consumed) and state 4 respiration (nmol of O₂ consumed) (18, 47).

**Measurement of ΔΨₚ**—Isolated mitochondria from stomach tissue of rats were used to detect ΔΨₚ. Equal amounts of mitochondria (25 μg) from control and experimental groups were taken in 100 μl of JC-1 assay buffer (100 mM MOPS, pH 7.5, containing 550 mM KCl, 50 mM ATP, 50 mM MgCl₂, 50 mM sodium succinate, 5 mM EGTA) and were incubated in the dark with JC-1 (300 nM) for 15 min at 37 °C. The fluorescence of each sample was measured using a Hitachi F-7000 fluorescence spectrophotometer (excitation 590 nm, emission 530 nm for JC-1 monomer or JC-1 aggregates). ΔΨₚ was calculated as the ratio of 590 nm/530 nm (4, 43).
nilide (200 μM final concentration). The mixture was incubated at 37 °C for 1 h, and absorbance was taken at 405 nm (4, 43).

RT-PCR for Proapoptotic and Antiapoptotic Genes—Equal amounts of stomach tissue (30 mg) from control, indomethacin-treated (48 mg kg⁻¹ b.w.), and SEGA (3a)-pretreated (50 mg kg⁻¹ b.w.) indomethacin-treated (48 mg kg⁻¹ b.w.) rats were used for total RNA isolation using a commercially available kit (RNaseasy kit, Qiagen). RNA (2 μg) was used to prepare cDNA using oligo(dT)₁₇. Equal amounts of cDNA were used for PCR amplification using specific forward and reverse primers of bcl-2, bax, and actin. The PCR-amplified products were resolved in 2% agarose gel and documented in a gel documentation system (Alpha Innotech). The intensity of each band was quantified with densitometric software (Lab Image beta version, Kapelan GmbH, Germany). The intensity of each band was normalized with that of actin (6).

Terminal Deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) Assay in Vitro in Cultured Gastric Mucosal Cells and in Vivo in Rat Gastric Mucosa—In vitro apoptosis was detected in the cultured gastric mucosal cells using a commercially available APO-BrdU™ TUNEL assay kit (Invitrogen). In brief, cultured cells were first divided into control, indomethacin-treated and SEGA (3a)-pretreated indomethacin-treated groups. After 16 h of incubation, the cells were washed with PBS twice and fixed with 1% paraformaldehyde in PBS (pH 7.4) followed by treatment with 70% ethanol in ice. The slides were then loaded with DNA labeling solution, containing deoxynucleotidyl transferase. Cells were stained with Alexa Fluor® 488 dye-labeled anti-BrdU antibody. The cells were then finally stained with propidium iodide (PI) solution containing RNase A. The cells were then visualized under a fluorescence microscope (Leica DM-2500) using appropriate filters for Alexa Fluor 488 and PI (4, 6, 43).

For the detection of apoptosis in vivo, the rats from control, indomethacin-treated, and SEGA (3a)-pretreated indomethacin-treated groups were killed after 30 min, 3 a, and 6 h of treatment. Then these tissues were fixed in 10% buffered formalin for 24 h at 25 °C and processed as described under “Immunohistological Studies.” The semithin sections (5 μm) were used for TUNEL staining using a commercially available kit (Promega).

Immunohistochemical Studies—The semithin sections (5 μm) of mucosal tissues from control and experimental groups were deparaffinized in xylene and rehydrated in graded ethanol. After antigen retrieval, slides were rinsed in water and washed twice with Tris-buffered saline (TBS) (pH 7.4) plus 0.9% sodium chloride to block the endogenous peroxidases, the slides were incubated with 0.3% H₂O₂. Then the slides were incubated with HRP-labeled anti-rabbit IgG secondary anti-rat antibody at a dilution of 1:1000 in 1% BSA in TBS for 2 h. Finally, the slides were rinsed three times in TBS. Slides were stained with diaminobenzidine and counterstained with hematoxylin. The slides were viewed under the 10× objective of a Leica DM-2500 microscope.

Radiolabeling of SEGA (3a)—SEGA (3a) was labeled with 99mTc by a standard stannous reduction method (48) as per Reaction 1. Nitrogen-purged water was used for the preparation of aqueous 99mTcO₄⁻ solution and stannous chloride solution. Briefly, aqueous 99mTcO₄⁻ (2 mCi/ml) was mixed with 0.03 ml of freshly prepared stannous chloride solution (1 mg/ml) at pH 3.2 and further mixed with 1 ml of SEGA (3a) solution (3 mg/ml). The mixture was incubated separately for 20 min at room temperature (30 °C).

The effect of stannous chloride on the labeling efficiency at different concentrations was studied to find the optimum concentration needed for maximum labeling. After adding SEGA (3a) to the mixture of 99mTcO₄⁻ and SnCl₂ adjusted at the optimum pH (pH 3.2), the plates were incubated for various time periods to see the effect of incubation time on the yield of labeling. The optimum pH (pH 3.2) and the optimum concentration of stannous chloride (0.5 mg/ml) were then selected. The plates were then developed in appropriate solvent systems. Acetone was used for the determination of free pertechnetate, whereas either methanol or brine solution was used for the determination of radiocolloid. After development, the TLC plate was dried at room temperature (30 °C).

Transchelation with Diethylenetriamine Pentaacetic Acid (DTPA)—This study was performed to check the stability and strength of binding of 99mTc with the SEGA (3a). Radiolabeled preparations of 0.5 ml were challenged against three different concentrations (10, 30, and 50 mCi) of DTPA in 0.9% saline by incubating at 37 °C for 2 h. The effect of DTPA on labeling was measured by TLC on a silica gel plate using normal saline and acetone as the mobile phase, which allowed the separation of free pertechnetate and DTPA chelate (Rₛ = 0.9) from that of the 99mTc-labeled SEGA (3a), which remained at the point of application (Rₛ = 0).

Determination of Mitochondrial Uptake of SEGA (3a)—Mitochondrial uptake was carried out according to the following method. All animal experiments were carried out in compliance with the relevant national laws relating to the conduct of animal experimentation and with the approval of institutional animal ethics committees. Sprague-Dawley rats (180–220 g) were used for this study. The animals were fasted for 24 h before the start of experiments as described above. After 24 h of fasting, all of the rats were well hydrated through an intraperitoneal route in each rat. After 30 min, indomethacin (48 mg kg⁻¹ b.w.) was adminis-
treated orally in each rat. The rats were sacrificed at 4 h postinjection. Mitochondria of stomach mucosa were isolated as described above. Mitochondrial protein was estimated, and the radioactivity of $^{99m}$Tc-chelate SEGA (3a) was counted with a $\gamma$-scintillation counter against suitably diluted aliquots of the injected solution as a standard. The data were expressed as percentage dose/mg of mitochondrial protein (Mean ± S.E).

**Stability Studies**—The stability of $^{99m}$Tc-labeled SEGA (3a) was determined in vitro using 0.9% sodium chloride and serum (from rat) by ascending TLC. The labeled complex (0.5 ml) was mixed with 1.5 ml of normal saline or rat serum and incubated at 37 °C. The samples were withdrawn at regular intervals up to 24 h, monitored by TLC, and analyzed in a $\beta$-counter.

**Statistical Analysis**—All data are presented as mean ± S.E. The level of significance was determined by unpaired Student’s $t$ test with one-way analysis of variance as applied. A $p$ value of $\leq 0.05$ was considered as significant.

**RESULTS**

**Synthesis of Tryptamine-Gallic Acid Hybrid Molecule**—A small molecule having the iron-chelating property and the capability of preventing MOS from entering into mitochondria is necessary to protect gastric mucosa against NSAID-induced gastric mucosal injury or gastropathy. To design such a small molecule, we began the synthesis using 5-hydroxytryptamine (5HT), a hydrophobic amine that enters inside mitochondria (49). However, 5HT is toxic at high concentration (50). In contrast, free amine and hydroxyl groups of 5HT offer a scope to conjugate a powerful antioxidant bearing an iron-chelating property to make a non-toxic antioxidant hybrid molecule retaining mitochondrial penetration. Gallic acid (GA), a natural polyphenol and antioxidant (4), possesses the iron-chelating property (4, 51), and that is why we selected it to make a hybrid molecule with 5HT. The strategy might give double benefits because the conjugation of GA with 5HT is expected to enhance the bioavailability of GA in body fluid (lack of bioavailability is a common problem of bioactive polyphenol), or 5HT may be detoxified by GA through toxic group protection. 5HT was conjugated with GA through amide linkage to synthesize SEGA (3a) and through ester linkage to synthesize GASE (4d) (supplemental Schemes S1 and S2). Both antioxidant property in vitro and DPPH free radical-scavenging activity is based on the measurement of the absorbance change due to reduction of Fe(III) to Fe(II); the greater the decrease in absorbance, the better the antioxidant property. Antioxidants reduce the colorless Fe(III)-TPTZ to a blue Fe(II)-TPTZ complex, which results in an increase in the absorbance at 595 nm, giving a FRAP value. A higher FRAP value indicates a greater reducing (i.e. antioxidant property) ability of the compound. FRAP values at 6 min were calculated from the equation described above. At 6 min, the absorbance change takes place abruptly due to reduction of Fe(III) into Fe(II). The results clearly indicate that SEGA (3a) shows a reducing ability (Fe(III) to Fe(II)) that is much better than GASE (4d) (Table 1). In the DPPH assay, the decrease of absorbance is correlated with the antioxidant potency of the compounds. The greater the decrease in absorbance, the higher is the DPPH scavenging potency (i.e. the antioxidant potency of different synthesized compounds). The results indicate that SEGA (3a) also shows greater DPPH scavenging potency compared with GASE (4d) (Table 1). These results indicate that when 5HT is conjugated with GA through amide linkage, it appears to be more effective than when conjugated by ester linkage.

Our next objective was to synthesize different types of tryptamine-antioxidant conjugates through amide linkage using other antioxidants replacing GA and to evaluate their activities for comparative efficacy. We replaced GA with 4-hydroxycinnamic acid and indole-3-acetic acid to synthesize other tryptamine-antioxidant conjugates, such as 2b and 2c, respectively (Fig. 1). These compounds were synthesized by successive condensation of 5HT with 4-hydroxycinnamic acid and indole-3-acetic acid, respectively through amide linkage.
Next, we searched to find out whether 5HT is the best possible tryptamine for our purpose. We replaced 5HT with other tryptamines to synthesize several other tryptamine-antioxidant conjugates, such as TRGA (3b), MEGA (3c), 2f, 2h, and 2i, by successive condensation of tryptamine, 5-methoxytryptamine with GA, 4-hydroxy cinnamic acid, and indole-3-acetic acid, respectively (Fig. 1) (supplemental Scheme S3). 5HT itself showed little antioxidant activity in vitro (Table 1). We were interested in investigating whether in SEGA (3a), 5HT has any individual antioxidant property. To explore this, we synthesized dimer of 5HT (Fig. 1) (supplemental Scheme S4). Now antioxidant potencies were evaluated of all of the synthesized compounds by FRAP and DPPH free radical scavenging assays in vitro. For the preliminary screening of antioxidant activity, all of the synthesized compounds were taken at high concentration (100 μM). From the above results, it is evident that SEGA (3a) shows antioxidant property in FRAP as well as in the DPPH assay in a concentration-dependent fashion (Table 1). We were interested in checking the antioxidant property of SEGA (3a) at different concentrations. Results indicate that SEGA (3a) shows excellent antioxidant property in the FRAP assay as well as in the DPPH assay in a concentration-dependent fashion. SEGA (3a) could chelate free iron ions as assessed by a TPTZ assay. When SEGA (3a) was added to the Fe(II)-TPTZ gave a broad absorbance peak at 595 nm, whereas when SEGA (3a) was added to the Fe(II)-TPTZ peak, no such broad peak was observed after the addition of TPTZ solution (Fig. 2). Thus, from this experiment, SEGA (3a) chelates free iron in vitro. Now, because of its maximum antioxidant potential and iron-chelating property, was subjected to further detailed biological evaluation and mechanistic studies on NSAID-induced gastropathy.

**SEGA (3a) Prevents Indomethacin (NSAID)-induced Gastric Mucosal Damage**—We tested whether SEGA (3a) could protect indomethacin (an NSAID)-induced MOS-mediated mitochondrial pathology and gastropathy in vivo. SEGA (3a) protected gastric mucosa from indomethacin-induced gastric injury in a dose-dependent manner (ED50 = 6.9 mgkg⁻¹ b.w.), as evident from the gastric injury index (Fig. 3A). For rapid visualization of the protective effect of SEGA (3a), we present the real morphological data obtained by opening the stomach interior. From the morphology, it is very clear that SEGA (3a) protected the injury, and the oozing out of blood (which appeared black due to oxidation of released hemoglobin under an acidic environment) in indomethacin exposed rat gastric mucosa (Fig. 3B). The gastroprotective effect of SEGA (3a) was also verified by following the changes in microscopic structure of the actual histology of the gastric mucosa. SEGA (3a) restored normal architecture of gastric mucosa from indomethacin-induced increased gastric mucosal cell death and cell shedding in the superficial mucosa (Fig. 3C). Excessive gastric mucosal injury by NSAID leads to the release of blood in the stomach. In an indomethacin-treated rat, a sharp Soret peak (417 nm) was observed, indicating the presence of hemoglobin in the stomach due to a mucosal injury. But in the case of SEGA (3a) pretreatment, we did not find any Soret peak. The data

| Synthesized compounds | FRAP value (μM) MeSD | DPPH radical scavenging activity ΔA597 MeSD |
|-----------------------|----------------------|---------------------------------------------|
| 5-Hydroxy tryptamine  | 39.51 ± 2.7          | 0.45 ± 0.08                                 |
| GEGA (3a)             | 24.02 ± 1.98         | 0.38 ± 0.07                                 |
| TRGA (3b)             | 14.32 ± 0.36         | 0.31 ± 0.06                                 |
| MEGA (3c)             | 15.32 ± 0.46         | 0.35 ± 0.08                                 |
| 2f                    | 2.45 ± 0.19          | 0.11 ± 0.02                                 |
| 2h                    | 3.09 ± 0.13          | 0.12 ± 0.01                                 |
| 2i                    | 1.87 ± 0.13          | 0.11 ± 0.01                                 |
| 2f                    | 2.44 ± 0.24          |                                            |
| 2h                    | 2.48                |                                            |
| 2i                    | 1.71 ± 0.02          |                                            |
further confirmed the gastroprotective effect of SEGA (3a) (Fig. 3D).

SEGA (3a) Scavenges Intramitochondrial O$_{2}^\cdot$ and Free Iron in Gastric Mucosal Cells and Prevents MOS—Intramitochondrial generation of ROS and subsequent oxidative stress play a critical role in NSAID-induced gastric injury. Because SEGA (3a) protects gastric mucosa from NSAID-induced damage, we tested the ROS-scavenging activity of SEGA (3a). Mitochondrial generation of O$_{2}^\cdot$ was detected by MitoSOX staining (red). B, SEGA (3a) (50 μM) scavenges intramitochondrial free iron in vitro in cultured gastric mucosal cells. Mitochondrial generation of free iron was detected by Phen Green SK staining (green). C, SEGA (3a) (50 mg·kg$^{-1}$·b.w.) prevents indomethacin-induced formation of protein carbonyl, peroxidation of lipid, and depletion of thiol content in mitochondria (***, $p < 0.001$ versus control; ###, $p < 0.001$ versus indomethacin ($n = 5$)). Detailed descriptions are given under “Experimental Procedures.” Error bars, S.E.
eration of $O_2^\cdot$, but pretreatment with SEGA (3a) significantly inhibited the generation of $O_2^\cdot$ as revealed by the decreased fluorescence of the $O_2^\cdot$-derived oxidation product of MitoSOX (Fig. 4A). Mitochondrial free iron was measured by Phen Green SK, a specific fluorescent probe used to assay chelatable iron (Fig. 4B). Mitochondria of gastric mucosal cells were tagged by Mitotracker Red, a specific fluorescent probe for mitochondria. From the experiment, it is evident that indomethacin treatment resulted in increased intramitochondrial free iron accumulation, but pretreatment with SEGA (3a) significantly inhibited indomethacin-mediated free iron accumulation as revealed by decreased fluorescence of Phen Green SK (Fig. 4B). Mitochondrial $O_2^\cdot$ and free iron are responsible for MOS (6). SEGA (3a) by scavenging $O_2^\cdot$ and free iron, protected mitochondria from MOS, we were interested to find out whether SEGA (3a) corrects indomethacin-induced mitochondrial dysfunction in gastric mucosal cells during indomethacin-induced gastropathy (Fig. 4C). SEGA (3a) significantly prevented indomethacin-induced mitochondrial lipid peroxidation, thiol depletion, and protein carbonyl formation (Fig. 4C), which are the markers for MOS.

**TABLE 2**

|                      | Mitochondrial respiratory function (RCR) (mean ± S.E.) | $\Delta \Psi_m$ (fluorescence ratio, 590 nm/530 nm) (mean ± S.E.) | Mitochondrial dehydrogenase activity (MTT reduction, absorbance 570 nm) (mean ± S.E.) |
|----------------------|------------------------------------------------------|---------------------------------------------------------------|-------------------------------------------------------------------------------------|
| Control              | 6.21 ± 0.8                                           | 5.79 ± 0.48                                                   | 0.82 ± 0.09                                                                         |
| Indomethacin         | 3.77 ± 0.41*                                         | 2.68 ± 0.32*                                                 | 0.42 ± 0.07*                                                                        |
| Indomethacin + SEGA (3a) | 5.35 ± 0.7*                                          | 4.29 ± 0.37*                                                 | 0.71 ± 0.09*                                                                        |

*p < 0.01 versus control (n = 6–8).

*p < 0.01 versus indomethacin (n = 6–8).

*p < 0.01 versus indomethacin (n = 6–8).

SEGA (3a) Prevents Activation of Mitochondrial Pathway of Apoptosis—The activation of the mitochondrial pathway of apoptosis in gastric mucosal cells is a consequence of indomethacin-induced MOS.Withdrawn November 22, 2019(135,781),(933,996)
immunohistochemistry using anti-active caspase-3 antibody (Fig. 6C). Active caspase-3 immunolabeled mucosal cells (dark brown staining, indicated by an arrow) were found after indomethacin treatment. But SEGA (3a) pretreatment significantly decreased the active caspase-3-immunolabeled cells, indicating the antiapoptotic role of SEGA (3a) (Fig. 6C). Thus, the data indicate that SEGA (3a) prevents indomethacin-induced gastric mucosal cell apoptosis.

SEGA (3a) Accelerates Healing of Indomethacin-induced Damage of Gastric Mucosa—Prevention of MOS and the mitochondrial pathway of apoptosis expedites the healing process (5). Because SEGA (3a) prevents both MOS and apoptosis, we were interested in discovering whether SEGA (3a) could accelerate the healing of indomethacin-induced already damaged gastric mucosa. Interestingly, in addition to the gastroprotective effect, SEGA (3a) also accelerated healing of already injured mucosa by indomethacin (Fig. 7). Although autohealing takes place in the case of damaged mucosa, SEGA (3a) treatment accelerates the healing process. SEGA (3a)-induced healing of gastric mucosal injury was checked by histological analysis (Fig. 7). At 4, 8, and 20 h, the mucosa shows gastric injury with an injury index of 52, 28, and 20, respectively, whereas after treatment with SEGA (3a), damage of gastric mucosa was gradually repaired, as evident from an injury index of 14, 8, and 0, respectively. At 20 h, SEGA (3a) completely restored normal architecture of gastric mucosa, whereas in the case of the indomethacin group, there was significant injury. The results indicate that mucosa shows a time-dependent autohealing of the indomethacin-induced gastric damage in the absence of SEGA (3a). However, SEGA (3a) treatment significantly expedites healing with the progress of time, as evident from the restoration of gastric mucosa (Fig. 7). In indomethacin-treated animals, the autohealing at 4 h was negligible, as evident from the distorted mucosal histology, but SEGA (3a) treatment restored healthy mucosal architecture at 4 h, with almost complete restoration at 20 h (Fig. 7).

Quantitation of SEGA (3a) Entering Mitochondria—Because SEGA (3a) scavenged intramitochondrial O₂⁻, chelated intramitochondrial iron, and prevented MOS, we were interested in quantitating how much of the administered SEGA (3a) entered the mitochondria under in vivo conditions. For this purpose, SEGA (3a) was radiolabeled with ⁹⁹ᵐTc isotope as reported (48).
and administered to rats. The data indicated that 0.05% of the administered dose of SEGA (3a) entered per mg of mitochondria of gastric mucosal tissue (Fig. 8A). The stability or the structural integrity of SEGA (3a) in physiological saline as well as in serum was checked. SEGA (3a) was found to be very stable at 37 °C (Fig. 8B).

**DISCUSSION**

The present study describes the designing and synthesis of a small molecule, tryptamine-gallic acid hybrid (SEGA (3a)), which prevents NSAID-induced mitochondrial pathology, apoptosis, and gastropathy by blocking MOS through scavenging and correcting mitochondrial dysfunction.

Iron, which play an important role in one of the pathological condition. A mitochondria-targeted molecule is required for this purpose. This molecule must be small and lipophilic and be an ROS scavenger in nature. Moreover, free iron is known to generate ROS through the Fenton reaction. Thus, the iron-chelating property would be an additional advantage in controlling oxidative stress. All of these criteria were considered while designing the molecule. Several antioxidants and iron chelators have been reported, but none of them can satisfy all of the above criteria. Thus, a new molecule is essential, which will satisfy all of these criteria in preventing NSAID-induced gastropathy. Keeping this in mind, we have synthesized a series of tryptamine-antioxidant hybrid molecules. GA, when conjugated with 5HT through amide linkage, shows greater activity both in vitro and in vivo. Thus, all other tryptamine-antioxidant hybrid molecules were generated through the amide linkage. For the structure-activity relationship studies, we synthesized different tryptamine-antioxidant derivatives. Because SEGA (3a) appears to be the most active among all of the tryptamine-antioxidant conjugates, it is suggested that the presence of the 5-hydroxy group in the indole moiety of SEGA (3a) plays an important role for its gastroprotective activity. When the 5-hydroxy group in the indole moiety of SEGA (3a) was replaced by hydrogen and the methoxy group in the molecules TRGA (3b) and MEGA (3c), the activity was decreased. Indomethacin was selected as the representative NSAID over others because it is the most frequently used NSAID in gastrointestinal toxicity studies in experimental animals (13). The dose of indomethacin was selected as 5 mM and 48 mg·kg⁻¹ b.w. for in vitro and in vivo studies.
and in vivo studies, as reported earlier (4, 6, 7). The role of MOS and consequent apoptosis behind NSAID-induced gastric mucosal injury is already well established and is considered to be the major player in the acid-independent (5) and COX-independent pathway of NSAID-mediated gastric injury (53, 54). Indomethacin with its acidic carboxyl group (pK$_a$ = 4.5) and lipid solubility has been found to damage both rat and human mitochondria (13). Moreover, indomethacin enhances mitochondrial ROS, which disrupts mitochondrial function (6).
Because the gastroprotective effect of SEGA (3a) is dependent on its iron-chelating and free radical-scavenging properties, we compared the gastroprotective effect of SEGA (3a) with that of the standard iron-chelating agent desferrioxamine (ED$_{50}$ = 100 mg·kg$^{-1}$·b.w.) and free radical scavenging agents gallic acid (ED$_{50}$ = 18.9 mg·kg$^{-1}$·b.w.), vitamin E (ED$_{50}$ = 45 mg·kg$^{-1}$·b.w.), phenyl-N-tet-butyl nitroline (ED$_{50}$ = 100 mg·kg$^{-1}$·b.w.), and quercetin (ED$_{50}$ = 125 mg·kg$^{-1}$·b.w.) (4, 55, 56). We found that SEGA (3a) (ED$_{50}$ = 6.9 mg·kg$^{-1}$·b.w.) is much more effective than these compounds. The gastroprotective efficacy (ED$_{50}$) of SEGA (3a) was also compared with those of ranitidine (histamine H2 receptor antagonist), omeprazole, and lansoprazole (proton pump inhibitors), the three most commonly used gastroprotective agents. The gastroprotective potency (ED$_{50}$) of SEGA (3a) in protecting indomethacin-induced gastric mucosal injury was found to be superior to that of ranitidine (ED$_{50}$ = 13.5 mg·kg$^{-1}$·b.w.) (55) but inferior to those of omeprazole (ED$_{50}$ = 5 mg·kg$^{-1}$·b.w.) (7) and lansoprazole (ED$_{50}$ = 5.4 mg·kg$^{-1}$·b.w.) (5). Although proton pump inhibitors are effective at a very low dose against NSAID-induced gastropathy (5), they have some adverse effects like diarrhea (57), linear mucosal defects, and friable mucosa associated with collagenous colitis (58, 59), subacute cutaneous lupus erythematosus (60), Leydig cell tumors (61), acute nephritis (62), and collagenous colitis (58, 59), subacute cutaneous lupus erythematosus (60), linear mucosal defects, and friable mucosa associated with collagenous colitis (58, 59), subacute cutaneous lupus erythematosus (60), Leydig cell tumors (61), acute nephritis (62), and collagenous colitis (58, 59), subacute cutaneous lupus erythematosus (60), linear mucosal defects, and friable mucosa associated with collagenous colitis (58, 59), subacute cutaneous lupus erythematosus (60), Leydig cell tumors (61), acute nephritis (62), and collagenous colitis (58, 59), subacute cutaneous lupus erythematosus (60), linear mucosal defects, and friable mucosa associated with collagenous colitis (58, 59), subacute cutaneous lupus erythematosus (60), Leydig cell tumors (61), acute nephritis (62), and collagenous colitis (58, 59), subacute cutaneous lupus erythematosus (60), linear mucosal defects, and friable mucosa associated with collagenous colitis (58, 59), subacute cutaneous lupus erythematosus (60), Leydig cell tumors (61), acute nephritis (62), and collagenous colitis (58, 59), subacute cutaneous lupus erythematosus (60), linear mucosal defects, and friable mucosa associated with collagenous colitis (58, 59), subacute cutaneous lupus erythematosus (60), Leydig cell tumors (61), acute nephritis (62), and collagenous colitis (58, 59), subacute cutaneous lupus erythematosus (60), linear mucosal defects, and friable mucosa associated with collagenous colitis (58, 59), subacute cutaneous lupus erythematosus (60), Leydig cell tumors (61), acute nephritis (62), and collagenous colitis (58, 59), subacute cutaneous lupus erythematosus (60), linear mucosal defects, and friable mucosa associated with collagenous colitis (58, 59), subacute cutaneous lupus erythematosus (60), Leydig cell tumors (61), acute nephritis (62), and collagenous colitis (58, 59), subacute cutaneous lupus erythematosus (60), linear mucosal defects, and friable mucosa associated with collagenous colitis (58, 59), subacute cutaneous lupus erythematosus (60), Leydig cell tumors (61), acute nephritis (62), and collagenous colitis (58, 59), subacute cutaneous lupus erythermato
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