Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information

Copper nitroprusside analogue nanoparticles against melanoma: Detailed *in vitro* and *in vivo* investigation

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2. Experimental Procedures

2.1. Materials and Methods

2.1.1. Chemicals

Sodium nitroprusside (Na_2 [Fe $(CN)_5NO$), copper sulfate ($CuSO_4.5H_2O$), sodium azide, chlorpromazine, Wortmannin, methyl-β-cyclodextrin, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide reagent (MTT), 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA), ribonuclease (RNase), bovine serum albumin (BSA), propidium iodide, cisplatin, rhodamine B, RIPA buffer, PVDF membrane, TBHP (tert-butyl hydroperoxide), Dulbecco's Phosphate Buffer Saline (DPBS), fluoreshield DAPI and methanol were purchased from Sigma Aldrich chemicals, USA. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), acrylamide, bis-acrylamide, trypsin, streptomycin penicillin were obtained from Himedia. TritonX and Bradford reagent were purchased from Genetix Biotech Asia Pvt. Ltd. Monensin and amiloride were obtained from Alfa-Aesar, Ward Hill, Massachusetts, USA. Dimethyl sulfoxide (DMSO) was obtained from Rankem (India). Mitotracker green and Lysotracker green were purchased from Thermo Fischer Scientific. [Methyl 3H]-thymidine was procured from Perkin-Elmer Life and Analytical Sciences.

2.1.2. Antibodies

Primary antibodies: anti-Bcl2 Rabbit mAb (#3498), anti-caspase 3 Rabbit mAb (#9662), anti-STAT 3 Mouse mAb (#83541), anti-cytochrome c Rabbit mAb (#11940), anti-E-cadherin Mouse mAb (#14472), anti-GPX4 Rabbit mAb (#52455), anti-NRF-2 Rabbit mAb (#12721), anti-SOD-2 Rabbit mAb (# 13141), anti-Bax Rabbit mAb(#2772), anti-cytochrome c Rabbit mAb (#11940), anti-cleaved caspase 3 Rabbit mAb (#9664), anti-p53 Mouse mAb (#2524), anti-Akt Rabbit mAb(#4691), anti-PI3K Rabbit mAb(#4249), anti-m-TOR Rabbit mAb(#2983), anti-β-actin Rabbit mAb (#8584), anti-Ki-67 Rabbit mAb (#9129), anti-CD31/PECAM-1 Mouse mAb (#3528), corresponding secondary antibodies: anti-mouse IgG (HRP) (#7076) and anti-rabbit IgG (HRP) (#7074) were purchased from Cell Signaling Technologies Danvers, Massachusetts, USA. Goat anti-rabbit IgG [H+L] Alexa Flour 488/594 and protein ladder were purchased from Puregene. ECL western blotting substrate was obtained from BIO-RAD, Berkeley, California.

2.1.3. Kits

TUNEL assay kit and JC-1mitochondrial staining kit were purchased from Sigma Aldrich, Burlington, Massachusetts, USA. Nitrate/Nitrite Colorimetric Assay Kit and Annexin V-FITC with PI Apoptosis Detection kit was acquired from Cayman, Ann Arbor, Michigan, USA. Alexa Fluor® 488 Phalloidin (#8878) were purchased from Cell Signaling Technologies Danvers, Massachusetts, USA

2.1.4. Cell lines

Murine melanoma cell line (B16F10) was purchased from ATCC.

2.1.5. Animal Experiments

All the animal experiments were carried out in female C57BL/6J mice (5-8 weeks old) with the approval of institutional animal ethics committee of CSIR-IICT (Approval no; IICT-IAEC-078 dated 29-12-2021).

2.2. Synthesis of copper nitroprusside analogue nanoparticles

The copper nitroprusside analogue nanoparticles (abbreviated as CuNPANP) were synthesized as per our published report. [1]Briefly, the CuNPANP were synthesized by the interaction of copper sulfate ($CuSO_4$) and FDA approved sodium nitroprusside (SNP) at room temperature with a molar ratio of 1:1 according to equation 1.

$$Na_2 [Fe (CN)_5 NO] + CuSO_4 \rightarrow Cu [Fe(CN)_5NO] \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

Initially, 30 mL of SNP stock solution (10^{-1} M) was taken in a 100 mL beaker and kept over a magnetic stirrer to which 30 mL of $CuSO_4$ stock solution (10^{-1} M) was added drop wise. The brown colour of aqueous SNP solution was turned into green colour suspension instantly. The resulting solution was stirred for 2 h at RT. Later, the particles were allowed to settle down and supernatant was discarded. The solution was washed several times to remove the un-reacted reactants. Finally, pellet was collected in a 50 mL tube and lyophilized to obtain powdered nanoparticles. For detail characterization of the nanoparticles readers can refer to our earlier report. [1]

2.3. Characterization of CuNPANP

2.3.1. X-ray diffraction (XRD) spectroscopy

The crystalline nature of the CuNPANP were analyzed through X-ray diffraction (XRD) analysis using Bruker AXS D8 Advance Powder X-ray diffractometer (using CuKαλ=1.5406 A radiation) in range 2θ = 20º to 60º.[1]

2.3.2. UV-VIS and transmittance spectroscopy

The absorbance and transmittance of the as-synthesized CuNPANP were measured using Hitachi U-2910 UV-VIS-NIR spectrophotometer. [1]

2.3.3. Transmission electron microscopy (TEM) analysis

The accurate size and morphology CuNPANP were analyzed using Tecnai G2 F30 S-Twin Microscope. CuNPANP were dissolved in MilliQ and drop casted on carbon coated copper grid for TEM analysis. [1]

2.3.3. Scanning electron microscopy (SEM) analysis

Surface topography (size and shape) of the synthesized CuNPANP were determined by SEM analysis using Hitachi S-3000 N, Japan.[1]

2.3.4. X-ray photo electron spectroscopy (XPS)

In order to know the presence of elements, functional groups and their oxidation state in CuNPANP, we have performed the XPS analysis [KRATOS AXIS 165 with a dual anode (Mg and Al) apparatus using the Mg Kα anode]. The CuNPANP pellets were drop casted on the glass plate, dried and submitted for the XPS analysis. [2]

2.3.5. Stability studies

The stability study of CuNPANP has been carried out in commercially available PBS: C-PBS, freshly prepared phosphate buffer under different pH conditions (P-buffer-pH5.8 and P-buffer-pH7.4) in a time dependent manner (2 h to 96 h) to analyze the size (hydrodynamic diameter) and charge (zeta potential) of the nanomaterials using DLS instrument Litesizer 500 particle analyzer (Anton Par, 2000). In this study, 100 µL (100 µg) of CuNPANP suspension was mixed with 900 µL of DMEM or FBS for each time point to know the particle size and charge.[1]

2.4. *In vitro* assays

The mouse specific melanoma cells (B16F10) cells were cultured in complete media (DMEM as per the manufacturer's instructions) supplemented with 1% & 10 % antibiotic-anti-mycotic

solution and FBS respectively. The cells were cultured by maintaining 5% CO_2 at 37º C in a humidified incubator.

2.4.1. [methyl 3H]-Thymidine incorporation assay

The inhibitory effect of CuNPANP on B16F10 cells was evaluated by performing [methyl 3H]-thymidine incorporation assay according to our earlier report. [2] Briefly, 40×10^3 B16F10 cells were seeded/well in a 24 well plate and maintained at 37ºC, 5% CO_2 in a humidified incubator. After reaching 70-80% confluency, cells were treated with CuNPANP in a concentration dependent manner (1-20μg/mL) for 24h. The cells were washed with PBS and incubated with DMEM media containing [methyl 3H]-Thymidine (1μC) for 4h. Following incubation, cells were washed with PBS and incubated with 0.1% SDS (100μL) for 1h for cell lysis. Then, the cells were scrapped and the cell lysates were transferred into a 96 well plate followed by addition of scintillating oil (1:1). The radioactivity was measured as counts per minutes using PerkinElmer MicroBeta2 system in a scintillation chamber.

2.4.2. Cellular uptake study through ICPOES analysis

To know the uptake pathway of the CuNPANP, B16F10 cells (15×10^5) were seeded in 100 mm cell culture dish under appropriate conditions at 37°C, 5 % CO_2 in a humidified incubator. Upon reaching 80–90 % confluency, the cells were washed with DPBS and pre-incubated with inhibitors of different pathway (i) clathrin dependent; chlorpromazine hydrochloride, ii) caveolae dependent: methyl-β-cyclodextrin, iii) macropinocytosis: amiloride, iv) energy dependent: sodium azide v) phagocytosis; Wortmannin vi) clathrin and caveolae independent: monensin for specific time period. After this, cells were treated with CuNPANP (10 μg/mL) for 6 h then processed (as described above) and digested with 70 % HNO_3 before submission for ICPOES analysis. Similarly, B16F10 cells (15×10^5) were seeded in a 100 mm cell culture dish and treated with CuNPANP for 6 h as control.[1]

2.4.3. Cell cycle assay

To evaluate the effect of CuNPANP on different phases of B16F10 cells, cell cycle assay was performed as per our previously published reports. [1, 3] Briefly, 3×10^5 B16F10 cells were seeded into a 60 mm cell culture dish and grown until 70-80% confluency. On the next day, cells were treated with CuNPANP (10μg/mL) and cisplatin (24μM, positive control) into the respective

plates for 18h. The cells were fixed using 70% ethanol and stored at -20ºC overnight. Then, the cells were processed using PI staining and cell cycle assay was carried out using FACS Canto II, Becton Dickinson, San Jose, CA, U.S.

2.4.4. Apoptosis assay

The apoptotic nature of CuNPANP towards B16F10 cells was evaluated through flow cytometry analysis using Annexin-V FITC staining as per our earlier published report. [1] Briefly, B16F10 cells were seeded at a concentration of 3×10^5 cells in a 60 mm cell culture dish. The cells were maintained until 80-90% confluency and then incubated with CuNPANP (10µg/mL) and cisplatin (positive control) for 18h. Then, AnnexinV –FITC/PI staining was performed to process the cells as per the manufacturer's instruction. The labeled cells from different group (UT, CuNPANP and cisplatin) were analyzed by FACS Canto II, Becton Dickinson, San Jose, CA, U.S.

2.4.5. Detection of ROS

The intracellular ROS generation potential of CuNPANP was evaluated in B16F10 cells using H_2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate), DHE (dihydroethidium) and DAF-2A reagent according to our earlier reports. [1, 2, 4] Briefly, B16F10 cells (8×10^3 cells/well) were cultured in a 96 well plate for 24h at 37ºC, 5% CO_2. On reaching 70-80% cell confluency, CuNPANP (10 µg/mL) were added to respective wells and incubated for 4h. Here, tertiary-butyl hydroperoxide (TBHP; 100µM) was used as positive control. Later, the cells were washed with DPBS and incubated with DCFDA (10µM), DHE (10µM) and DAF-2A reagent for 35 min. Then, images were taken using Nikon Eclipse confocal microscope (TE2000-E, 20X, DCFDA; green fluorescence λex: 480 nm; λem: 535 nm, DHE; λex: 480-520nm; λem: 561 nm, DAF-2A; λex: 480 nm; λem: 535 nm) at 20 × magnification.

2.4.6. Mitochondrial JC-1staining

To evaluate the mitochondrial membrane potential in B16F10 cells upon CuNPANP treatment, JC-1 staining was performed as per our earlier reports. [1] To this, B16F10cells (8×10^3cells/well) were cultured in a 96 well plate at 37ºC, 5% CO_2 under appropriate cell culture conditions. After 24h, B16F10 cells were treated with CuNPANP (10µg/mL) for 18h. Valinomycin was used as

positive control. The cells were stained with JC-1 staining mixture for 20 min and observed under confocal microscopy (λ_{ex}: 490; λ_{em}: 525 nm for monomeric JC-1 and λ_{ex}: 540; λ_{em}: 590 nm for aggregated JC-1) at 20 × magnification.

2.4.7. Immunocytochemistry analysis

The anti-proliferative nature of CuNPANP was analyzed in B16F10 cells through immunofluorescence (cytochrome-c, Ki-67, E-cadherin) studies as per our published report. [2] To this, B16F10 cells were seeded on coverslip with a density of 1×10^5cells/well and grown up to 60-70% confluency. Next day, cells were treated with CuNPANP (10µg/mL) for 18h. Cisplatin was used as positive control. Then, the cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min. Again the cells were washed with PBS followed by permeabilization with 0.2% triton X (5 min) and blocking with 3% BSA (1h). After this, cells were washed with PBS and incubated with anti-Ki-67, anti-cytochrome c and anti-E-cadherin primary antibodies for overnight. Next day, cells were washed with TBST and incubated with Alexa Fluor 488 (for Ki-67) and Alexa Fluor 594 (for cytochrome c and E-cadherin) conjugated secondary antibody for 1h under dark condition. Then the cells were washed with TBST and mounted with fluorosheild DAPI. The images were captured using Nikon TiEclipse confocal microscope at 60 × magnification.

2.5. *In vivo* anti-cancer studies

2.5.1. Immunohistochemistry analysis in melanoma tissue sections

The anti-proliferative effect of CuNPANP was analyzed in the melanoma tissue sections through immunofluorescence studies as per our published reports. [1, 4] To this, the tumor tissue containing slides were dipped in xylene solution for 10 min (3 times). Further, the sections were processed by dipping with decreasing concentration of ethanol (100, 90, 70 %) followed by lukewarm heating with sodium citrate buffer (10 mM: pH=6) for 10 min (antigen retrieval). Then, the tissue sections were blocked with 5% BSA for 1 h followed by overnight primary antibody incubation (anti-Ki-67, anti-CD31/PECAM-1, anti-E-cadherin). Then, the slides were washed with TBST for 3 times followed by secondary antibody (Alexa Fluor 488) incubation for 1h under dark condition. Then, the slides were washed with TBST and mounted with fluorosheild DAPI. The

images were captured using Nikon TiEclipse confocal microscope (TE 2000-E, Japan) at 60 × magnification.

2.5.2. TUNEL assay

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL assay) was performed to analyze the presence of fragmented DNA in the tumor tissue sections upon CuNPANP (1 & -5mgkg-1) treatment as per our published reports. [1, 4] To this, the tumor tissue containing slides were dipped with xylene for 10 min each (3 times). Then, the slides were dipped with decreasing concentration of ethanol (100, -90 & -70 %) followed antigen retrieval through lukewarm heating with sodium citrate buffer (10 min). Then, the slides were washed with TBST followed by blocking with 5% BSA for 1 h. Further, the slides were incubated in dark with TUNEL reaction mixture for 1 h. Then, the slides were washed with TBST and mounted with fluorosheild DAPI. The images were captured using Nikon Eclipse confocal microscope (TE 2000-E, Japan) at 60 × magnification.

2.5.3. Western Blot analysis

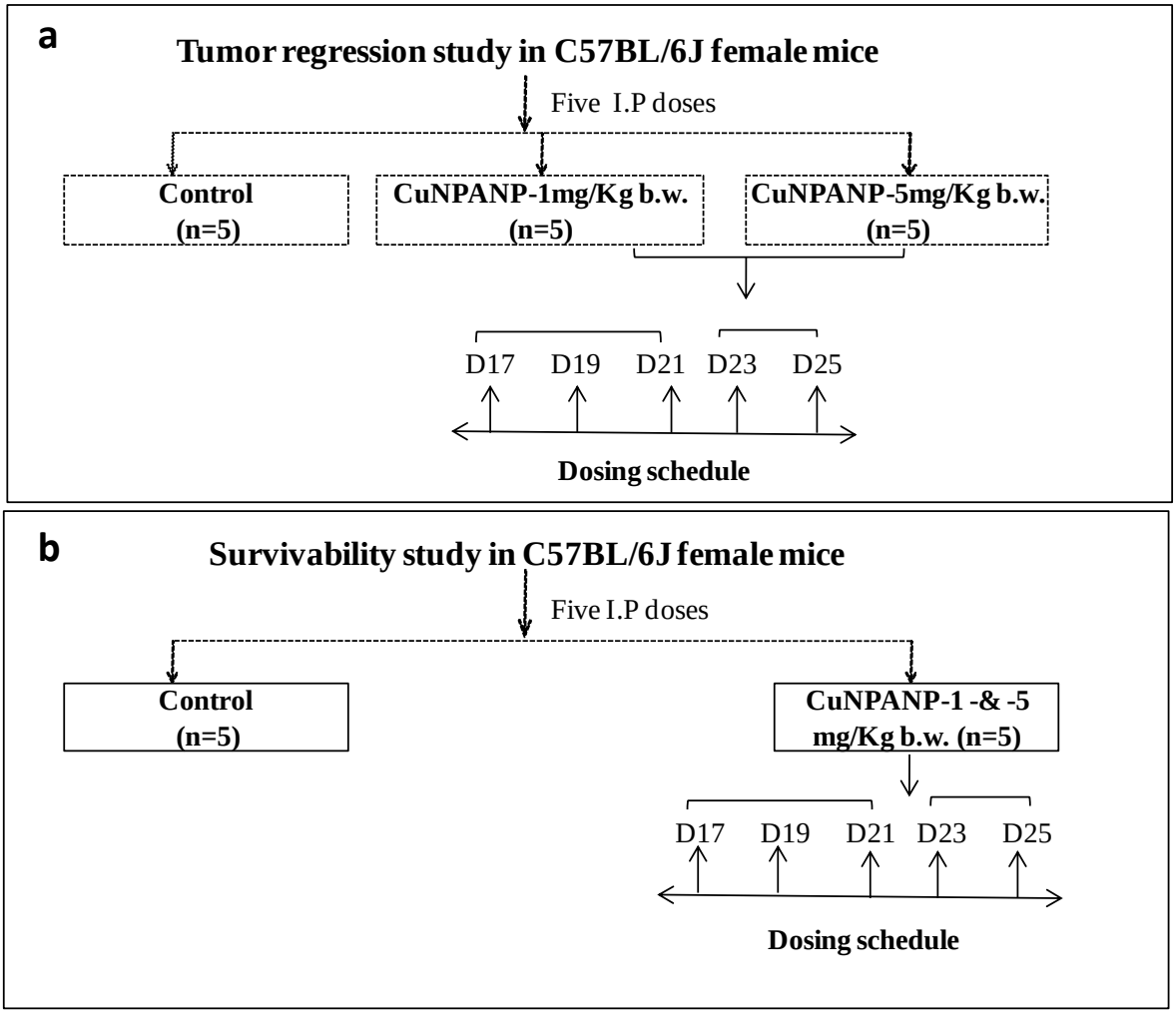
The molecular mechanism behind the anti-cancer activities of CuNPANP towards melanoma was evaluated through western blot analysis in both cell (B16F10) and tissue lysate as per our earlier published reports.[1,2,4]

Preparation of cell lysate: To this, 3×10^5 B16F10 cells were seeded in a 60 mm sterile dish until 70-80% confluency. On the next day, cells were treated with CuNPANP and cisplatin (positive control) for 18h. Then, the cells were scrapped with RIPA: PIC (1000:10) and centrifuged at 12000 rpm at 4°C for 20 min in order to obtain the protein samples.

Preparation of tissue lysate: The tumor samples were collected in PBS after sacrificing the mice from different treatment (1 & -5mgkg-1) and untreated groups. From the tumor samples, approximately 50 mg of tissue was taken from different groups. Then the tissues samples were homogenized using a tissue homogenizer and incubated with RIPA: PIC (1000:10µL) for 45min. After digestion, the tissue samples were centrifuged at 12,000 rpm for 30min in order to collect the protein samples.

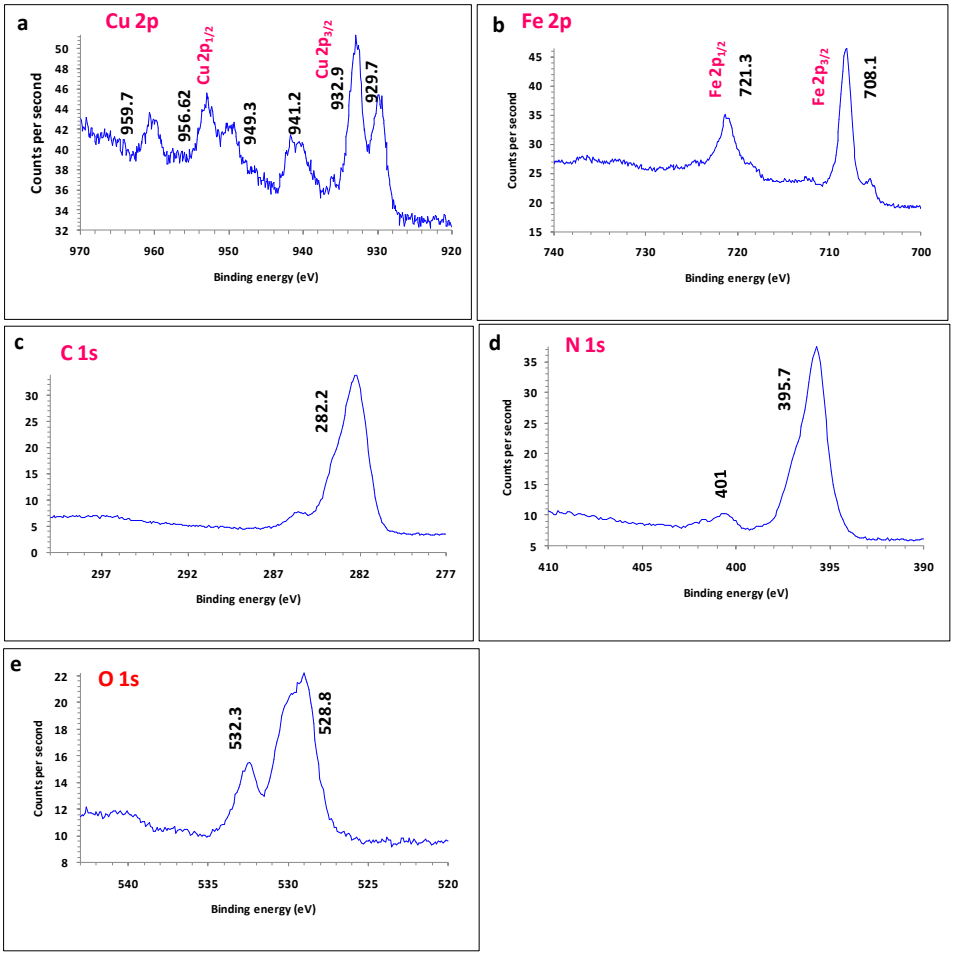
Initially, Bradford assay was performed in order to calculate the total concentration of proteins in both cell and tissue lysates. The protein samples (both for cell and tissue; 50 μg) were separated on 15% SDS-PAGE gel. Then, the non-specific sites were blocked using 3% BSA followed by TBST washing and overnight primary antibody incubation (anti-Bcl2, anti-Bax, anti-caspase 3, anti-STAT 3, anti-GPX4, anti-NRF2, anti-SOD2, anti-cytochrome c, anti-cleaved caspase 3, anti-E-cadherin, anti-p53, anti-Akt, anti-PI3K, anti-mTOR and anti-β-actin). Further, the membranes were washed (with TBST) and incubated with secondary antibody for 1h and blot was developed using ECL substrate in the chemidoc (BIORAD) instrument.

Scheme S1



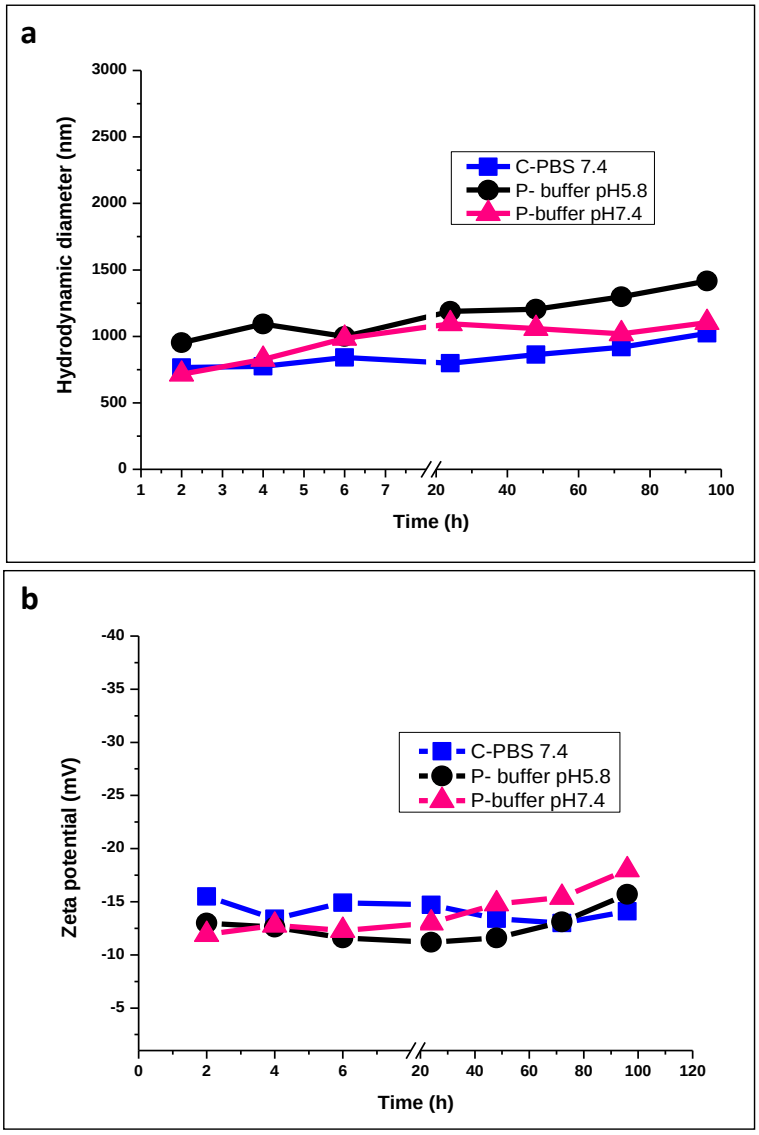
Scheme S1. Dosing schedule for orthotopic melanoma mouse model a) tumor regression and b) survivability study.

SI Fig.1



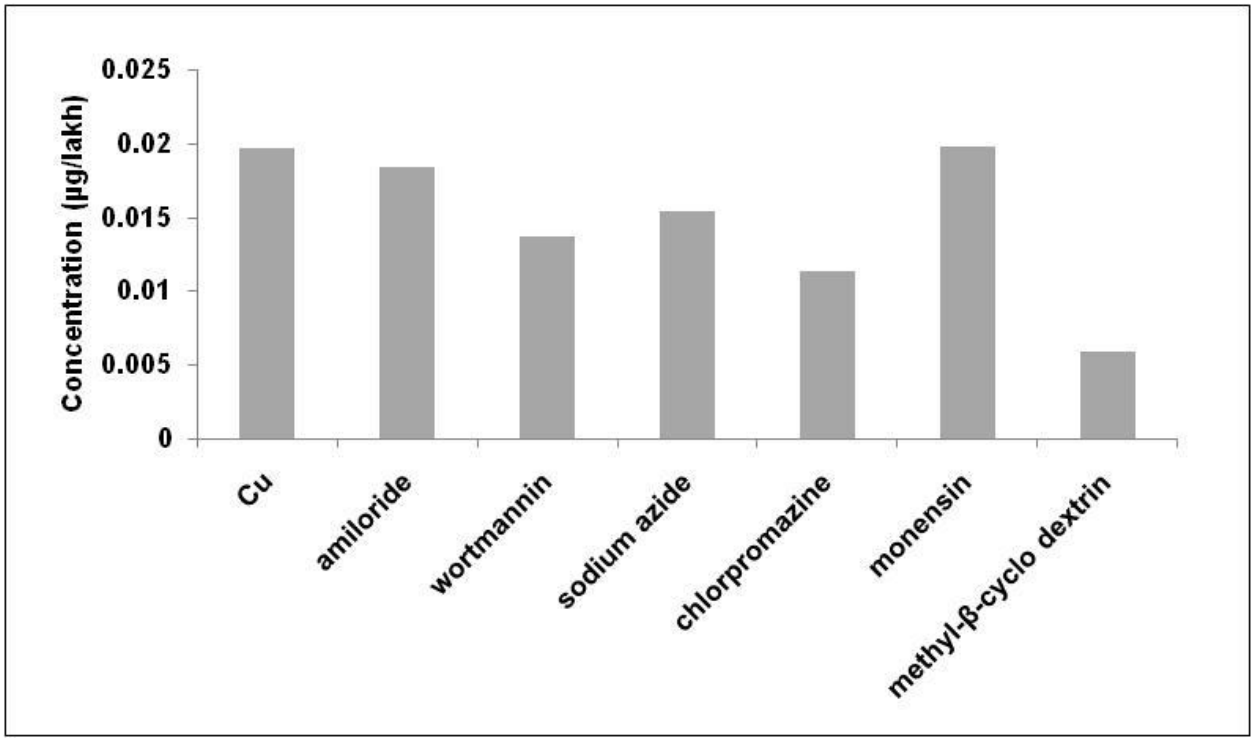
SI Fig.1 High-resolution XPS spectra of a) Cu 2p, b) Fe 2p, c) C 1s, d) N 1s e) O 1s.

SIFig.2



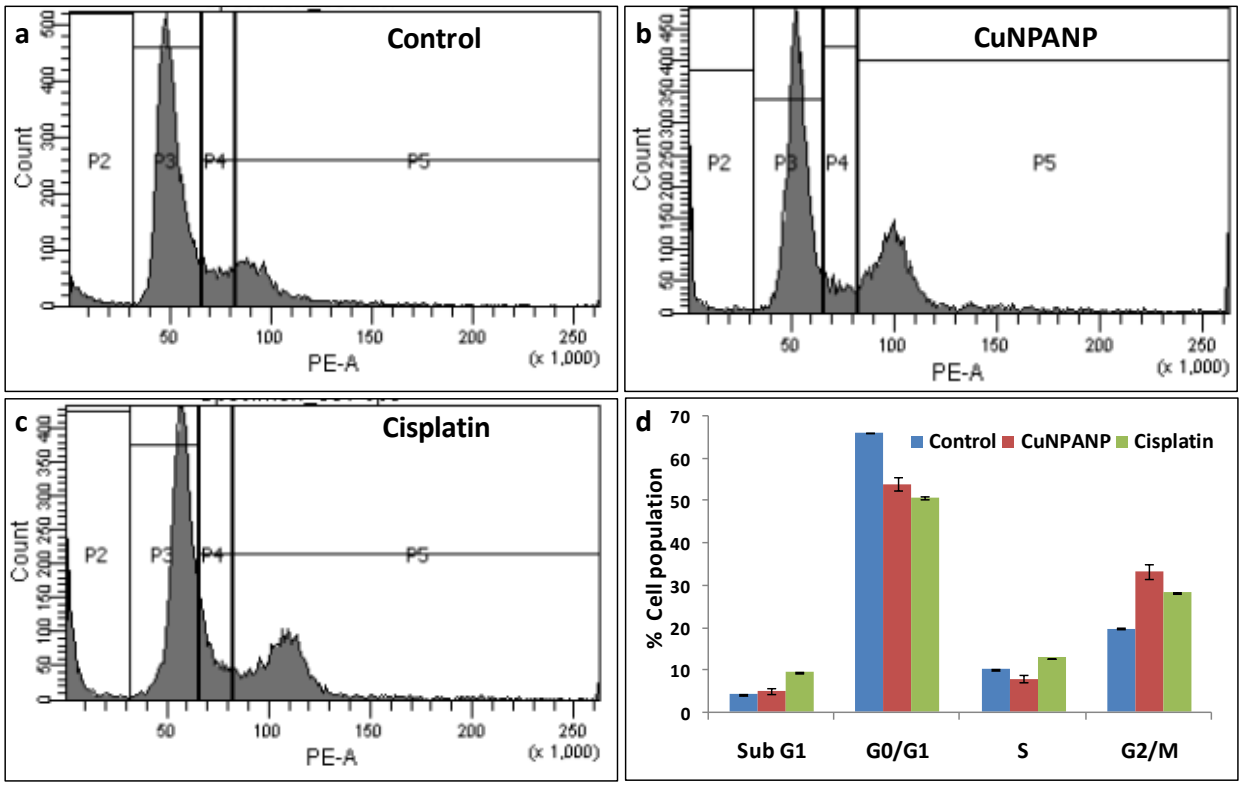
SI Fig.2 a-b) DLS analysis of CuNPANP suspension in different buffers or saline (commercially available PBS: C-PBS, prepared phosphate buffer under different pH conditions (P-buffer-pH5.8 and P-buffer-pH7.4) at different time points (2,-4,-24,-48,-72 & 96h) exhibiting no significant changes of hydrodynamic diameter and zeta potential value.

SI Fig. 3



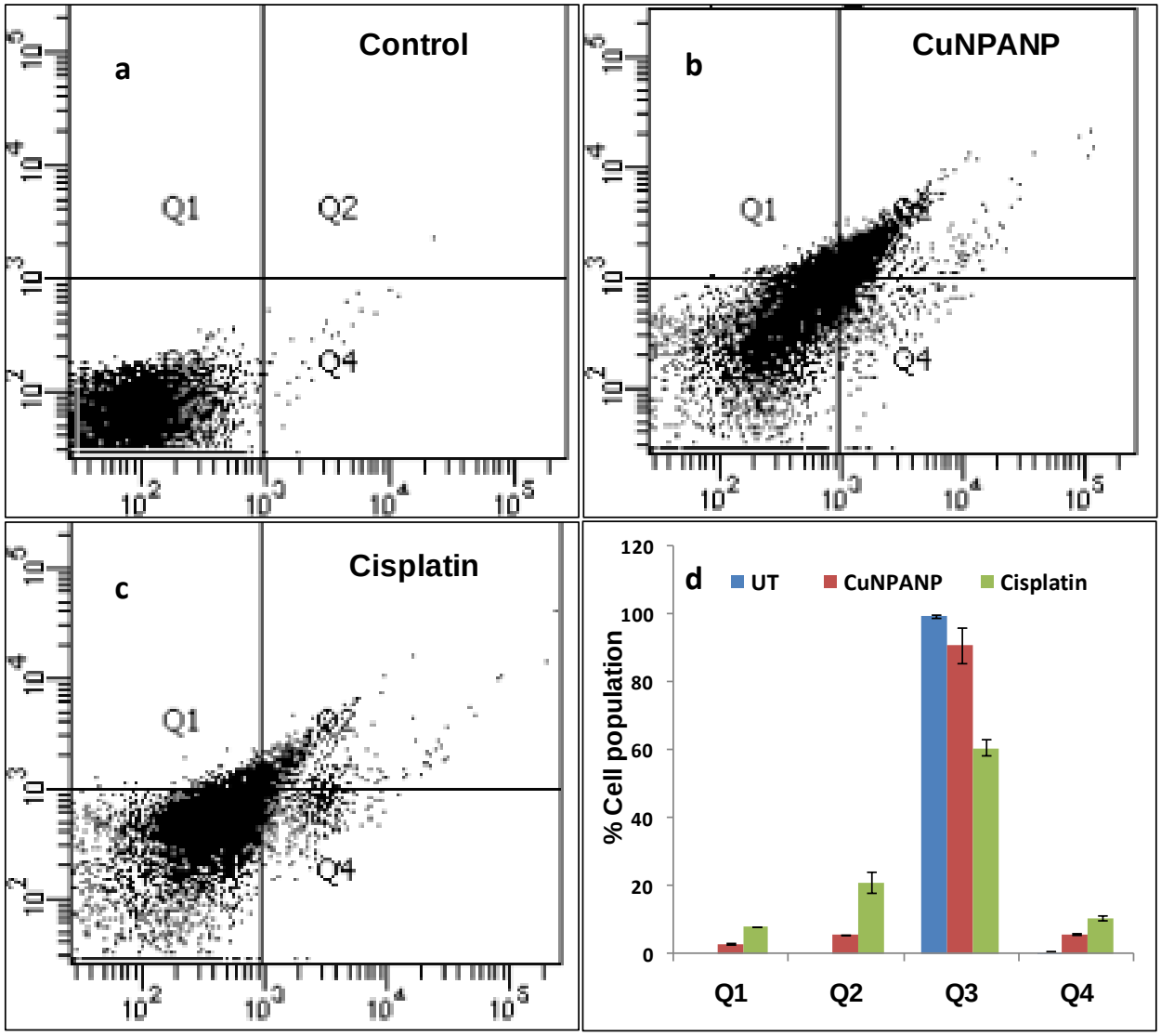
SI Fig. 3 Cellular internalization pathway study of CuNPANP in B16F10 cells by incubating the cells with inhibitors of different pathway (clathrin dependent: chlorpromazine hydrochloride; caveolae dependent: methyl-β-cyclodextrin; macropinocytosis: amiloride; energy dependent: sodium azide; phagocytosis: Wortmannin; clathrin and caveolae independent: monensin) for specific time period followed by CuNPANP treatment for 6h. Data are represented as µg/cell × 10^5. These are pooled data from various experiments therefore statistical significance was not provided.

SI Fig. 4



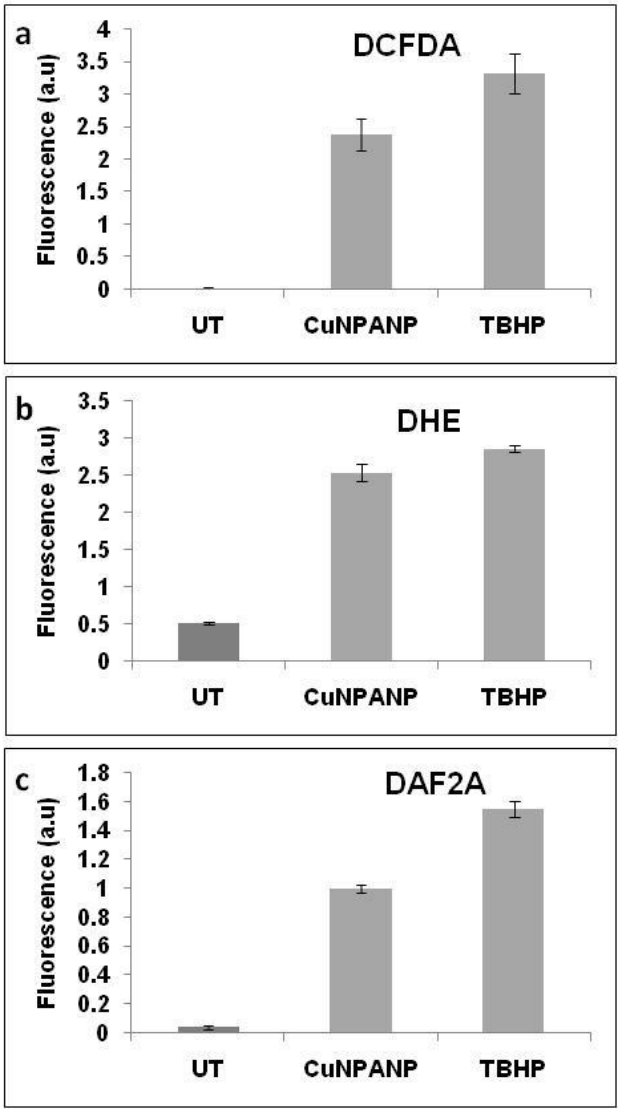
SI Fig. 4 Cell cycle assay in B16F10 cells using flow cytometry where a) control b) CuNPANP c) cisplatin d) graphical presentation of cell cycle phases. CuNPANP show significant cell cycle arrest in G2/M phase in B16F10 cells as compared to untreated control. Cisplatin is used as positive control. Three independent experiments are performed and represented as mean ± SD.

SI Fig. 5



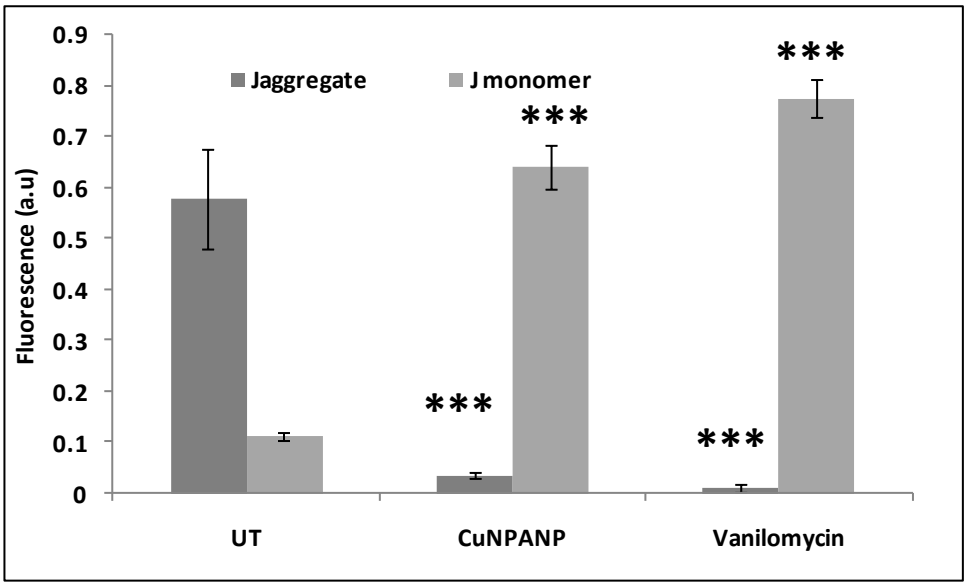
SI Fig. 5 Apoptosis analysis in B16F10 cells using Annexin-V-FITC staining where a) control b) CuNPANP c) cisplatin d) graphical presentation of apoptosis assay. CuNPANP exhibhit both early and late apoptotic cell death. Cisplatin is used as positive control. Three independent experiments are performed and represented as mean ± SD.

SI Fig. 6



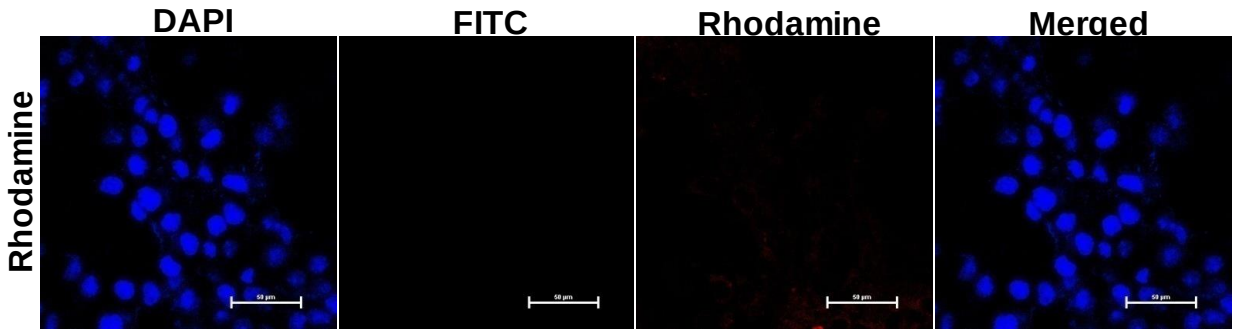
SI Fig. 6 The quantification of ROS (reactive oxygen species) generation in B16F10 cells using a) DCFDA b) DHE c) DAF2A reagent. The CuNPANP induces more ROS in B16F10 cells as compared to the untreated control. Quantification was performed from 3 independent images and represented as mean ± SD.

SI Fig. 7



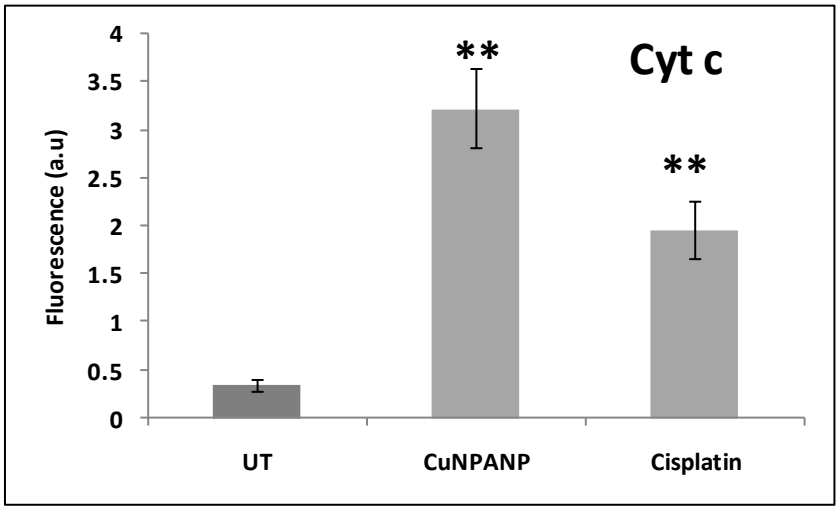
SI Fig. 7 The quantification of JC-1 staining in B16F10 cells. The CuNPANP treated cells showed mitochondrial membrane depolarization as compared to the untreated control. Vanilimycin is used as positive control. Quantification was performed from 3 independent images and represented as mean ± SD.

SI Fig. 8



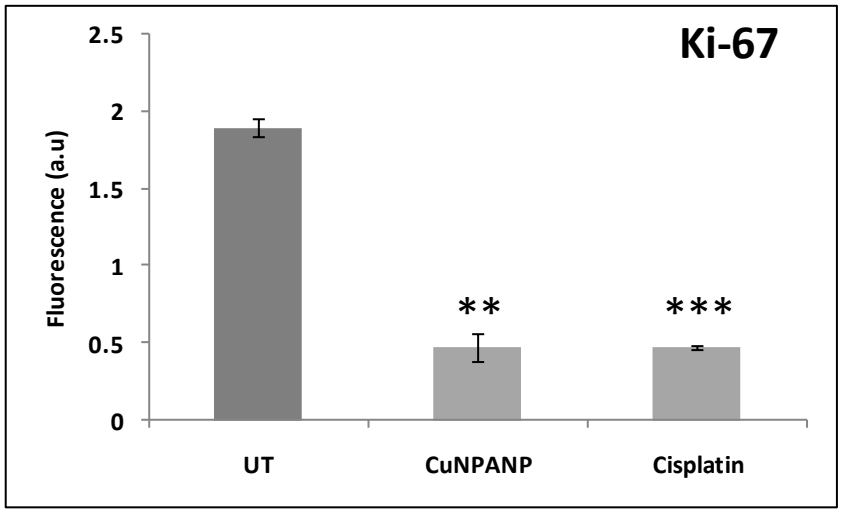
SI Fig.8 Representative images of cellular localization of rhodamine in B16F10 cells where Column I: DAPI; II:FITC; III:Rho-red; IV: merged.

SI Fig. 9



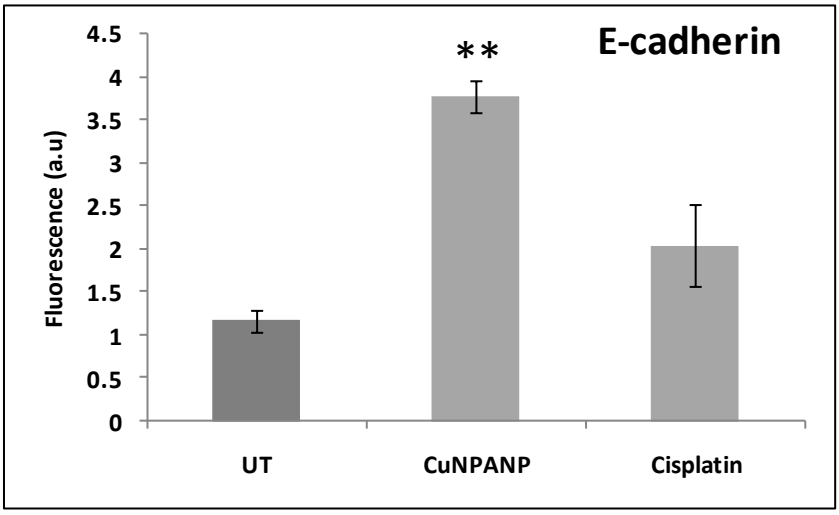
SI Fig. 9 The quantification of the immunofluorescence images indicates higher expression of cytochrome c in CuNPANP treated group as compared to the untreated control. Cisplatin is used as positive control. Quantification was performed from 3 independent images and represented as mean ± SD.

SI Fig. 10



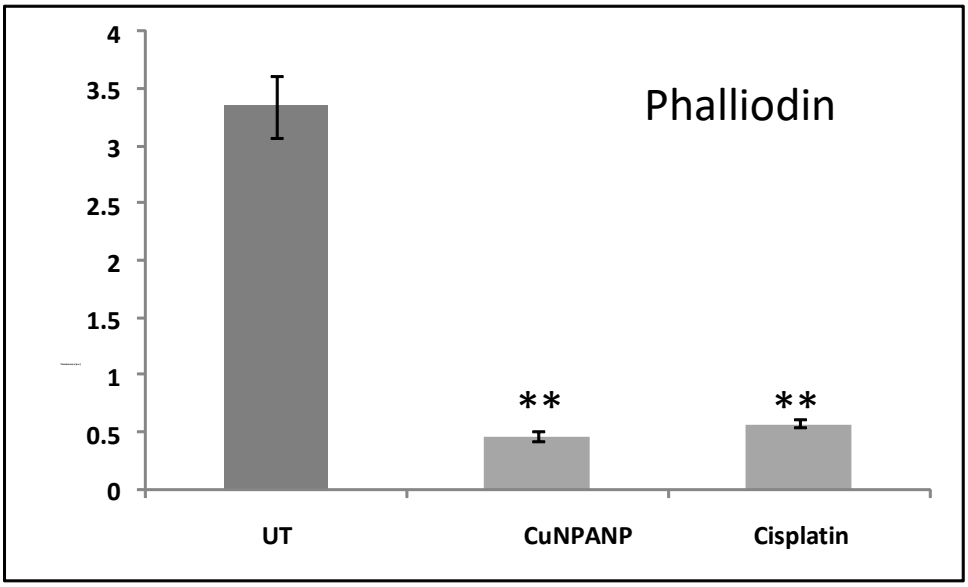
SI Fig. 10 The quantification of the immunofluorescence images of Ki-67 staining inB16F10 cells. The CuNPANP treated group showed lower expression of KI-67 as compared to the untreated control. Cisplatin is used as positive control. Quantification was performed from 3 independent images and represented as mean ± SD.

SI Fig. 11



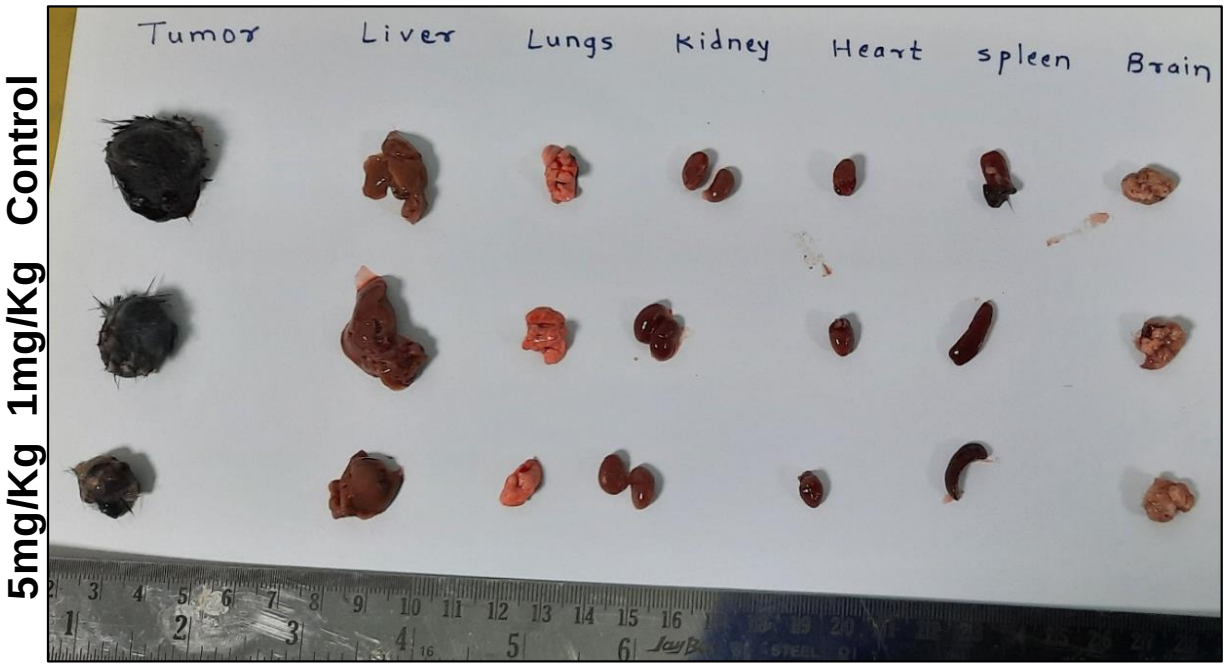
SI Fig. 11 The quantification of the immunofluorescence images indicates higher expression of E-cadherin in CuNPANP treated group as compared to the untreated control. Cisplatin is used as positive control. Quantification was performed from 3 independent images and represented as mean ± SD.

SI Fig. 12



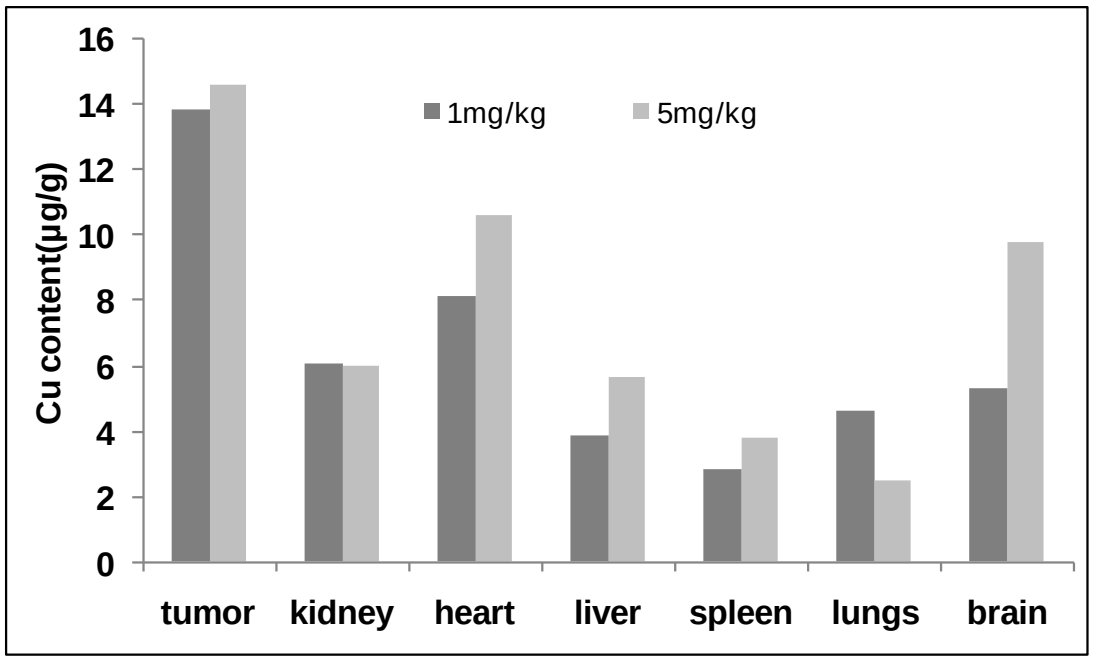
SI Fig. 12 The quantification of the phalloidin staining in B16F10 cells. There is lower expression of cytoskeletal components in the CuNPANP treated group as compared to the untreated control. Cisplatin is used as positive control. Quantification was performed from 3 independent images and represented as mean ± SD.

SI Fig. 13



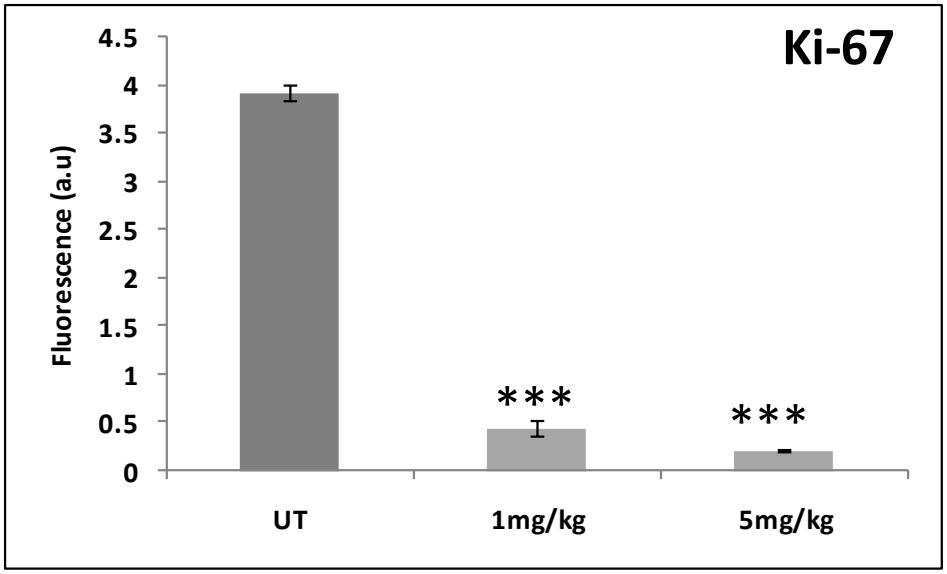
SI Fig. 13 Image of tumor and other organs (liver, lungs, kidney, heart, spleen and brain) from control untreated and CuNPANP treatment (1 -& -5mgkg-1) group.

SI Fig. 14



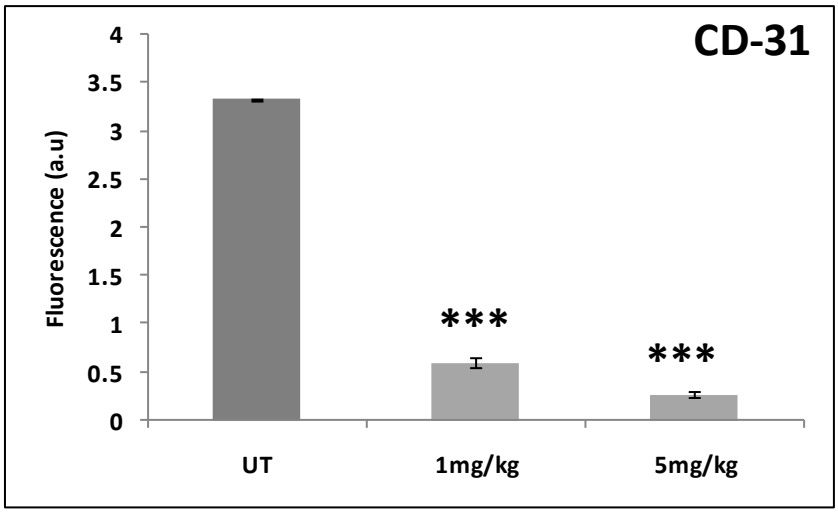
SI Fig. 14 Biodistribution of copper (Cu) in tumors and major organs treated with CuNPANP. These are pooled data from various experiments therefore statistical significance was not provided.

SI Fig. 15



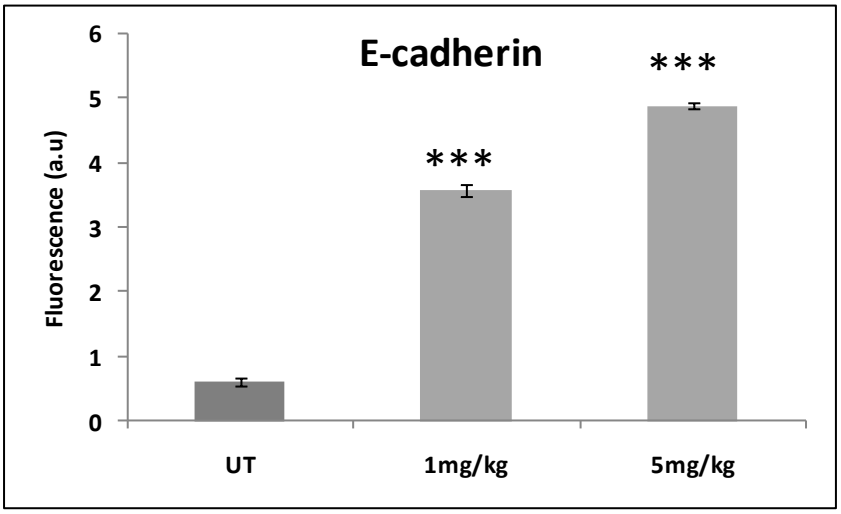
SI Fig 15. The quantification of the immunofluorescence images indicates higher expression of Ki-67 in untreated group as compared to CuNPANP ($1mgkg^{-1}$& $5mgkg^{-1}$) treated group. Quantification was performed from 3 independent images and represented as mean ± SD.

SI Fig. 16



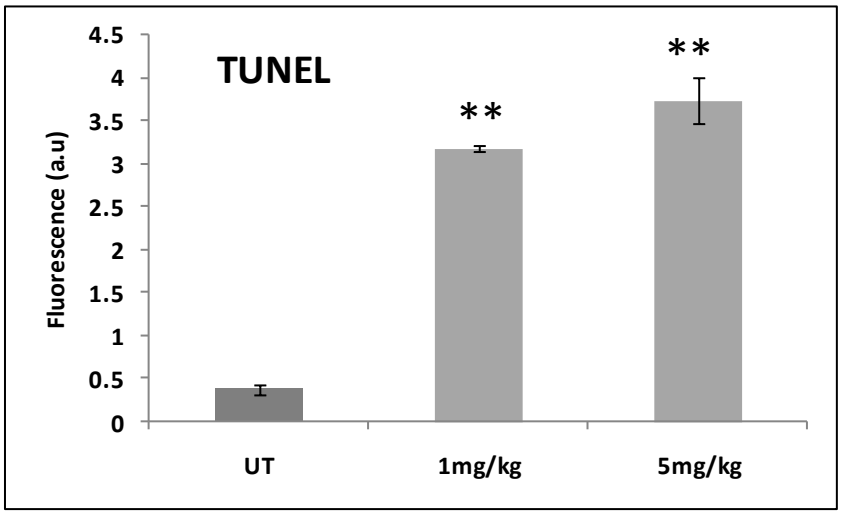
SI Fig.16 The quantification of the immunofluorescence images indicates higher expression of CD31/ PECAM-1 in untreated group as compared to CuNPANP ($1mgkg^{-1}$& $5mgkg^{-1}$) treated group. Quantification was performed from 3 independent images and represented as mean ± SD.

SI Fig. 17



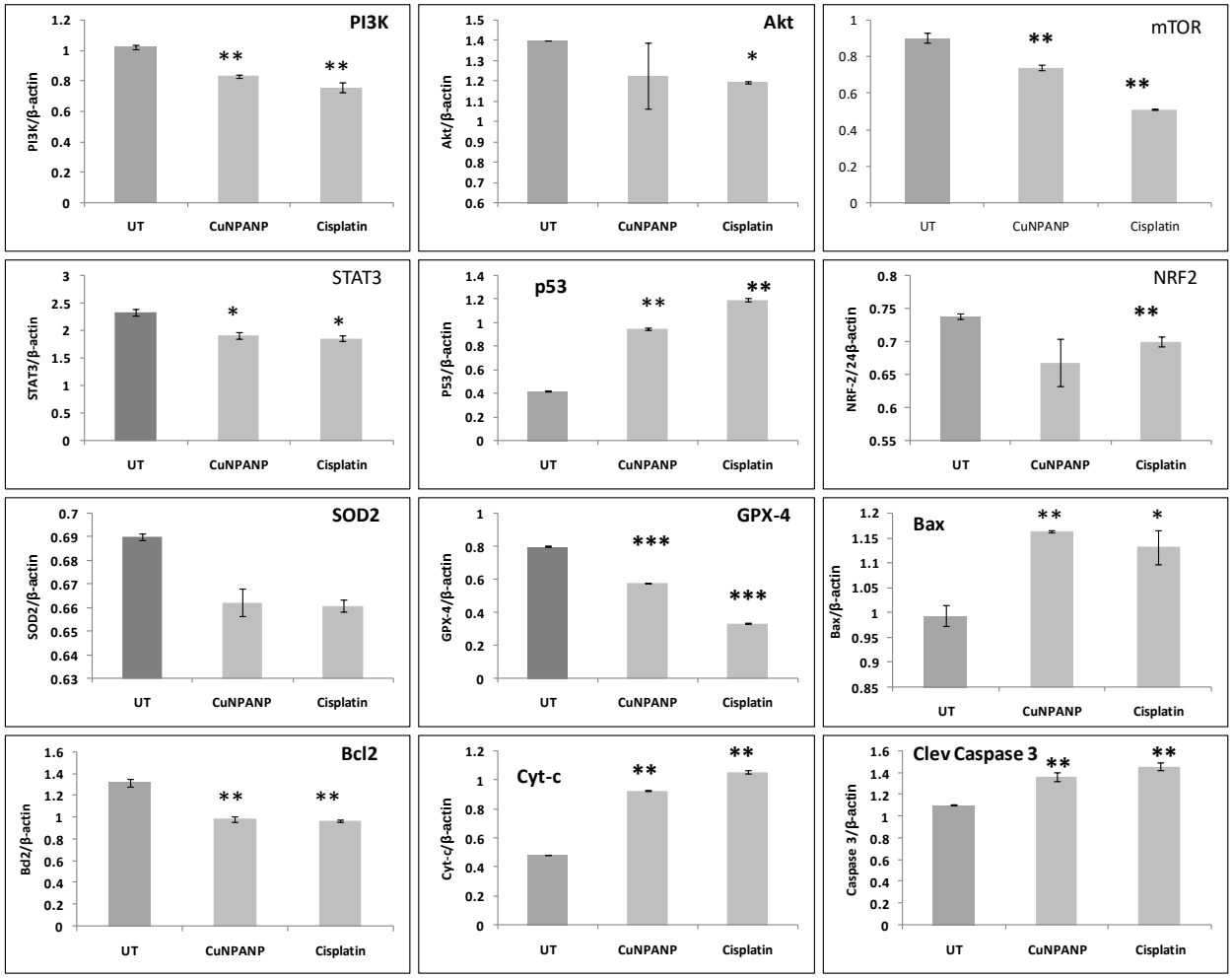
SI Fig.17 The quantification of the immunofluorescence images indicates higher expression of E-cadherin in CuNPANP ($1mgkg^{-1}$& $5mgkg^{-1}$) treated group as compared to the untreated control. Quantification was performed from 3 independent images and represented as mean ± SD.

SI Fig. 18



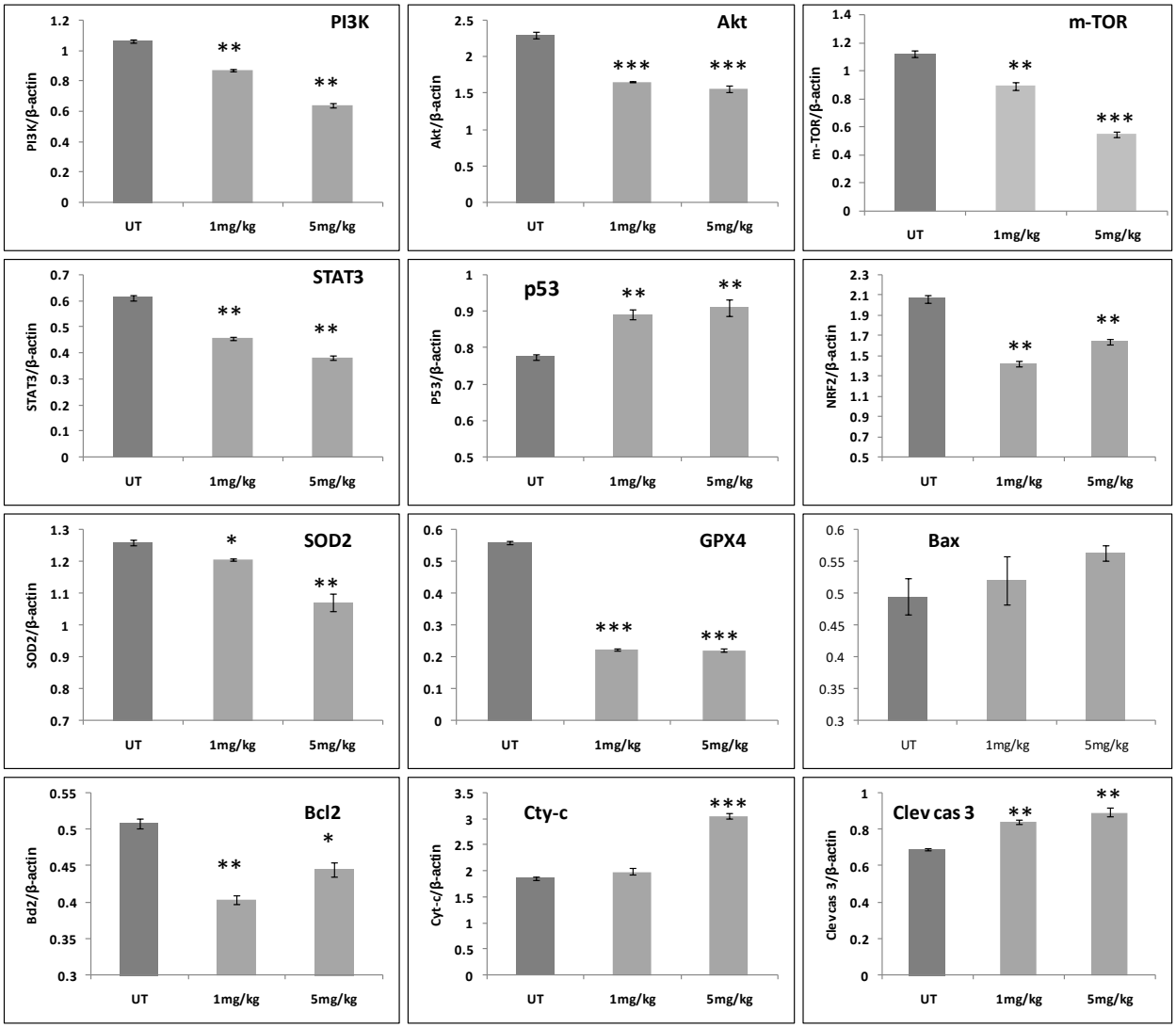
SI Fig.18 The quantification of the immunofluorescence images of TUNEL assay indicates higher DNA fragmentation in CuNPANP ($1mgkg^{-1}$& $5mgkg^{-1}$) treated group as compared to untreated group. Quantification was performed from 3 independent images and represented as mean ± SD.

SI Fig. 19a



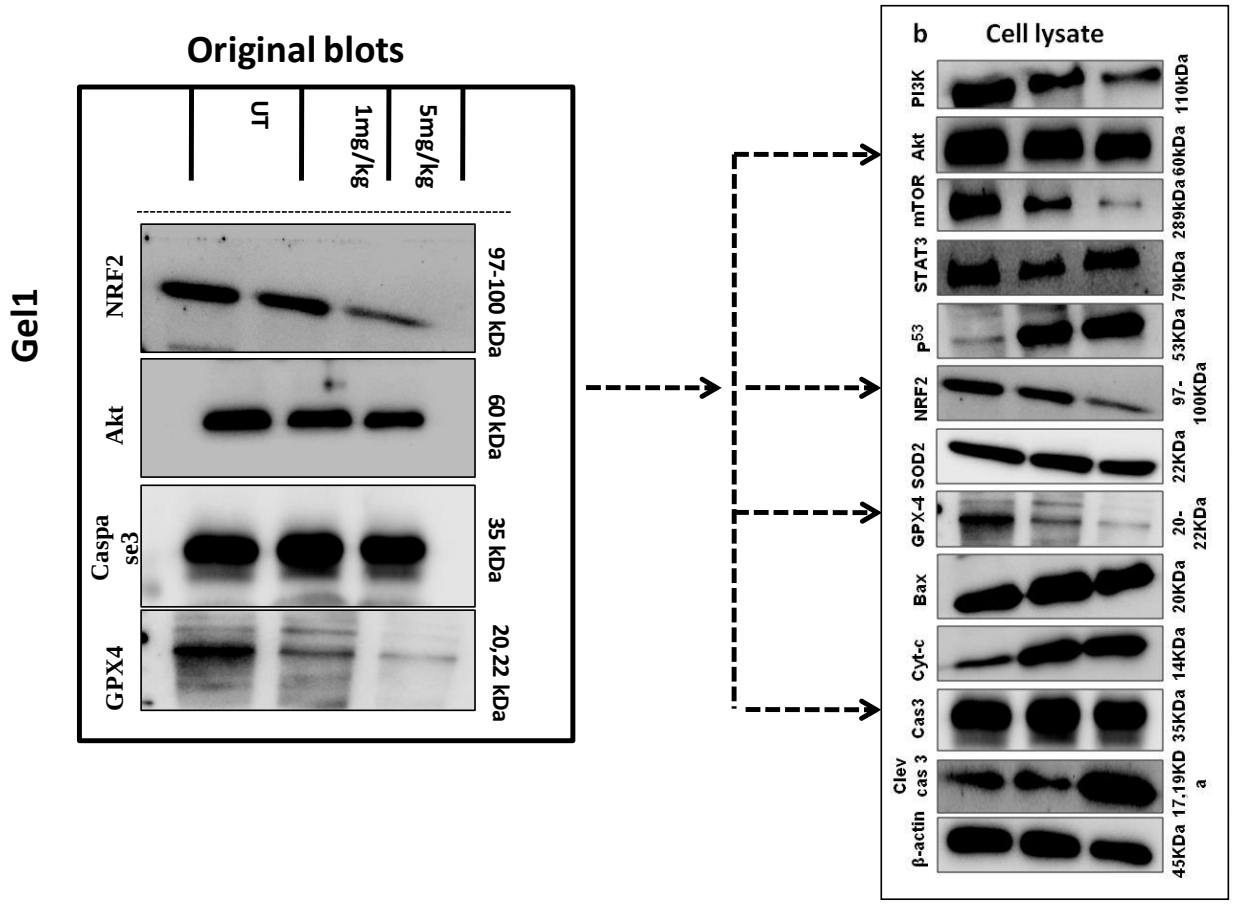
SI Fig. 19a The quantification data of expression of proteins (Western blot in cell lysate) such as PI3K, Akt, , m-TOR, STAT3, p53, NRF-2, SOD2, GPX4, Bax, Bcl2, cytochrome c, cleaved caspase 3, using image J analysis. The data collected for three times from the same image and presented as mean ± SD.

SI Fig. 19b



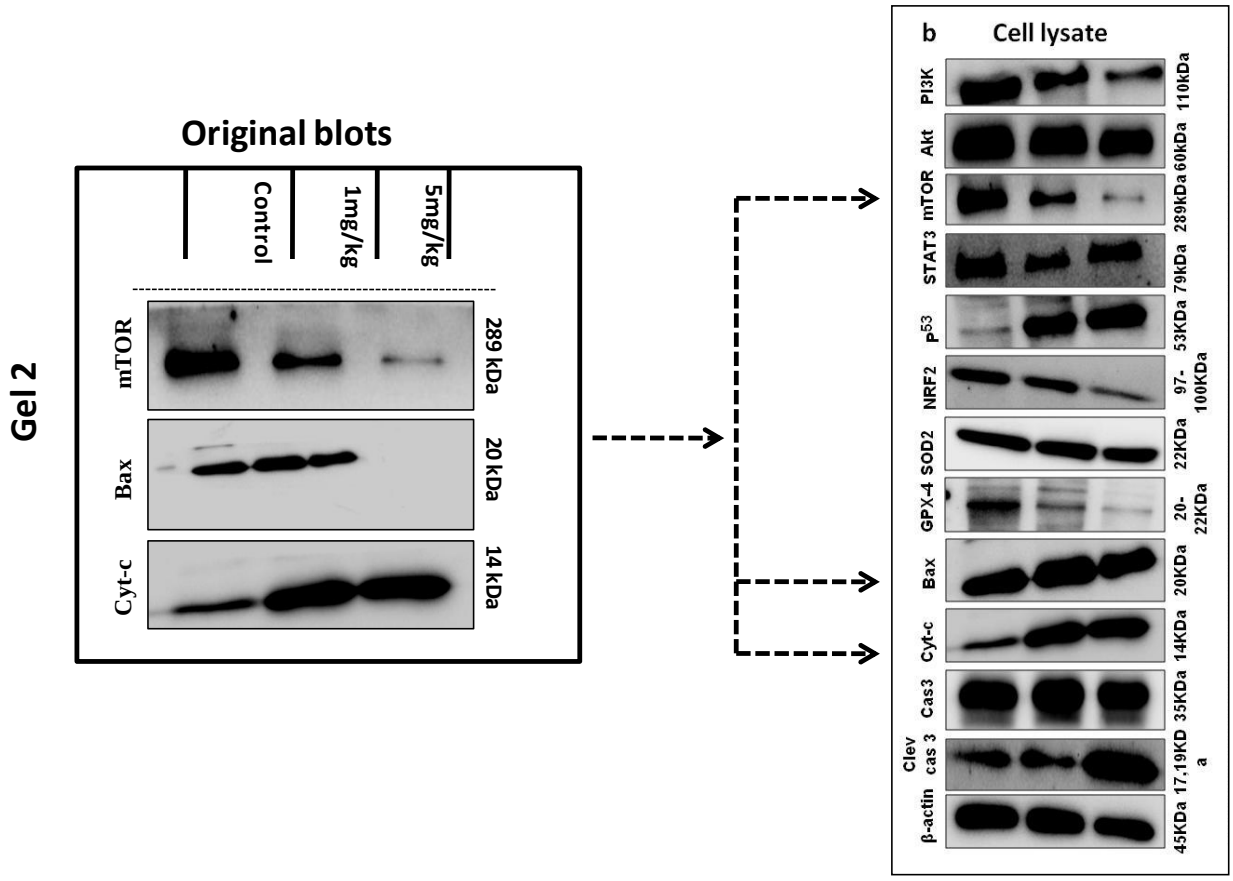
SI Fig. 19b The quantification data of expression of proteins (Western blot in cell lysate) such as PI3K, Akt, , m-TOR, STAT3, p53, NRF-2, SOD2, GPX4, Bax, Bcl2, cytochrome c, cleaved caspase 3, using image J analysis. The data collected for three times from the same image and presented as mean ± SD.

SI Fig. 20a



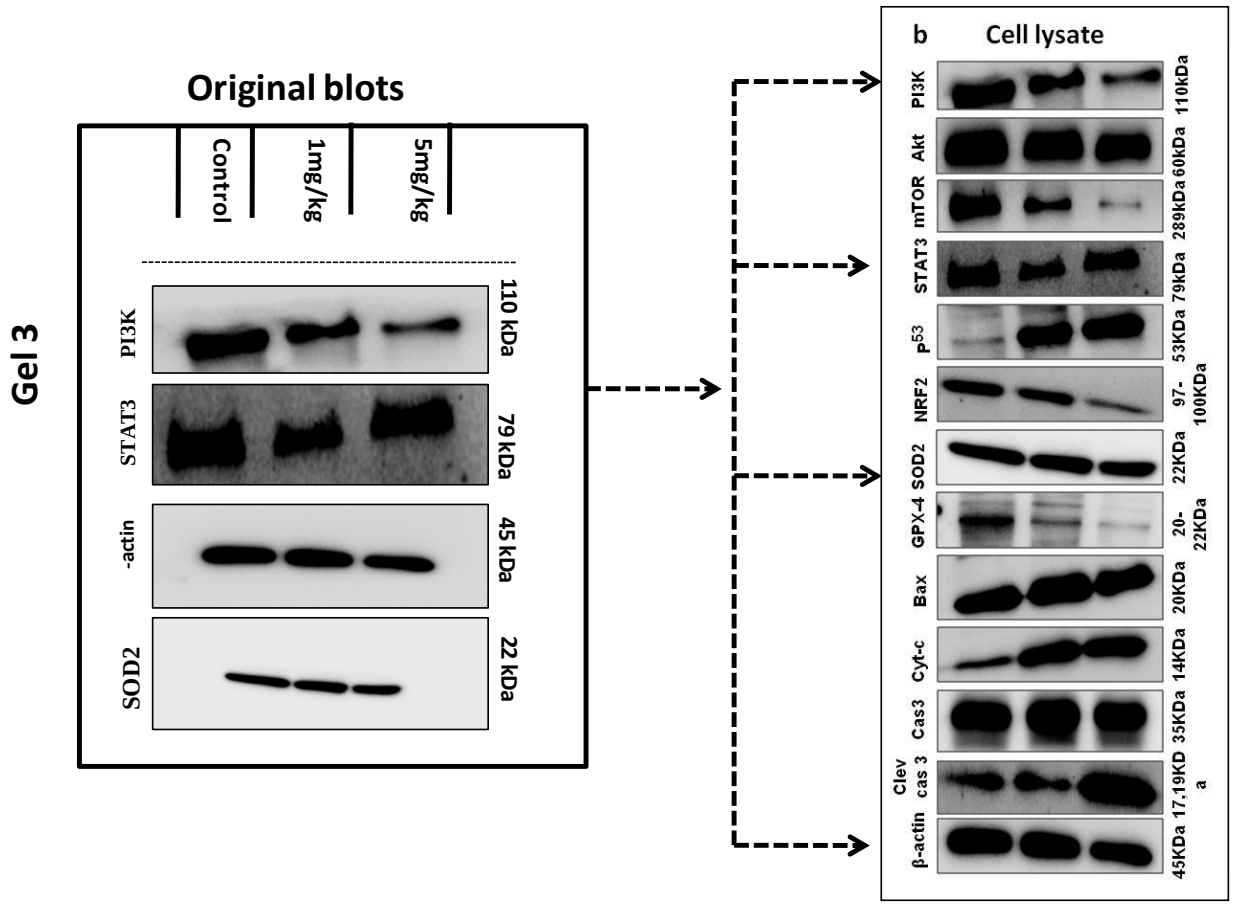
SI Fig. 20a Original Western blot images of B16F10 cell lysate in different CuNPANP treated, untreated control and cisplatin treated groups. Same source of tissue lysate was used for all protein expressions in the Western blot experiments.

SI Fig.20b



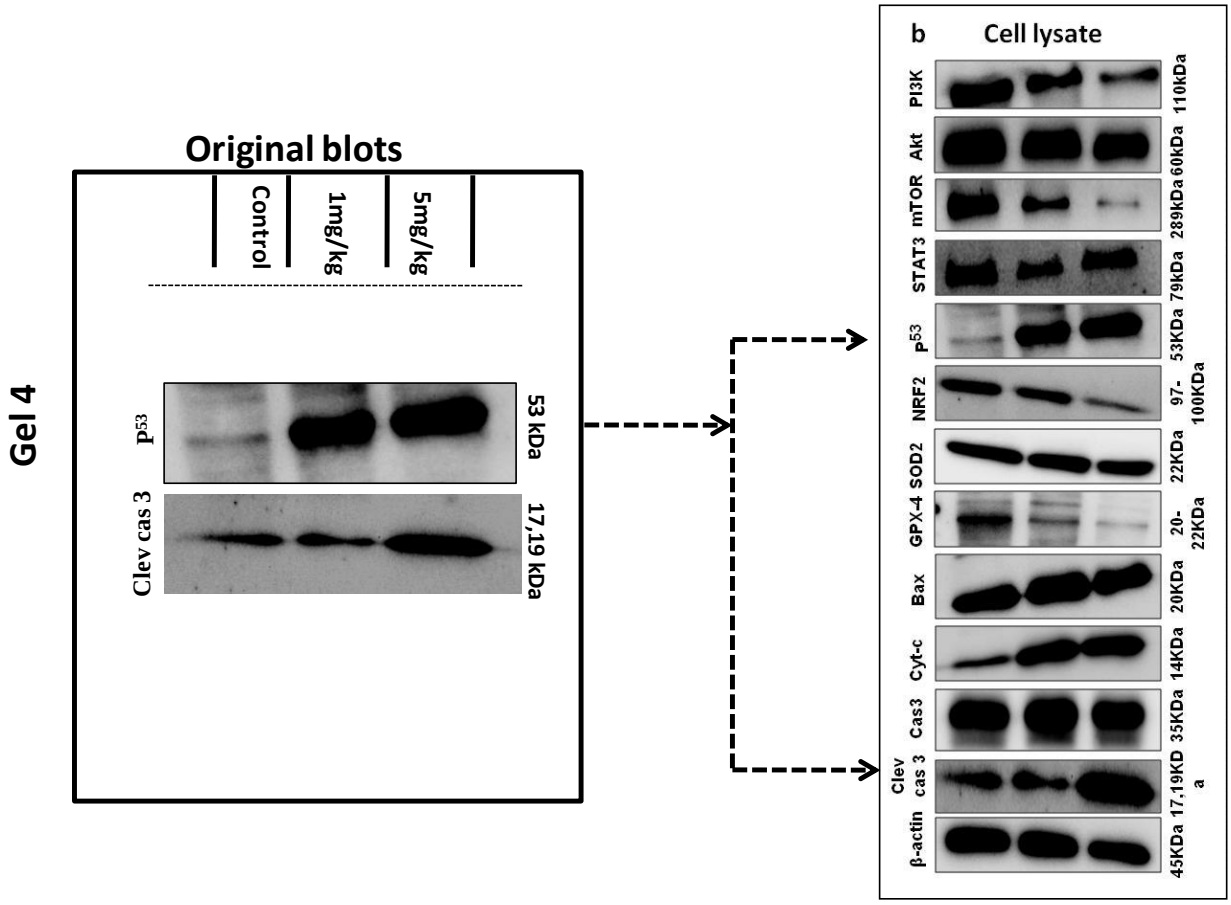
SI Fig. 20b Original western blot images of B16F10 cell lysate in different CuNPANP treated, untreated control and cisplatin treated groups. Same source of tissue lysate was used for all protein expressions in the Western blot experiments.

SI Fig. 20c



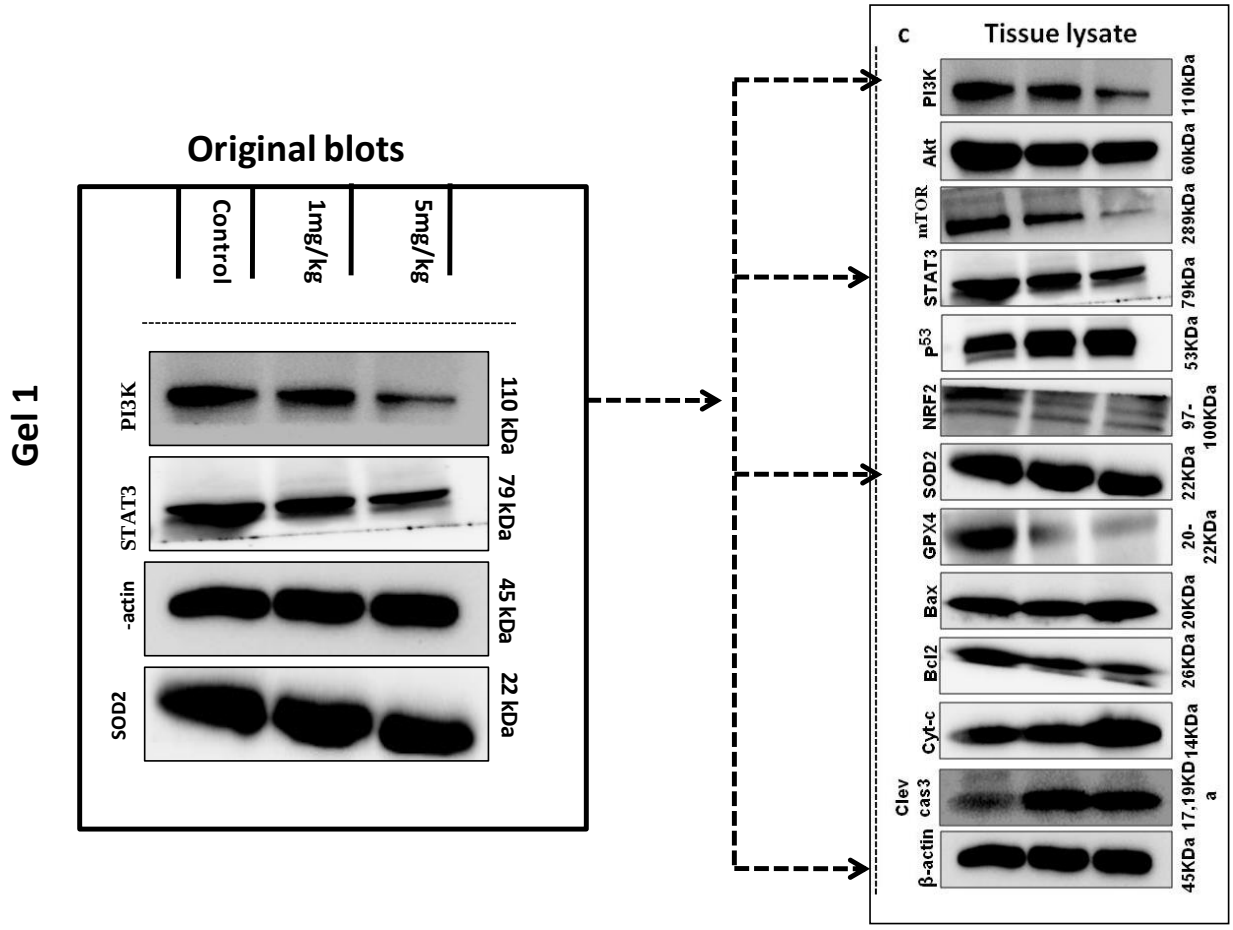
SI Fig. 20c Original western blot images of B16F10 cell lysate in different CuNPANP treated, untreated control and cisplatin treated groups. Same source of tissue lysate was used for all protein expressions in the Western blot experiments.

SI Fig.20d



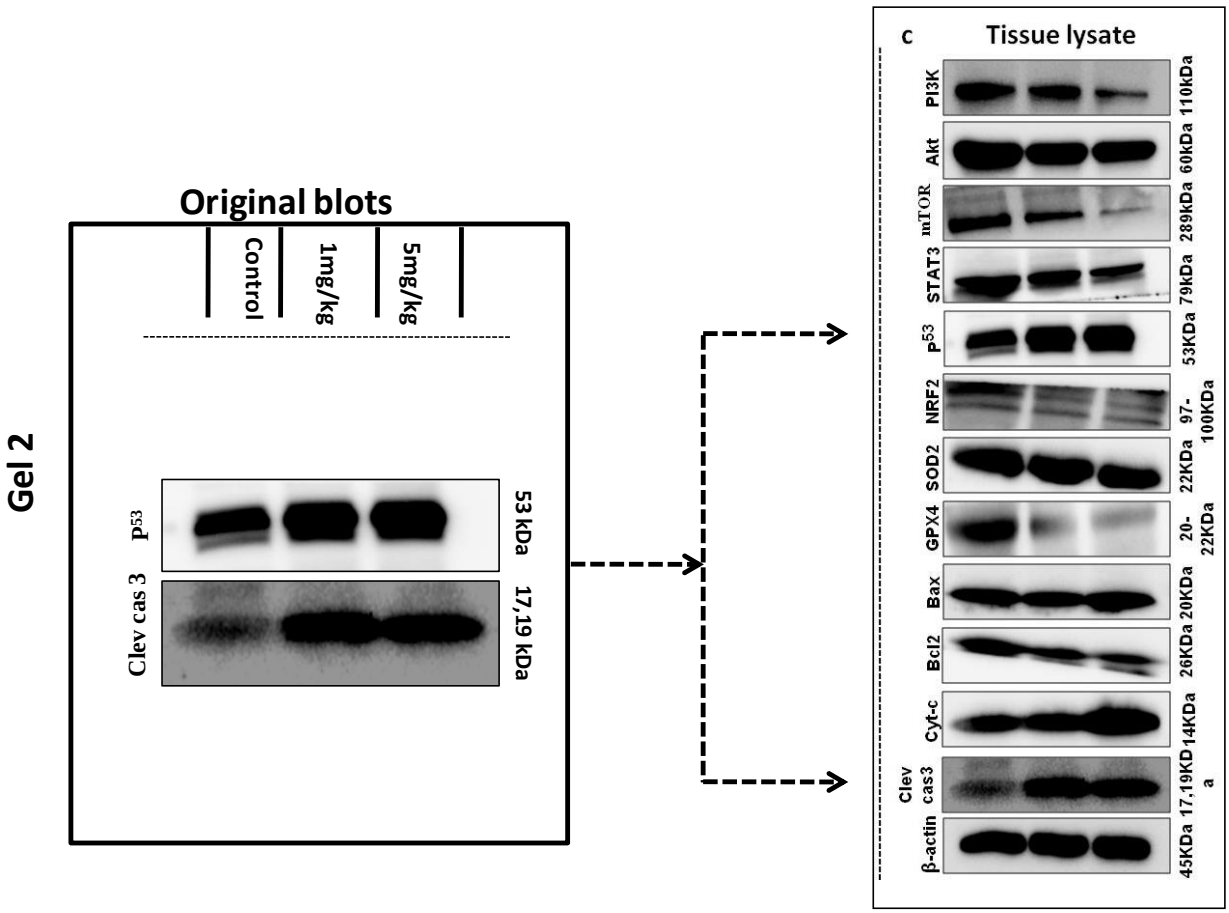
SI Fig. 20d Original western blot images of B16F10 cell lysate in different CuNPANP treated, untreated control and cisplatin treated groups. Same source of tissue lysate was used for all protein expressions in the Western blot experiments.

SI Fig.20e



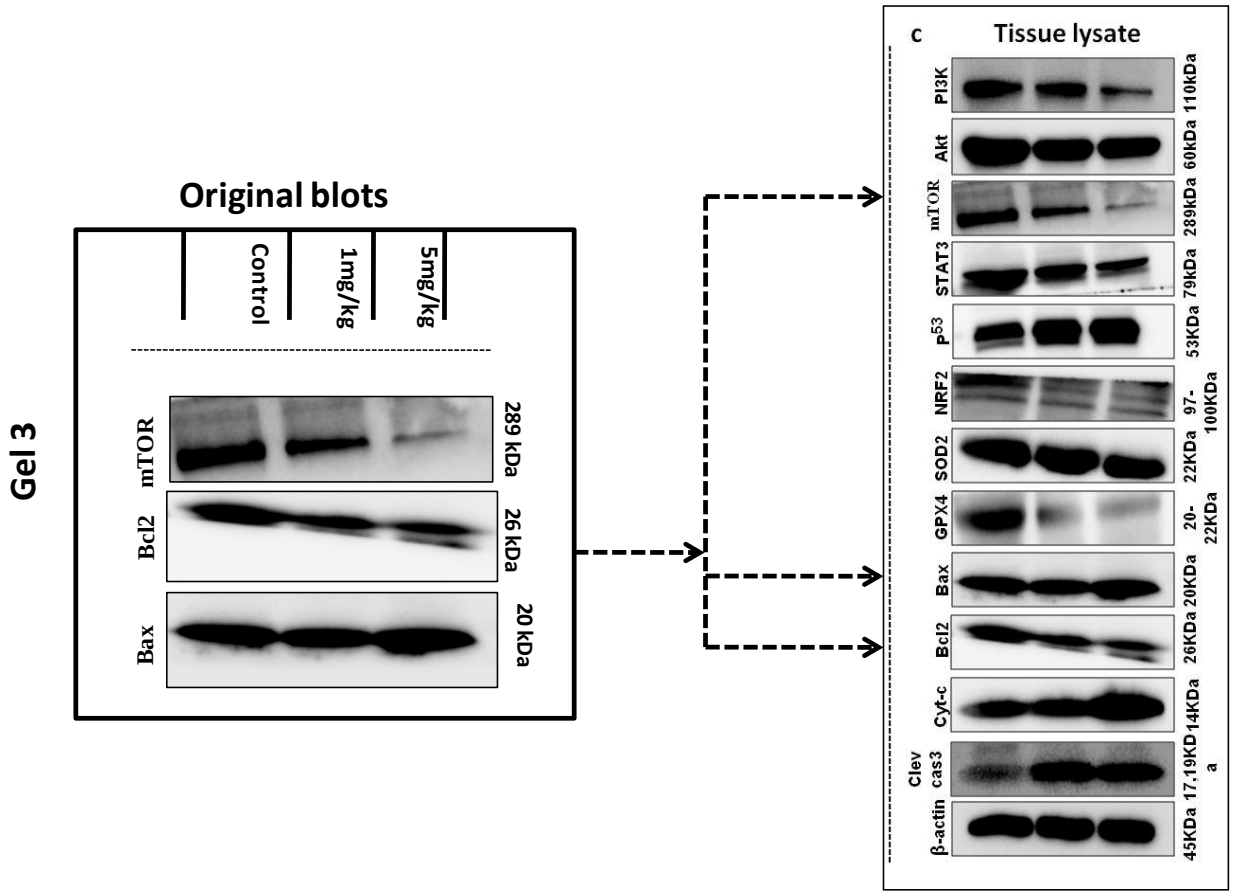
SI Fig. 20e Original western blot images of melanoma tissue in different CuNPANP treatment (1- & -5mgkg^{-1}) and untreated control groups. Same source of tissue lysate was used for all protein expressions in the Western blot experiments.

SI Fig.20f



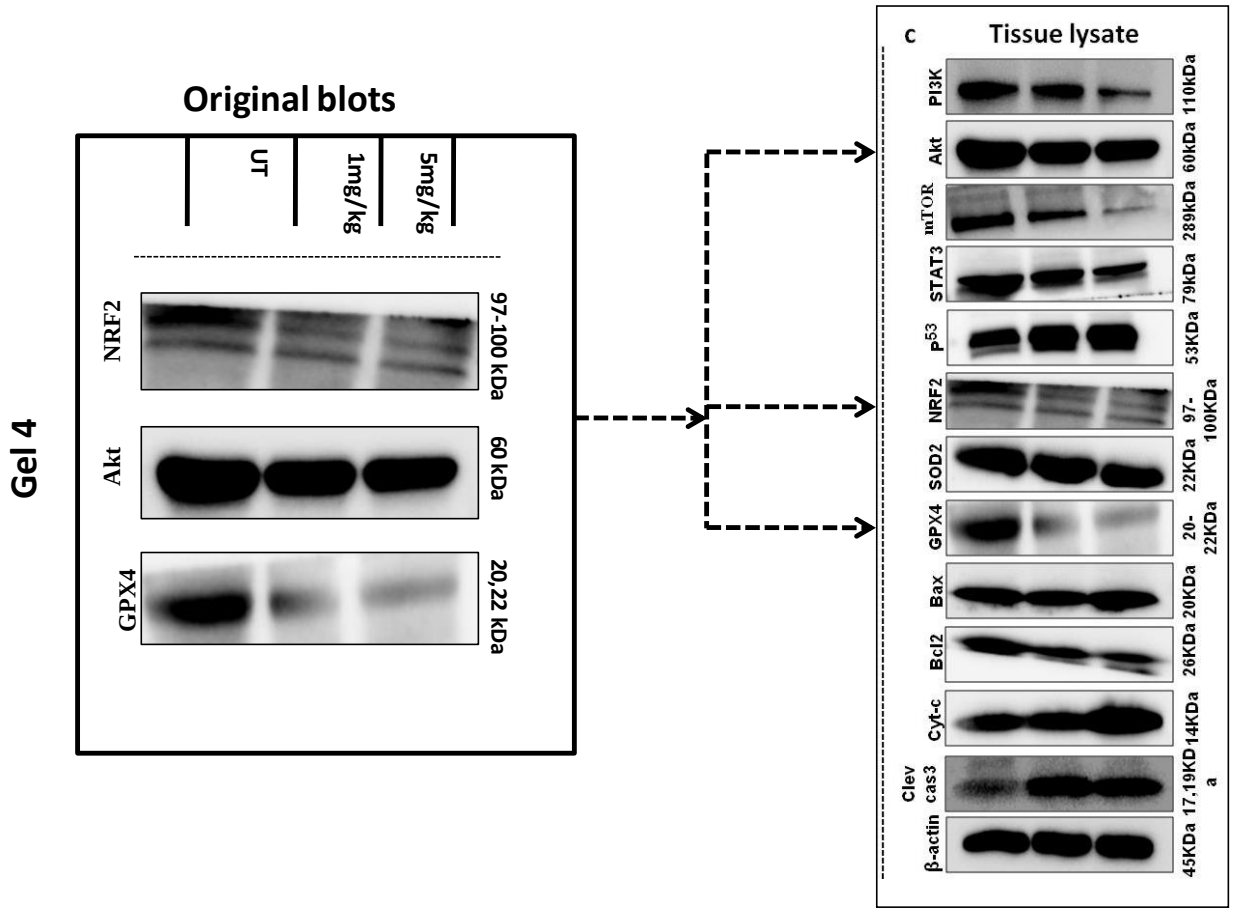
SI Fig. 20f Original western blot images of melanoma tissue in different CuNPANP treatment (1- & -5mgkg^{-1}) and untreated control groups. Same source of tissue lysate was used for all protein expressions in the Western blot experiments.

SI Fig.20g



SI Fig. 20g Original western blot images of melanoma tissue in different CuNPANP treatment (1- & -5mg kg^{-1}) and untreated control groups. Same source of tissue lysate was used for all protein expressions in the Western blot experiments.

SI Fig.20h



SI Fig. 20h Original western blot images of melanoma tissue in different CuNPANP treatment (1- & -5 $mgkg^{-1}$) and untreated control groups. Same source of tissue lysate was used for all protein expressions in the Western blot experiments.

References

1. S. Tripathy, S. Haque, S. Londhe, S. Das, C. C. Norbert, Y. Chandra, B. Sreedhar and C. R. Patra, *Biomaterials Advances*, 2024, 213832.
2. S. Haque, R. Kotcherlakota, P. Bhamidipati, K. Muralidharan, B. Sreedhar, R. Amanchy and C. R. Patra, *Advanced Therapeutics*, 2023, **6**, 2300142.
3. S. Das, S. Tripathy, B. Sreedhar, S. Mukherjee and C. R. Patra, *Advanced Therapeutics*, 2023, **6**, 2200293.
4. S. Londhe, S. Haque, S. Tripathy, S. Bojja and C. R. Patra, *Nanoscale*, 2023, **15**, 10017-10032.