Electronic supplementary information (ESI)

Comparative biological activity study of non-radical vs stable radical of pyridazine-sulfonamide aminophenol type compound

Experimental section

Materials and physical measurements.

Analytical grade reagents were obtained from Sigma Aldrich and used without further purification. Spectroscopic grade solvents were used for spectroscopic and electrochemical measurements. The C, H, and N contents of the compounds were obtained from a Perkin-Elmer 2400 Series II elemental analyzer. Infrared spectra of the samples were measured from 4000 to 400 cm^{-1} with KBr pellets at room temperature on a Perkin-Elmer Spectrum RX 1 FT-IR spectrophotometer. ^1H NMR spectra in $CDCl_3$ were obtained on a Bruker DPX 300 MHz spectrometer. ESI mass spectra were recorded on a micro mass Q-TOF mass spectrometer. Electronic absorption spectra in solution were obtained on a Perkin-Elmer Lambda 750 spectrophotometer in the range of 3300-175 nm. The X-band EPR spectra were measured on a Magnettech GmbH MiniScope MS400 spectrometer, where the microwave frequency was measured with an FC400 frequency counter. The electro analytical instrument, BASi Epsilon-EC for cyclic voltammetric experiments in CH_2Cl_2 containing 0.2 M tetrabutylammonium hexafluorophosphate as supporting electrolyte was used. The BASi platinum working electrode, platinum auxiliary electrode, Ag/AgCl reference electrode were used for the measurements. The redox potential data are referenced *vs.* ferrocenium/ferrocene, Fc^+/Fc, couple.

4-(3,5-di-tert-butyl-2-hydroxyphenylamino)-N-(6-methoxypyridazin-3-yl)benzenesulfonamide ($LSO_{AP}H_2$): To a solution of 3,5-di-*tert*-butylcatechol (1.1 g, 5 mmol)and triethylamine (0.3 ml) in *n*-hexane (30 ml) and CH_3CN (20 ml), 4-amino-N-(6-methoxypyridazin-3-yl)benzenesulfonamide(1.0 g, 5 mmol) was added. The resulting mixture was stirredin air for 7 days. A whitish ppt was obtained, which waspoured on a basic alumina column. Elution with a mixture of chloroformand *n*-hexane solvents (1:5, v/v) afforded pure **$LSO_{AP}H_2$** ligand, yield 1.72 g (ca. 84 % with respect to catechol). ^1H NMR

(300 MHz, $CDCl_3$): δ = 11.2 (s, 1H), 7.62 (d, 1H, J = 8.4 Hz), 7.37–7.25 (m, 3 H), 6.99 (d, 2 H, J = 9.6 Hz), 6.66 (d, 2H, J = 8.4 Hz), 5.81 (s, 1H), 5.42 (s, 1H), 3.89 (s, 3H), 1.39 (s, 9 H), 1.22 (s, 9 H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 211.1 (C1), 210.5 (C21), 209.9 (C18), 208.5 (C15), 208.5 (C2), 208.2 (C4), 185.5 (C6), 135.22 (C23), 134.21(C22), 111.7 (C17, C19), 111.4 (C20, C16), 111.3 (C3), 110.2 (C5), 66.7 (C25), 35.0 (C7), 34.4 (C11), 31.5 (C12), 29.7 (C13, C14, C12), 29.5 (C8, C9, C10) ppm. IR (KBr): ν˜ = 3435(m, NH), 3058 (s, *t*Bu), 2957 (s, *t*Bu), 1587 (s), 1477 (s), 1421 (s), 1310(m), 1221 (m), 1024 (s), 738 (s), 690 (s) cm^{-1}.

Single crystal X-ray structure determinations of the compound. Single crystal of **$LSO_{AP}H_2$**was picked up with nylon loops and were mounted on Bruker AXS D8 QUEST ECO diffractometer equipped with a Mo-target rotating-anode X-ray source and a graphite monochromator (Mo-Kα, λ = 0.71073 Å). Final cell constants were obtained from least-squares fits of all measured reflections. Intensity data were corrected for absorption using intensities of redundant reflections. The structures were readily solved by direct methods and subsequent difference Fourier techniques. The crystallographic data were listed in Table S1.The molecular structure was solved using the SHELXL-2016/6[1]package. All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were placed at the calculated positions and refined as riding atoms with isotropic displacement parameters.The molecular structures of the complexes were confirmed by the single crystal X-ray structure determinations of **$LSO_{AP}H_2$**.

Table S1. Crystallographic Data for $\mathbf{LSO_{AP}H_2}$

	$\mathbf{LSO_{AP}H_2}$
formula	$C_{25}H_{32}N_4O_4S$
CCDC No	2130713
fw	484.60
cryst color	colourless
cryst system	triclinic
space group	*P -1*
a (Å)	9.3204(8)
b (Å)	15.1349(11)
c (Å)	20.1462(15)
α ()	68.822(2)
β ( )	88.431(3)
γ ( )	81.237(3)
V (Å)	2617.8(4)
Z	4
T (K)	273(2)
refl. collected ($2\Theta_{max}$)	49.98
*ρ*calcd (g cm^{-3})	1.230
uniquerefl.	72684
ref (I>2σ)	6404
μ (mm^{-1})	0.160
λ (Å)	0.71073
F(000)	1032
R1[b]/goodness of fit [c]	0.0705/ 1.088
wR2[d] [I >2*σ* (**I**)]	0.1333
no. of params/restr.	629/0
residual density ($eÅ^{-3}$)	0.497/-0.471
[a]Observation criterion*: I >2σ(I).* [b]$R1 = \Sigma\|\|F_o\| - \|F_c\|\|/\Sigma\|F_o\|$. [c]GOF = $\{\Sigma[w(F_o^2 - F_c^2)^2]/(n-p)\}^{1/2}$. [d]wR2 = $\{\Sigma[w(F_o^2-F_c^2)^2]/\Sigma[w(F_o^2)^2]\}^{1/2}$ where $w = 1/[\sigma^2(F_o^2) + (aP)^2+bP]$, $P = (F_o^2+2F_c^2)/3$	

DFT Computation

Gas phase geometry of $\mathbf{LSO_{AP}H_2}$ and $\mathbf{LSO_{ISQ}}$were optimized with coordinates obtained from single crystal structure using DFT/B3LYP method with 6-31+G as basis set (O, N, S, C, H) of Gaussian Program Package 09.[2-4]GAUSSSUM employed for calculation of fractional contribution of groups to the spin density.[5]$\mathbf{LSO_{AP}H_2}$was optimized with the singlet spin state while $\mathbf{LSO_{ISQ}}$was optimized with doublet spin state.

Biological studies

Chemicals and reagents:

Chemicals and reagents were obtained from Himedia (India), Invitrogen (India), SRL (India) and Sigma-Aldrich (USA). DCFDA (# D6883) was purchased from Sigma-Aldrich (India). Fetal bovine serum (#16000044) was obtained from Gibco, USA and MEM sodium pyruvate, MEM non-essential amino acids L-glutamine and Gentamicin were procured from Hi-Media, India.

Cell line culture:

In the present study three cancerous cell lines namely human liver cancer cell line HepG2, human triple negative breast cancer cell line MDA-MB 231 and human cervical cancer cell line Helawere procured from the repository of National Center for Cell Science (NCCS), Pune, India. All the above cell lines were cultured in a T25 with DMEM, supplemented with10% Fetal bovine serum, non-essential amino acids, 1mM sodium pyruvate, 2mM L-glutamine, 100 mg/L streptomycin, 100 units/L penicillin,and 50 mg/L gentamycin in a 37°C humidified incubator containing 5% CO_2.

Cytotoxicity assay:

The viability of Hela, MDA-MB 231 and HepG2 cells after exposure to various concentrations of $\mathbf{LSO_{AP}H_2}$ and $\mathbf{LSO_{ISQ}}$ were determined by The MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay.[6, 7]Briefly, around 1×10^4 cells per well of 96-well plates were exposed to $\mathbf{LSO_{AP}H_2}$ and $\mathbf{LSO_{ISQ}}$ ligands at the concentrations of 10,20, 40, 60, 80, 100 µg/ml for 24 h of incubation at 37°C and 5% CO_2. Following this, the cells were incubated again with 10 µl MTT solution (stock 1 mg/ml) for 4 h at 37°C and 5% CO_2 following a wash with 1× phosphate-buffered saline (PBS), and the resulting formazan crystals were dissolved in 100 µl DMSO to measure the absorbance at 570 nm by using a microplate reader (Biorad). The data were formulated comparing with the control ones.

Annexin V-FITC/PI staining for apoptosis assay:

Induction of apoptosis was quantified via flow cytometric analysis of control and $\mathbf{LSO_{AP}H_2}$ and $\mathbf{LSO_{ISQ}}$ treated Hela cells (15µg/ml, 30 µg/ml and 45µg/ml) that were stained with annexin V-FITC/PI, using the Annexin V-FITC apoptosis detection kit according to the manufacturer's protocol (BD Bioscience).[8]Briefly post treatment cells were harvested with 1X Trypsin and washed in ice cold 1x PBS followed by re-suspended in 100µL of 1X binding buffer solution supplied within the kit. Finally cells were incubated with 5 µL of annexin V-FITC and 5 µL of PI for 15 min at room temperature in dark before acquiring data using BD FACS Verse flow cytometer (BD Biosciences, San Jose, CA). Annexin V/FITC positive cells were regarded as apoptotic cells analyzed using Cell Quest Software (BD Biosciences).

Measurement of cellular ROS using DCFDA:

Normally, the DCFDA enters the cell and reacts with the reactive oxygen to give a green fluorescent colour compound dichlorofluorescein (DCF). Briefly, a stock solution of DCFDA (10 mM) was prepared in methanol and was further diluted with PBS to a working concentration of 100 µM. Hela cells were treated with $\mathbf{LSO_{AP}H_2}$ and $\mathbf{LSO_{ISQ}}$ at a concentration of 15µg/ml, 30 µg/mland 45µg/ml at 37°C, for 24h time period and washed with ice-cold 1× PBS followed by an incubation with 10µM of DCFDA for 30 min in the dark at 37°C and directly imaged under fluorescent microscope (Leica). For flowcytometric analysis cells were then trypsinized, washed with 1x PBS and collected in centrifuge tubes. DCF fluorescence was then measured using BD FACS Verse flow cytometer (BD Biosciences) and analyzed using Cell Quest Software (BD Biosciences).[9]

Apoptotic Nuclear Morphology Study by DAPI Staining:

To identify any morphological changes within the nucleus of Hela cells upon treatment with synthesized compounds, DAPI (4′,6-diamidino-2-phenylindole) staining was used. Briefly Hela cells were seeded in 6-well plates containing coverslips and treated with respective doses of compound $\mathbf{LSO_{AP}H_2}$ and $\mathbf{LSO_{ISQ}}$ for 24 hours. Post treatment cells were washed three times with 1x PBS and DAPI staining was done and finally washed with 1× PBS. Coverslips with stained cells were mounted on slides and nuclear morphology was observed under fluorescence microscope (Leica).

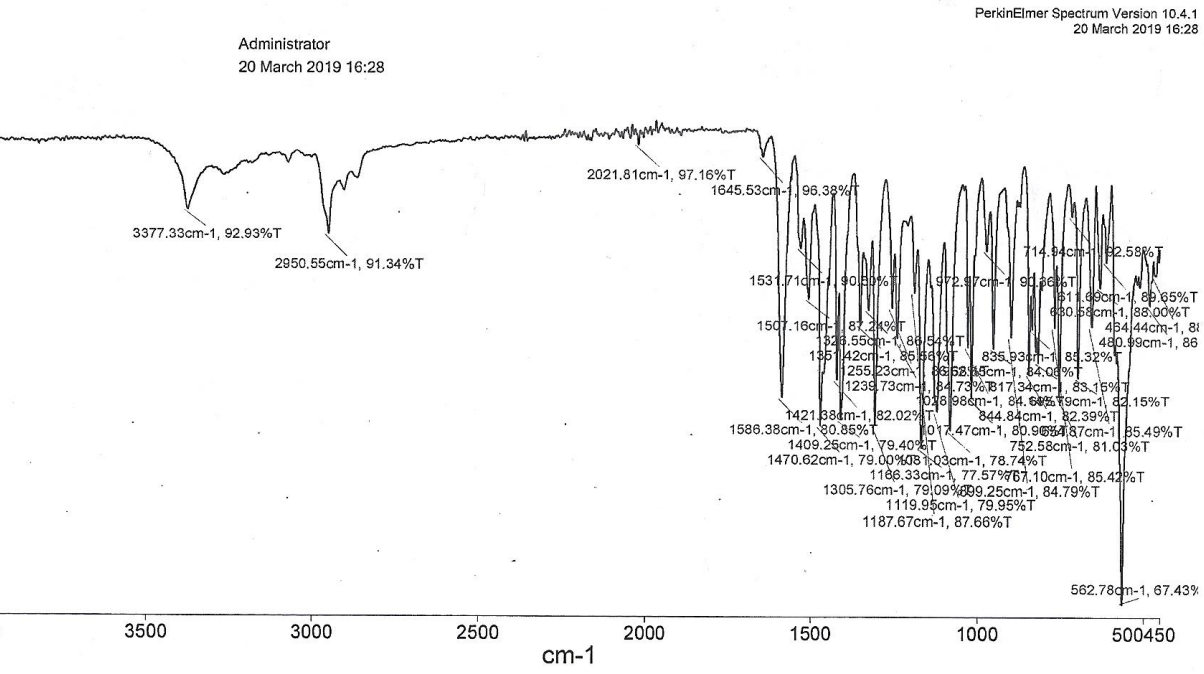

Fig. S1.IR spectrum of $\mathbf{LSO_{AP}H_2}$

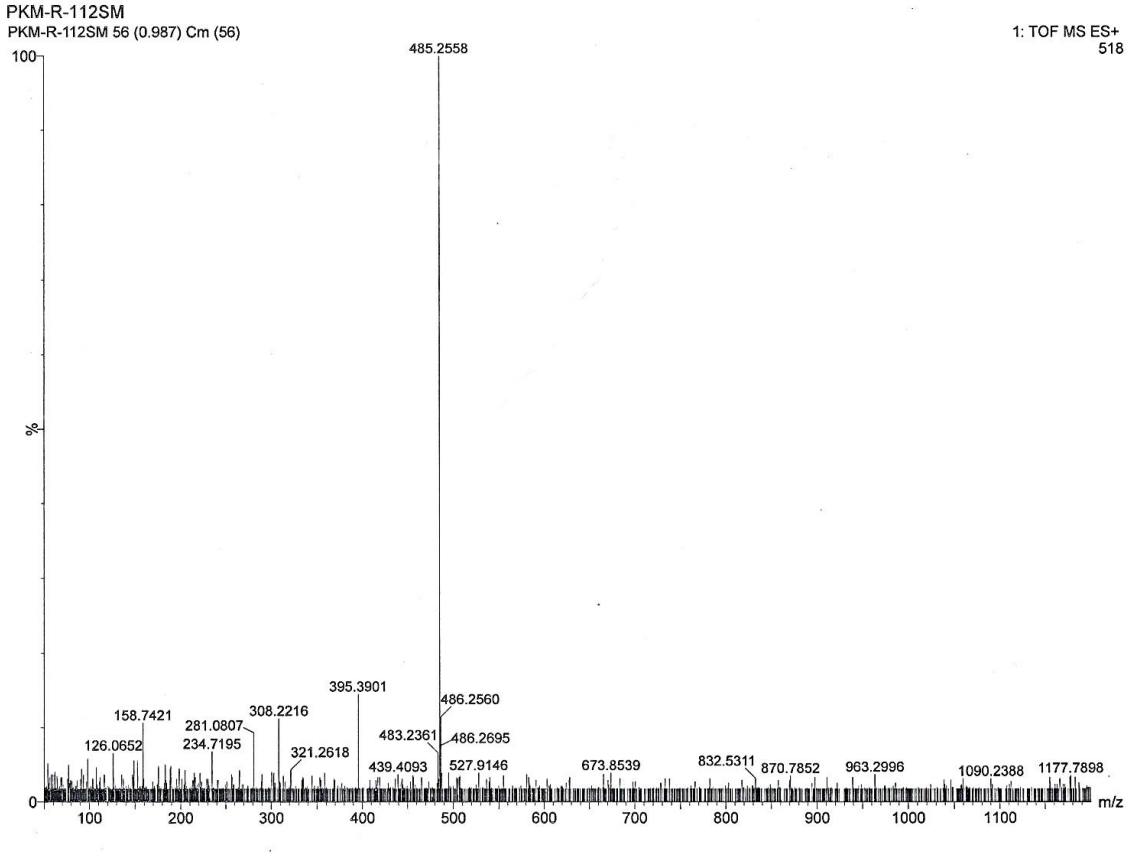

Fig. S2. ESI mass spectra of $\mathbf{LSO_{AP}H_2}$

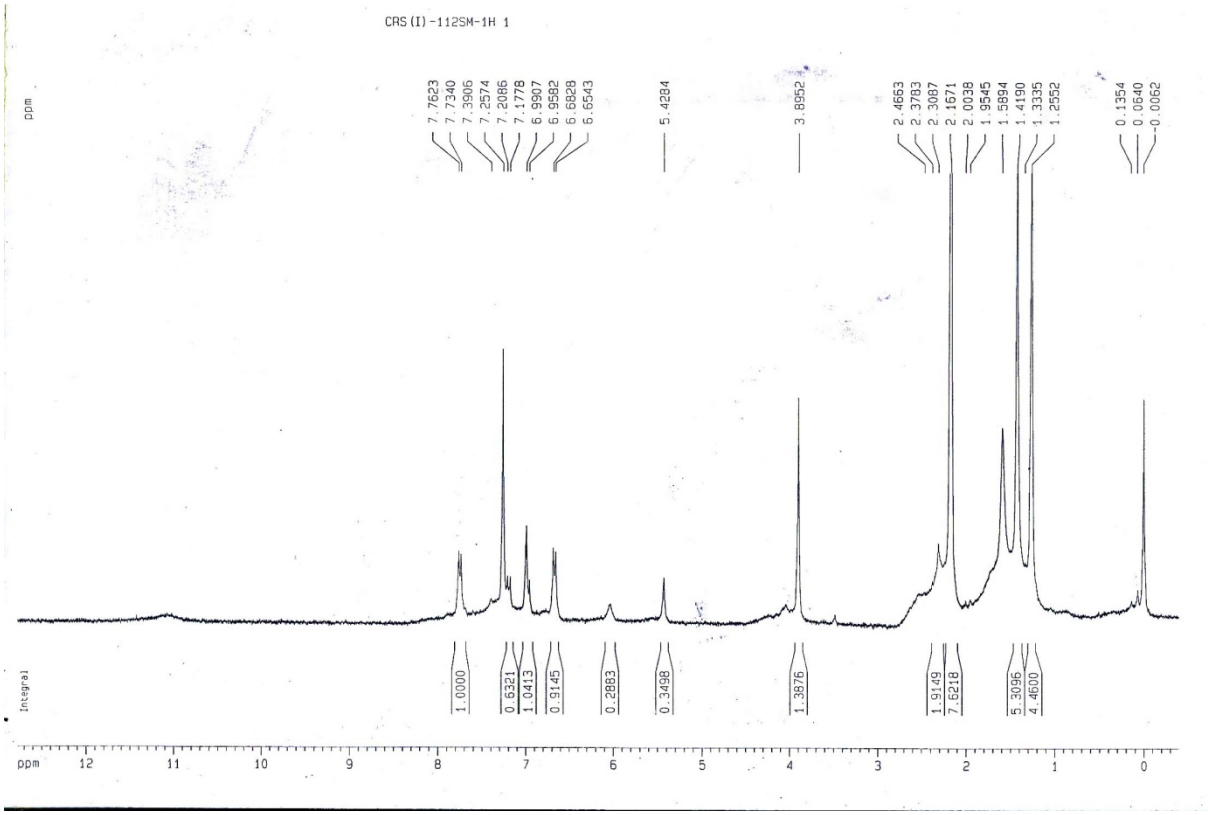

Fig. S3. ^{1}H NMR spectra of $\mathbf{LSO_{AP}H_2}$in $CDCl_3$

Table S2. Selected Experimental Bond parameters (Å) of $\mathbf{LSO_{AP}H_2}$

O(1)-C(1)	1.373(4)
N(1)- C(6)	1.419(4)
O(4)-C(26)	1.371(4)
N(2)-C(31)	1.431(4)
C(1)-C(2)	1.395(5)
C(2)-C(3)	1.388(5)
C(4)-C(3)	1.381(5)
C(5)-C(4)	1.387(5)
C(6)-C(5)	1.392(5)
C(1)-C(6)	1.387(5)
N(1)-C(15)	1.385(4)
S(2)-C(18)	1.746(3)
S(2)-O(2)	1.424(2)
N(3)-C(21)	1.391(4)
C(21)-C(22)	1.398(5)
C(22)-C(23)	1.345(5)
C(23)-C(24)	1.389(5)
N(5)-C(24)	1.303(5)
N(4)-N(5)	1.361(4)
N(4)-C(21)	1.310(4)

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