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A Modified Sesamol Derivative Inhibits Progression of Atherosclerosis

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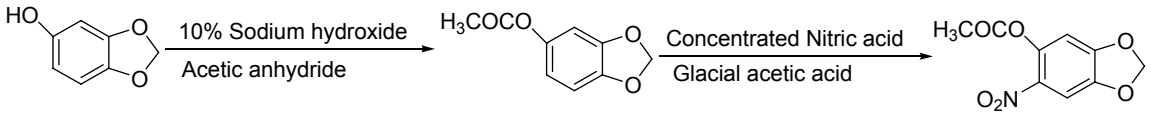
Methods:

The Institutional Animal Care and Use Committee (IACUC) at The Ohio State University approved the experimental animal protocols.

Synthesis of 3,4-methylenedioxy-6-nitrophenyl acetate

The rationale for simple chemical modification on 3,4-methylenedioxy phenol is to provide additional therapeutic benefits by way of introducing acetyl group at phenolic OH and nitro group at the 6th position. The acetyl group was introduced with the intent of providing chemical stability to sesamol by preventing aerial oxidation rendering longer shelf life. The nitro group was added to provide additional "NO" release functionality in-vivo. The synthesis of INV 403 is achieved in two step process starting from 3,4-methylenedioxyphenol which is schematically represented below:



Acetylation of 3,4-methylenedioxy phenol using fresh sample of acetic anhydride in presence of 10% NaOH yielded 3,4-methylenedioxyphenl acetate in 75% chemical yield. The nitration of 3,4-methylenedioxyphenyl acetate using concentrated nitric acid in presence of glacial acetic acid provided 3,4-methylenedioxy-6-nitro phenyl acetate in 70% yield as a pale yellow solid. The final compound was further purified by recrystallization from 95% ethanol to get crystalline yellow material.

Animal Models of Atherosclerosis

Rabbit Model of Atherosclerosis: Ten male Watanabe Heritable Hyperlipidemic WHHL rabbits (WHHL, 2 months old) were obtained from the Brown Family in Alabama and allowed to acclimate for 2 weeks prior to be being fed with high cholesterol chow (fat: 2.7% wt/wt; cholesterol: 0.5% wt/wt; Harlan Teklad TD87251) for 6 weeks, at which time point they were randomized to control or INV-403 groups (20 mg/kg/d for 12 weeks).

INV-403 was dissolved in 90% ethanol and sprayed onto to the high cholesterol chow. The diet-drug mixture was then vacuum dried overnight to remove ethanol and then used to feed rabbits. The dose of 20 mg/kg was selected according to published data [Food Chem Toxicol. 2008 46(8):2736-41; Shock. 2008;30(4):456-62.], which indicated that around 10 mg/kg sesamol was safe and effective. Considering the different molecular weight (224.15 for INV-403 and 138.12 for sesamol), 20 mg/kg INV-403 was used to treat animals.

In vivo Magnetic Resonance Imaging (MRI) to Assess Atherosclerosis Progression

Atherosclerotic plaque in the abdominal aorta of WHHL rabbits was analyzed by *in vivo* MRI scans using a 1.5T Siemens clinical scanner at the indicated time points. Kidneys were used as anatomical landmarks and were depicted using a coronal gradient echo, T1-weighted localizing sequence (TR/TE 800/1.0). Forty-eight, axial slices spanning the iliac bifurcation to the superior pole of the topmost kidney were obtained using a T1-weighted gradient echo turbo FLASH protocol (FOV 288/384; TR/TE 230/5.6; NEX=3; BW 100MHz; time of acquisition of ~11 minutes). No respiratory or cardiac gating was necessary as the abdominal aorta is relatively free from motion artifact. Plaque burden was determined by manually tracing the external elastic lamina (EEM) and the luminal border (L) and determining the area within each boundary using Siemens Viewer software. Slice volume (V) was calculated as V = (EEM-L)*4.0 and total wall volume per animal was calculated using the formula TWV = Σ [(EEM-L)*4] and expressed in mm^3. TWVs for each animal at each time point were normalized for varying numbers of readable slices with the formula NWV = (TWV/n)*m; n=number of slices readable in the individual animal and m=mean number of slices readable in all animals over all time points. All normalized wall volumes were reported in mm^3.

Immunohistochemistry and Morphometry

Analysis of Atherosclerosis: Segments of descending thoracic aorta were embedded in Optimal Cutting Temperature compound (Tissue-Tek, Sakura Finetek USA Inc, Torrance, Calif) and frozen on dry ice. En face sections were then prepared. To analyze atherosclerotic burden, 8-12 sections (4 μm thick) were collected at intervals of 20 μm. After H&E staining and Oil-red O staining, each section was analyzed in a blinded manner after digitizing the images. The images were analyzed under a research microscope (Zeiss Axioskop with Spot I digital camera, Jena, Germany) with National Institutes of Health (NIH) Image software version 1.61 (Wayne Rasband, NIH, http://rsb.info.nih.gov/nih-image). Results were expressed as mm^2.

Immunohistochemistry: Immunohistochemical staining was performed by using the primary antibodies and a detection system (Immunoperoxidase Secondary Detection System; Chemicon International, Temecula, Calif), and quantified with software (NIH Image) after digitization of the images with a camera system (Zeiss Axioskop with Spot I digital camera). Antibodies against CD68 were purchased from Santa Cruz Biotechnology Incorporated (Santa Cruz, Calif). A polyclonal anti-α-Actin antibody was obtained from Upstate Cell Signaling Solutions (Lake Placid, NY). T cell receptor β antibody was obtained from Biolegend (San Diego, CA). Anti-VCAM1 antibody was bought from R & D systems. To quantify the staining, the mean density of 4 negative control (no primary antibody) sections was set as the threshold, and positive area was acquired by software. Results were finally normalized by the area subtended by the external elastic lamina to the luminal interface or the intimal-medial volume.

Vascular Physiology Studies

Rabbits were euthanized by injection of lethal doses of pentobarbital. The ascending aortas was removed and 3 mm thoracic aortic rings were suspended in individual organ chambers filled with physiological salt solution buffer (sodium chloride, 130 mEq/L; potassium chloride, 4.7 mEq/L; calcium dichloride, 1.6 mEq/L; magnesium sulfate, 1.17

mEq/L; potassium diphosphate, 1.18 mEq/L; sodium bicarbonate, 14.9 mEq/L; EDTA, 0.026 mEq/L; and glucose, 99.1 mg/dL [5.5 mmol/L]; pH, 7.4), aerated continuously with 5% carbon dioxide in oxygen at 37°C. Vessels were allowed to equilibrate for 1 hour at a resting tension of 30 mN before being subjected to graded doses of agonists as described previously[1, 2]. The vasoconstrictor agonists included phenylephrine (PE), endothelin-1 (ET-1), or angiotensin II. Responses were expressed as a percentage of the peak response to 120 mEq/L of potassium chloride. The vessels subjected to PE were washed thoroughly and allowed to equilibrate for 1 hour before beginning experiments with acetylcholine or SNP. After a stable contraction plateau was reached with PE (0.1 μM), the rings were exposed to graded doses of the endothelium-dependent agonist acetylcholine or the endothelium-independent agonist SNP. Results were expressed as a percentage of pre-contraction by PE (0.1 μM). The rings exposed to acetylcholine were thoroughly washed and allowed to equilibrate for 1 hour. After a stable contraction plateau was reached with PE (0.1 μM), insulin was then added in an accumulative manner. Results were expressed as a percentage of pre-contraction by PE (0.1 μM).

In-situ Detection of $O_2^{\bullet-}$

Briefly, in situ detection of $O_2^{\bullet-}$ was performed in snap-frozen aortic tissues embedded in OCT compound (Tissue-Tek®, Sakura Finetek USA Inc, Torrance, CA) as previously described.[3] Tissue samples were cryosectioned at 4 μm of thickness, collected onto Superfrost Plus slides (Fisher Scientific, Pittsburg, PA), and stored at -80°C until needed. Four slides that were randomly chosen from each rat (tissue block) were placed into phosphate buffered saline (PBS) for 30 min at room temperature and then stained with dihydroethidium (DHE, 10 μM, Molecular Probes, Inc., Eugene, OR) in PBS for 20 min in a moist chamber in the dark. The slides were rinsed extensively with PBS,

coverslipped, and digitally imaged with a research microscope (Zeiss Axioskop with a Spot I digital camera, Jena, Germany). To quantify the staining, the mean density of the area subtended by the external elastic lamina to the luminal interface or the intimal-medial volume is acquired.

8-epi Prostaglandin F2α (8-Isoprostane) Enzyme Linked Immunoassay

Stat-8-Isoprostane ELISA kit (Cayman Chemical, , Ann Arbor, Mich) was used to determine 8-isoprostane level in plasma and liver. Both samples were prepared according to the manufacturer's instruction, and underwent affinity purification (Cayman Chemical, Ann Arbor, Mich). The 8-isoprostane levels in the liver were adjusted by protein amount, which were determined by BCA Protein Assay (Pierce, Rockford, IL.), while 8-isoprostane levels in the plasma were normalized by the starting volume of plasma.

Western Blot Analysis

Samples were homogenized and solubilized in radioimmunoprecipitation assay buffer (RIPA, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride, with 0.25% sodiumdeoxycholate and 1.0% Nonidet P-40) and centrifuged at 10,000g and 4°C for 30 min. The supernatant was collected and subjected to western blot analysis. In brief, 40 μg of protein was separated by SDS-polyacrylamide gel electrophoresis and subsequently transferred to nitrocellulose membrane. The membrane was then incubated with: mouse anti-β-actin (Sigma, St. Louis, MO), mouse anti-VCAM1, monoclonal anti-ICAM1 (R&D Systems, Minneapolis, MN), mouse anti-phospho-IκBα(Ser32/36), rabbit anti-IκBα (Cell Signaling Technology, Danvers, MA). Finally, the membranes were incubated with a horseradish peroxidase-linked secondary antibody and visualized with an enhanced

chemiluminescence kit (Amersham Biosciences Inc., Piscataway, NJ). Band density was quantified by densitometric analysis using ImageJ.

NF-κB Activation Assays

Nuclear proteins were extracted from rabbit aorta with NE-PER Nuclear and Cytoplasmic Extraction Reagents (PIERCE, Rockford, IL) and subject to electrophoretic mobility shift assay (EMSA) with lightshift Chemiluminescent EMSA Kit (PIERCE, Rockford, IL) according to the manufacturer's instruction. Oligonucleotides (Sequences: NF-kappaB sense, AgT TgA ggg gAC TTT CC Cag gC; NF-kappaB antisense, gCC Tgg gAA AgT CCC CTC AAC T) were synthesized by Invitrogen, and labeled with Biotin 3′ End DNA labeling Kit (PIERCE, Rockford, IL). Binding Reactions were performed in 20 µl solution containing 10x binding buffer (2 µl), Glycerol (2.5%), $MgCl_2$ (5 mM), Poly(dI·dC) (50 ng/µl), NP-40 (0.05%), Nuclear extract (8 µg proteins), and Biotin-DNA (20 fmol). The binding action products were then resolved by 6% native polyacrylamide gel and transferred to nylon membrane. After cross-linking, biotin–DNA was visualized by chemiluminescence.

IKK2 Activity Assay

HTScan® IKKβ Kinase Assay Kit (Cell Signaling Technology) was used to determine the effect of INV-403 on the IKK2 activity. Briefly, a biotinylated peptide substrate of IKK2 was phosphorylated by recombinant human IKK2 kinase for 1 hour at 37°C, using the following reaction conditions: 60 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 µM Na-orthovanadate, 1.2 mM DTT, 200 µM ATP, 2.5 µg/50 µl PEG20.000, Substrate: Rb CTF 1.5 µg/50 µl, recombinant IKKbeta: 50 ng/50 µl, INV-403 (variable). The phosphorylated substrate was then Colorimetric ELISA detection methods.

Quantitative RT-PCR Analysis

Total RNA was isolated with TRIzol reagent (Invitrogen). Four microgram of total RNA was reverse transcribed by random hexamers and ThermoScript RT-PCR System (Invitrogen). Quantitative real-time PCR was performed with the Stratagene Mx3005 using SYBER Green PCR Master Mix (Applied Biosystems, Foster City, CA). Relative expression level compared to GAPDH was obtained as previously described[4]. The primers used in the experiment are depicted in the table below.

Gene	Sense/Antisense	Sequence
Rabbit ICAM-1	Sense	5'-GCC TGA GGT CCA GTT CTG TG-3'
	Antisense	5'-GCG GAC ACA GCT CTC AGT AG-3'
Rabbit GAPDH	Sense	5'-GCC TGG AGA AAG CTG CTA AG-3'
	Antisense	5'-CCA GCA TCG AAG GTA GAG GA-3'
Rabbit VCAM-1	Sense	5'-TGC CGA GCT AAA TTA CAT ATC G-3'
	Antisense	5'-TCA TTG TCA CAG AGC CAC CT-3'
Rabbit P-selectin	Sense	5'-CGG ACC AGA AAG ACT GGA CT-3'
	Antisense	5'-GTT CCT CAC ATG GTG CTG GAC-3'
Rabbit L-selectin	Sense	5'-GCT CAG AAG GAG CCG AGT TA-3'
	Antisense	5'-TTA CCA TGA CTG CCA CAG GA-3'
Rabbit MCP-1	Sense	5'-AGC ACC AAG TGT CCC AAA GA-3'
	Antisense	5'-TGT GTT CTT GGG TTG TGG AA-3'
Rabbit CD68	Sense	5'-CCTTGCTGGGGCTACACGC-3'
	Antisense	5'-CCAGTGGTAGGAGTGGC-3'

Reference:

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3. Miller FJ, Gutterman DD, Rios CD, Heistad DD, Davidson BL. Superoxide Production in Vascular Smooth Muscle Contributes to Oxidative Stress and Impaired Relaxation in Atherosclerosis. *Circulation research.* 1998;82:1298-1305.

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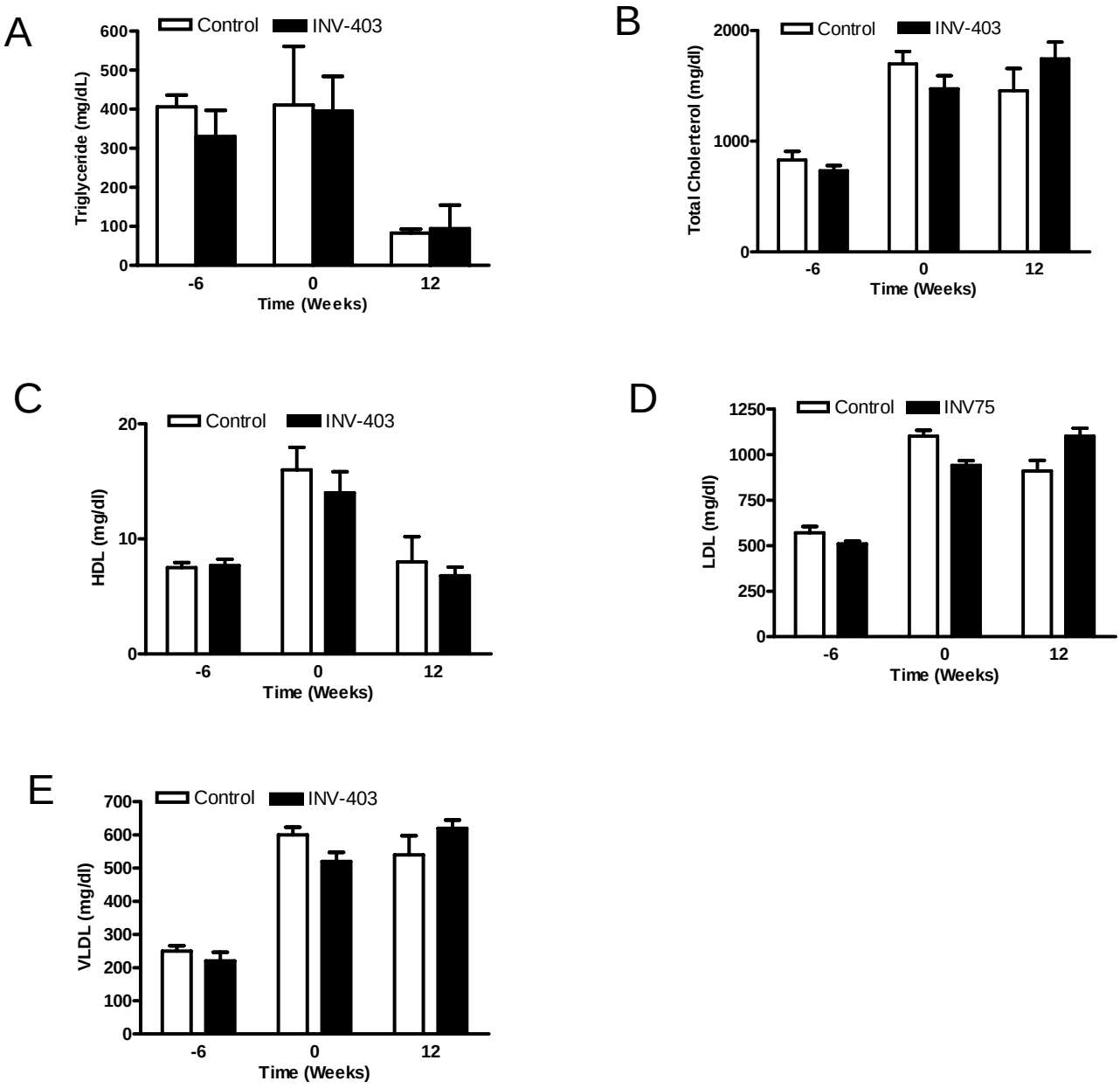
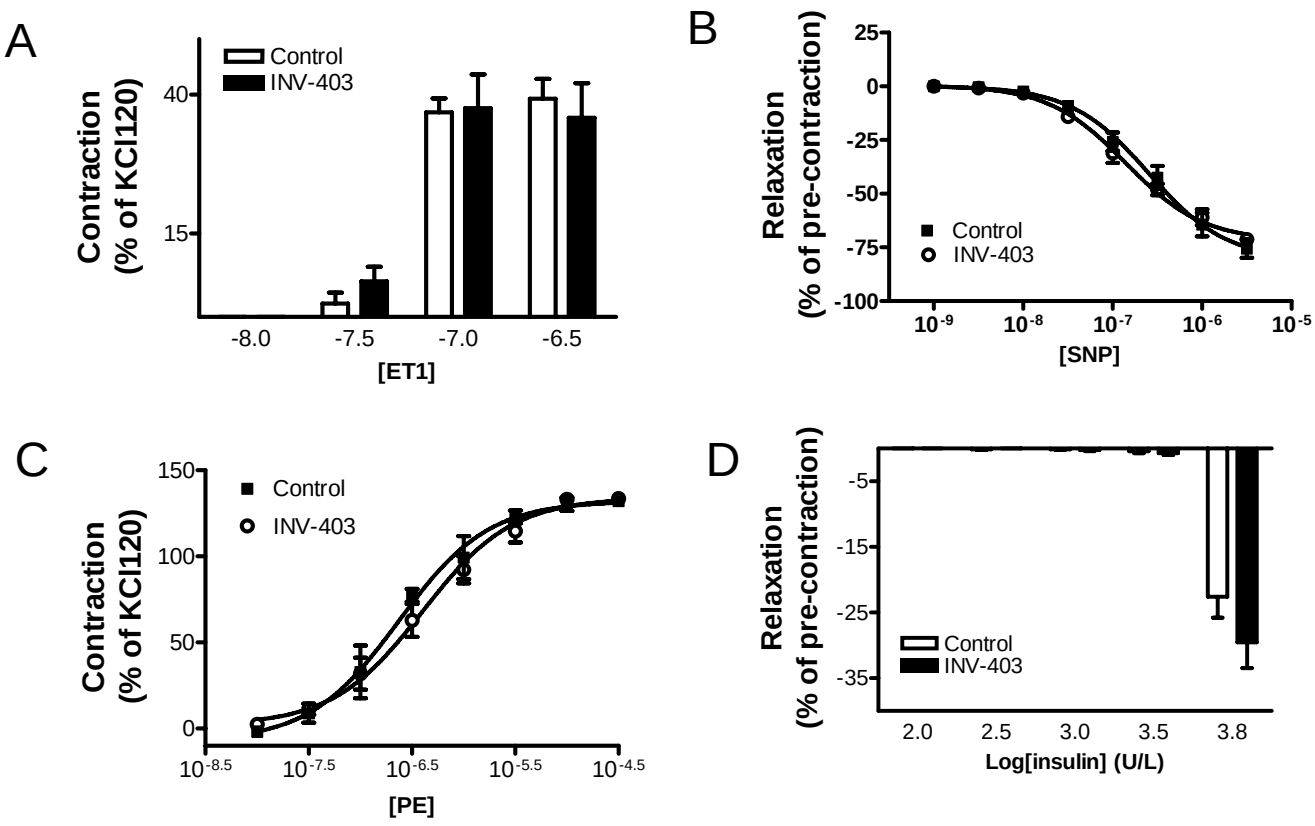
Supplementary Figures



Supplementary Figure I: INV-403 did not change plasma lipoprotein profile in high cholesterol-fed WHHL rabbits.

Supplementary Figures



Supplementary Figure II: INV-403 did not change aortic responses to endothelin (A), SNP (B), PE (C), and insulin (D).