Supporting Information for

NOSH-aspirin is a novel nitric oxide- and hydrogen sulfide-releasing hybrid: A new class of anti-inflammatory and anti-cancer pharmaceuticals

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Page

Chemistry:

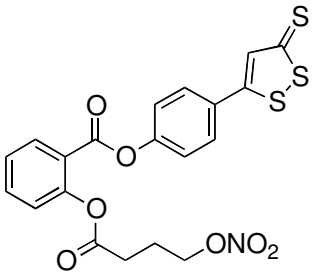
Experimental section:

All moisture-sensitive reactions were performed under an argon atmosphere using oven-dried glassware and anhydrous solvents. Anhydrous solvents were freshly distilled from sodium benzophenone ketyl, for THF and DCM was distilled from calcium hydride. Extracts were dried over anhydrous Na_2SO_4 and filtered prior to removal of all volatiles under reduced pressure. Unless otherwise noted, commercially available materials were used without purification. Silica gel chromatography was performed using 100-200 mesh silica gel (Natland). Thin layer chromatography was performed using precoated 250 μ plates (Analtech). Nuclear magnetic resonance (NMR) splitting patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), and broad (b); the value of chemical shifts (δ) are given in ppm relative to residual solvent (chloroform δ=7.27 for ^{1}HNMR or δ=77.23 for proton decoupled ^{13}C NMR), and coupling constants *(J)* are given in hertz (Hz). The mass spectra were recorded on AB SCIEX 4000 QTRAP LC-MS/MS instrument (EI).

Synthesis of NOSH compounds:

General Procedure for the Preparation of NOSH-1 (NBS-1120) and NOSH-3 (NBS-1121): To the solution of 2-[[4-nitroxy) butanoyl] oxy] benzoic acid (238.0 mg, 0.88 mmol) in dichloromethane was added DCC (201.0 mg, 0.97 mmol), DMAP (10.8 mg, 0.09 mmol) at 0^{o}C under argon atmosphere. Then added ADT-OH ((5-(4-hydroxyphenyl)-3H-1, 2-dithiole-3-thione) (200.0 mg, 0.88 mmol) and the whole reaction mixture was stirred at room temperature for overnight. After completion of the reaction as checked by TLC, filtered off and water was added then extracted into dichloromethane (2x75 ml). Organic solvent was removed under reduced pressure to get the crude product. Further it was purified by column chromatography to afford pure orange solid (NOSH-**1**). (298.0 mg, 78 % yield). NOSH-1: 4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenyl 2-((4-(nitrooxy)butanoyl)oxy)benzoate.

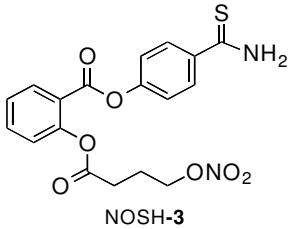
^{1}H-NMR ($CDCl_3$, 500 MHz): δ 2.18 (m, 2H), 2.78 (t, *J* = 6.8 Hz, 2H), 4.56 (t, *J* = 6.3 Hz, 2H), 7.22 (d, *J* = 8.3 Hz, 1H), 7.32 (d, *J* = 8.8 Hz, 2H), 7.44 (s,1H), 7.45 (t, *J* = 7.8 Hz, 1H), 7.72 (t, *J* = 7.8 Hz, 1H), 7.75 (d, *J* = 8.8 Hz, 2H), 8.27 (d, *J* = 7.8 Hz, 1H).
^{13}C-NMR ($CDCl_3$, 125 MHz): δ 22.08, 30.21, 71.91, 121.83, 123.23, 124.31, 126.68, 128.57, 129.76, 132.37, 135.43, 136.33, 151.48, 153.52, 162.34, 171.24, 171.75, 215.71.
ESIMS: *m/z* 478 (M^++1), 500 (M^++Na).



NOSH-**1**

NOSH-3:

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 2.11-2.16 (m, 2H), 2.75 (t, *J* = 7.2 Hz, 2H), 4.53 (t, *J* = 6.5 Hz, 2H), 7.19 (d, *J* = 7.5 Hz, 1H), 7.20 (bs, 1H), 7.21 (d, *J* = 8.0 Hz, 2H), 7.42 (t, *J* = 8.0 Hz, 1H), 7.66 (bs, 1H), 7.68 (dt, *J* = 8.5 Hz, 1.47 Hz, 1H), 7.94 (d, *J* = 8.8 Hz, 2H), 8.24 (dd,

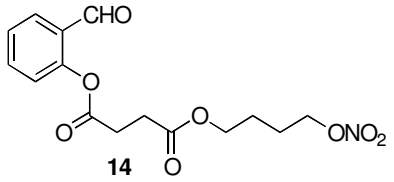


NOSH-**3**

J = 8.5 Hz, 1.47 Hz, 1H). ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 22.08, 30.22, 72.0, 121.94, 122.02, 124.24, 126.68, 128.74, 132.43, 135.30, 135.37, 137.37, 151.40, 153.49, 162.54, 171.33, 201.81. ESIMS: *m/z* 405 (M^++1), 427 (M^++Na), 450(M^++2Na).

NOSH-3: 4-carbamothioylphenyl 2-((4-(nitrooxy)butanoyl)oxy)benzoate

2-formylphenyl (4-(nitrooxy) butyl) succinate (14):

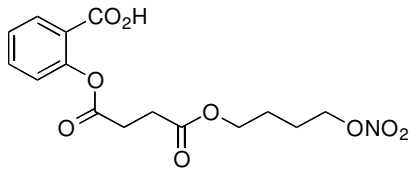


14

To the solution of salicyladehyde (1.0 g, 8.19 mmol) in methylene chloride added succinic anhydride (0.819 g, 8.19 mmol) and catalytic amount of DMAP (0.1 g, 0.819 mmol), and stirred for 24h at room temperature. Then added hydroxyl butyl nitrate (**13,** 1.1 g, 8.19 mmol**)** followed by addition of DCC (1.69 g, 8.196 mmol), at 0^{o}C under argon atmosphere. The whole reaction mixture was stirred at room temperature for 6h. After completion of the reaction as checked by TLC, filtered off and water was added then extracted into dichloromethane (2x75 ml). Organic solvent was removed under reduced pressure to get the crude product. Further it was purified by column chromatography to afford pure compound 2-formylphenyl (4-(nitrooxy) butyl) succinate 1.8 g (65 % yield).

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 1.75-1.82 (m, 4H), 2.78 (t, *J* = 6.8 Hz, 2H), 2.99 (t, *J* = 6.8 Hz, 2H), 4.17 (t, *J* = 6.35 Hz, 2H), 4.46 (t, *J* = 6.35 Hz, 2H), 7.19 (d, *J* = 7.8 Hz, 1H), 7.41 (t, *J* = 7.32 Hz, 1H), 7.64 (dt, *J* = 8.3, 1.95 Hz, 1H), 7.88 (dd, *J* = 7.32, 1.45 Hz, 1H). 10.10 (s, 1H). ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 23.57, 24.98, 28.96, 29.14, 64.0, 72.72, 123.47, 126.62, 128.12, 131.19, 135.42, 151.47, 170.95, 172.10, 189.0. ESIMS: *m/z* 340 (M^++1), 362 (M^++Na).

2-((4-(4-(nitrooxy) butoxy)-4-oxobutanoyl) oxy) benzoic acid (15):

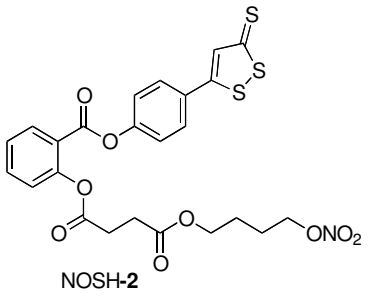


15

$KMnO_4$ (0.96 g, 6.084 mmol) was added to a stirred solution of aldehyde 2-formylphenyl (4-(nitrooxy) butyl) succinate (1.375 g, 4.056 mmol) in acetone (50 ml) at 0^{o}C. The reaction mixture was allowed to reach room temperature and was stirred for 3h. After completion of the reaction as checked by TLC, oxalic acid was added and filtered off. The filtrate was diluted with dichloromethane and washed with water, dried and concentrated under reduced pressure to get the crude product of 2-((4-(4-(nitrooxy)butoxy)-4-oxobutanoyl)oxy)benzoic acid (1**5).**

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 1.76-1.83 (m, 4H), 2.77 (t, *J* = 6.8 Hz, 2H), 2.99 (t, *J* = 6.8 Hz, 2H), 4.18 (t, *J* = 6.35 Hz, 2H), 4.47 (t, *J* = 6.35 Hz, 2H), 7.17 (d, *J* = 7.8 Hz, 1H), 7.37 (t, *J* = 7.8 Hz, 1H), 7.65 (t, *J* = 7.8 Hz, 1H), 8.13 (dd, *J* = 7.81, 1H). ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 23.74, 25.15, 29.12, 29.44, 64.07, 72.79, 122.37, 124.16, 126.48, 132.68, 135.10, 151.25, 169.59, 171.29, 172.38. ESIMS: *m/z* 355 (M^++1), 378 (M^++Na).

NOSH 2 (NBS-1130): Procedure followed as for the preparation of NOSH-1.

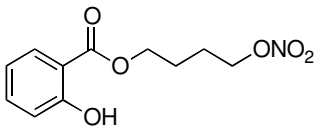


NOSH-**2**

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 1.73-1.80 (m, 4H), 2.71 (t, *J* = 6.8 Hz, 2H), 2.94 (t, *J* = 6.8 Hz, 2H), 4.13 (t, *J* = 6.3 Hz, 2H), 4.45 (t, *J* = 5.8 Hz, 2H), 7.22 (d, *J* = 7.8 Hz, 1H), 7.33 (d, *J* = 8.8 Hz, 2H), 7.41 (d, *J* = 8.3 Hz, 1H), 7.42 (s,1H), 7.68 (dt, *J* = 7.8 Hz, 1.96 Hz, 1H), 7.73 (d, *J* = 8.3 Hz, 2H), 8.22 (dd, *J* = 7.8 Hz, 1.46 Hz, 1H). ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 23.78, 25.15, 29.10, 29.37, 64.03, 72.74, 121.94, 123.35, 124.41, 126.62, 128.55, 129.74, 132.34, 135.37, 136.32, 151.49, 153.63, 162.42, 171.23, 171.73, 172.18, 215.74. ESIMS: *m/z* 564 (M^++1), 586 (M^++Na).

NOSH-2: 4-(nitrooxy)butyl (2-((4-(3-thioxo-3*H*-1,2-dithiol-5-yl)phenoxy)carbonyl)phenyl) succinate

4-(nitrooxy) butyl 2-hydroxybenzoate (17):

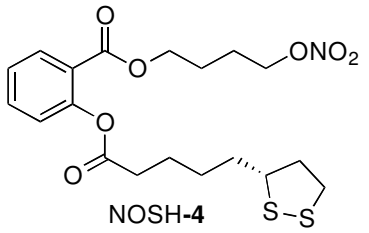


17

To the solution of compound 4-(nitrooxy) butyl 2-acetoxybenzoate (**16**, 0.5 g, 1.68 mmol) in MeOH/THF (1:1) 20mL was added K_2CO_3 (0.025 mmol) and stirred at room temperature for 15 min. solvent was removed, water was added and extracted into ethyl acetate. The crude product was purified by column chromarography to afford 4-(nitrooxy) butyl 2-hydroxybenzoate (0.3 g, 72 %) (**17**).

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 1.94 (m, 4H), 4.41 (bt, 2H), 4.54 (bt, 2H), 6.90 (t, *J* = 7.8 Hz, 1H), 7.0 (d, *J* = 8.3 Hz, 1H), 7.48 (t, *J* = 7.8 Hz, 1H), 7.83 (d, *J* = 7.8, 1H), 10.75 (bs, 1H). ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 23.83, 25.19, 64.52, 72.69, 112.39, 117.86, 119.43, 129.90, 136.09, 161.88, 170.25. ESIMS: *m/z* 256 (M^++1), 278 (M^++Na).

Preparation of NOSH-4 (NBS-1131):



NOSH-**4**

To the solution of compound 4-(nitrooxy) butyl 2-hydroxybenzoate (**17**, 0.3 g, 1.176 mmol) in methylene chloride was added *(R)*-lipoic acid **(18,** 0.24 g, 1.176 mmol) followed by addition of DCC (0.24 g, 1.176 mmol) and DMAP (0.024 g, 0.1176 mmol) stirred for 6h at room temperature. After completion of the reaction as monitored by TLC, filtered off concentrated under reduced pressure to obtain the crude product. Further the crude product was purified by column chromatography to afford the pure compound NOSH-**4** (0.35 g, 68 %). NOSH-4: (*R*)-4-(nitrooxy)butyl 2-((5-(1,2-dithiolan-3-yl)pentanoyl)oxy)benzoate

^{1}H-NMR ($CDCl_3$, 500 MHz): δ 1.52-1.68 (m, 3H), 1.76-1.98 (m, 8H), 2.46-2.53 (m, 1H), 2.66 (t, *J* = 7.8 Hz, 2H), 3.11-3.23 (m, 2H), 2.62 (q, *J* = 6.3 Hz, 1H), 4.32 (t, *J* = 5.3 Hz, 2H), 4.52 (t, *J* = 5.3 Hz, 2H), 7.12 (d, *J* = 8.3 Hz, 1H), 7.33 (t, *J* = 6.8 Hz, 1H), 7.58 (t, *J* = 6.8 Hz, 1H), 7.99 (d, *J* = 7.3 Hz, 1H). ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 23.63, 24.31, 25.07, 28.71, 33.92, 34.61, 38.52,

40.25, 56.41, 64.07, 72.66, 123.24, 123.86, 125.97, 131.44, 133.95, 150.74, 164.24, 172.0. ESIMS: *m/z* 466 (M^++Na).

Biology

***Cell culture*:** HT-29, SW-480 and HCT-15 human colon adenocarcinoma, MIA PaCa-2 and BxPC-3 human pancreatic cancer, LNCAP human prostate cancer, A549 human lung cancer, MCF-7 (estrogen receptor positive), MDA-MB 231 and SK-BR-3 (estrogen receptor nrgative) human breast cancer, and Jurkats human leukemia cell lines were obtained from American Type Tissue Collection (Manassas, VA). All cells lines were grown as monolayers except for the Jurkats which were grown in suspension. The pancreatic and breast cancer cells were grown in Dulbecco's modified Eagle's medium, the prostate, Jurkat, SW-480 and HCT-15 colon cells were grown in RPMI 1640 medium, the lung cells were grown in F-12 and the colon HT-29 cells were grown in McCoy 5A. All media were supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA) penicillin (50 U/ml), and streptomycin (50 µg/ml) (Invitrogen, Carlsbad, CA). Cells were seeded on culture dishes at a density of 25×10^3 cells/cm^2 and incubated at 37°C in 5% CO_2 and 90% relative humidity. Single cell suspensions were obtained by trypsinization (0.05% trypsin/EDTA), and cells were counted using a hemocytometer. Viability was determined by the trypan blue dye exclusion method.

MTT Assay: Cell growth inhibitory effect of NOSH compounds were measured using a colorimetric MTT assay kit (Roche, Indianapolis, IN). Cancer cells were plated in 96-well plates at a density of 50,000 cells/well. The cells were incubated for 24 h with different concentrations of NOSH compounds. After the indicated time, 10µl of MTT dye (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide, 5 mg/ml in phosphate buffered saline), was added to each well, and the plates were incubated for 2 hours at 37ºC. Then, the media was aspirated, and Add 100 µl of the solubilization solution (10% SDS in 0.01 M HCl) was added to each well to solubilize the formant crystals. The absorbance of the plates was measured on an ELISA reader at a wavelength of 570 nm. Each sample was performed in triplicate, and the entire experiment was repeated three times.

LDH release assay: For determination of lactate dehydrogenase (LDH) activity, HT-29 cells ($1x10^5$ cells/well) were incubated in 96-well plates with different concentrations of NOSH-1. After incubation for 2, 4, 8, 12 and 24 h, LDH activity in the supernatant was assessed using the LDH Cytotoxicity Assay Kit (Cayman Chemical Ann Arbor, MI), according to the manufacturer's instructions. Cytotoxicity was calculated as a percentage based on the LDH activity released from cells that had been treated with NOSH-1 compared with LDH activity from cultures incubated with Triton X-100. The % of LDH release was determined using the formula (E-C)/(T-C) x 100, where E is the experimental absorbance of cell cultures, C is the control absorbance of cell-free culture medium, and T is the absorbance corresponding to the maximal (100%) LDH release of Triton-lysed cells.[1]

Determination of plasma TNF-α: Fresh samples of blood from the animals were taken by cardiac puncture into heparin-containing vials. The determination of plasma TNF-α was carried out by an enzyme immunoassay kit from R&D systems (Minneapolis, MN) as described previously.[2] Briefly, each sample (50 µL) was incubated with antibodies specific for rat TNF-α

and washed three times with assay buffer. An enzyme-linked polyclonal antibody specific for rat TNF-α conjugated to horseradish peroxidase was then added to the wells. Following washing of unbound antibody-enzyme reagent, a substrate solution (containing tetramethylbenzidine, TMB, plus hydrogen peroxide) was added to the wells. The enzyme reaction yielded a blue product (oxidized TMB) that turned yellow when the stop solution (dilute hydrochloride acid) was added. The intensity of the color was determined by measuring the OD of the yellow color in a standard ELISA plate reader at 450 nm. Sensitivity of this TNF-α assay was determined by adding two standard deviations to the mean optical density value of 20 x zero standard replicates and calculating the corresponding concentration. The kit contains all reagents and standards needed for the TNF-α sensitivity assay. The results are expressed as pg/mL. Sensitivity for TNF-α is estimated to be around 1.6 pg/mL.

Inflammatory Edema Model: Carrageenan (1%, 100 µL, suspended in sterile saline solution, type IV lamda; Sigma-Aldrich) was subcutaneously injected into the plantar surface of the right hind paw in rat following the protocol described by Winter et al.[3] Paw volume was measured using a water displacement plethysmometer (model 520; IITC/Life Sciences Instruments, Woodland Hills, CA) before carrageenan injection and thereafter at 1-h intervals for 6 h. The paw volume measured just before carrageenan injection was used as the control volume. Data are expressed as the change in paw volume (milliliters) at each time point.

Determination of PGE_2 in rat paw exudates: Rats were euthanized by asphyxiation in a CO_2 chamber. After cutting each hind paw at the level of the calcaneus bone, exudates (oedema fluid) and some tissue were collected, weighed and placed in a test tube containing 5 mL of 0.1 M phosphate buffer (pH7.4), 1 mM EDTA, and 10 µM indomethacin. The mixture was homogenized and centrifuged for 10 min at 12,000 r.p.m. at 4°C. PGE_2 content in supernatant was determined in duplicate by an enzyme immunoassay kit following the protocol described by the manufacturer (Cayman Chemical, Ann Arbor, MI) and reported.[2] Briefly, standard (50 µL) or homogenate (50 µL), enzymatic tracer (50 µL) and specific antiserum (50 µL) were mixed. After incubation for 17 h (overnight) at 4°C, the plates were washed with wash buffer and Ellman's reagent (200 µL) was added into each well. The absorbance at 412 nm was measured after 1 h incubation at room temperature. Results are expressed as pg of PGE_2 per mg of protein. Proteins were determined by Biorad assay.

Western blot analysis: Exudates (oedema fluid) and some tissue were homogenized in lysis buffer (0.1% Triton X-100, 50 µM pepstatin, 0.2 mM leupeptin, 1 µg/mL aprotinin, 10 mg/ml phenylmethylsulfonyl flouride, 50 mM Tris, and 10 mM EDTA). Samples were then centrifuged, and the protein concentration of the supernatant was determined by colorimetric assay (Bio-Rad, Hercules, CA). Protein (30 µg) was separated on a 10% polyacrylamide gel and then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Proteins were probed with monoclonal mouse antibody against COX-1 and COX-2 (1:500; Cayman Chemical, Ann Arbor, MI). The membrane was then incubated with a goat anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (Santacruz Biotechnology, Santa Cruz, CA). A chemiluminescence reagent (Amersham Pittsburg, PA) was added to visualize the labeling according to the manufacturer's instructions.

Determination of plasma NO content: Plasma concentration of NO was quantified indirectly as the concentration of nitrate (NO_3^-) and nitrite (NO_2^-) levels in plasma, by the Griess reaction using an assay kit and following the protocol described by the manufacturer. Rat plasma was filtered using a 10 KD molecular weight cut-off filter from Millipore (Bedford, MA) before each analysis, to reduced background absorbance due to the presence of haemoglobin. After centrifugation for 10 min at 3000 rpm, samples (40 µL/well) were mixed with 10µL nitrate reductase mixture and incubated for 3 h after which Griess reagents 1 and 2 (50 µL each) were added. Absorbance was read after 10 min at 540nm using a plate reader. The concentration of nitrate/nitrite was calculated graphically from a calibration curve prepared from $NaNO_2$ standard solution, and it is expressed as micromolar nitrate**.**

Measurement of H_2S levels: H_2S levels were measured as previously described.[4, 5] Aliquots (100 µL) of rat plasma from above were mixed with distilled water (100 µL), Zinc acetate (1% w/v, 250 µL), trichloroacetic acid (10% w/v, 250 µL), N, N-dimethyl-*p*-phenylenediamine sulfate (133 µL, 20 µM) in 7.2M HCl and $FeCl_3$ (133 µl, 30 µM) in 7.2M HCl. The absorbance of the resulting mixture (300 µL) was determined after 15 min using a 96-well microplate reader at 670 nm. All samples were assayed in duplicate and H_2S levels were calculated against a calibration curve of NaHS (1-250 µM). This method overestimates H_2S levels as it measures free H_2S, HS^- (hydrosulfide anion), and S^{2-} (sulfide).[6] Therefore, our results presented here indicate the sum total of these species.

Statistical analysis: In vitro: data are presented as mean ± SEM for at least three different sets of plates done in triplicate. In vivo: treatment groups and number of animals in each group are indicated in the figure legends. Comparison between treatment groups was performed by one-factor analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. $P < 0.05$ was regarded as statistically significant.

References

(1) Decker T, Lohmann-Matthes ML. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J Immunol Methods* **1988,**115, 61-9.

(2) Chattopadhyay, M.; Velazquez, C. A.; Pruski, A.; Nia, K. V.; Abdellatif, K. R.; Keefer, L. K.; Kashfi, K., Comparison between 3-Nitrooxyphenyl acetylsalicylate (NO-ASA) and O^2-(acetylsalicyloxymethyl)-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (NONO-ASA) as safe anti-inflammatory, analgesic, antipyretic, antioxidant prodrugs. *J Pharmacol Exp Ther* **2010,** 335, (2), 443-50.

(3) Winter, C. A.; Risley, E. A.; Nuss, G. W., Carrageenin-induced edema in hind paw of the rat as an assay for antiiflammatory drugs. *Proc Soc Exp Biol Med* **1962,** 111, 544-7.

(4) Li L, Rossoni G, Sparatore A, Lee LC, Del Soldato P, Moore PK. Anti-inflammatory and gastrointestinal effects of a novel diclofenac derivative. *Free Radic Biol Med* **2007,** 42, 706-19.

(5) Huang S, Chua JH, Yew WS, Sivaraman J, Moore PK, Tan CH, et al. Site-directed mutagenesis on human cystathionine-gamma-lyase reveals insights into the modulation of H_2S production. *J Mol Biol* **2010,** 396, 708-18.

(6) Lee ZW, Zhou J, Chen CS, Zhao Y, Tan CH, Li L, et al. The slow-releasing hydrogen sulfide donor, GYY4137, exhibits novel anti-cancer effects in vitro and in vivo. *PLoS One* **2011,** 6,(6), e21077.