Supporting Information

Targeted delivery of novel peptide-docetaxel conjugate to MCF-7 cell through Neuropilin-1 receptor: Reduced toxicity and enhanced efficacy of docetaxel

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Materials and methods:

Reagents: Dimethylsulfoxide (DMSO), Thioanisole and Methanol (MeOH) were purchased from Spectrochem. Triton-X-100 and Dichloromethane were purchased from SRL. N, N′-dimethylformamide (DMF), Acetonitrile, Phenol, 1,2-Ethanedithiol (EDT) and Trifluoroacetic acids (TFA) were purchased from Merck. 2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid (HEPES) was purchased from Himedia. Docetaxel, Bovine Serum Albumin (BSA), 3-(maleimido) propionic acid N-hydroxysuccinimide ester,5(6)-Carboxyfluorescein,3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Dulbecco's Modified Eagle's Medium **(**DMEM), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), Kanamycin sulfate,Trypsin-EDTA solution, Dimethylsulfoxide for cell culture and Formaldehyde solution for molecular biology were purchased from Sigma Aldrich. Penicillin-Streptomycin, Neutravidin and Fetal Bovine Serum (FBS) were purchased from Invitrogen. Rabbit monoclonal anti-alpha Tubulin IgG (EP1332Y) antibody and goat polyclonal anti-Rabbit IgG H&L (Cy3.5 ®) preadsorbed were purchased from Abcam. Goat anti-mouse IgG-RPE conjugated was purchased from Southern Biotech. Propidium iodide (PI), p53 (F-8) mouse monoclonal IgG, p21 (F-5) mouse monoclonal IgG and annexin V apoptosis detection kit were purchased from Santa Cruz Biotechnology. Polyclonal IgG Neuropilin 1 primary antibody was purchased from Thermo Fisher Scientific Pierce. Bisbenzimide H 33258 (hoechst) and RNase A were purchased from Calbiochem. Cover glass bottom dishes were purchased from SPL. Tissue culture flask, 96 well plates, 6 well plates and FACS tubes were purchased from BD (Becton Dickinson). Wang resin (100-200 mesh) and all amino acids for solid phase synthesis of CGNKRTR peptide were

purchased from Novabiochem.The peptide was purified in Shimadzu HPLC system (LC 20AP) using reverse phase C18 column (Waters).All compounds were used without further purification.

Data Analysis: Microscopic images were analyzed using Image J software.

Synthesis of Peptide Modified Docetaxel (PMDX):

(a) Synthesis of CGNKRTR peptide and purification: A 7-mer CGNKRTR peptide for neuropilin-1-dependent CendR internalisation pathway was synthesized in Microwave Peptide Synthesiser equipped with Liberty 1 system (Discovery, CEM). Wang resin (100-200 mesh) was used as a solid support having substitution level 0.9 mmole/gm.DMF and DCM were used as solvent and all the amino acids, used in the synthesis ofCGNKRTR were N-terminal fluorenylmethyloxycarbonyl (Fmoc) protected.The side chain protections of all the amino acids were stable in basic medium but were labile in acidic medium. Wang resin was swelled in 1:1 mixture of DMF and DCM solvent for overnight. N, N′-diisopropylethylamine (DIPEA) in DMF having concentration 2M was used as a base and HBTU in DMF having concentration 0.5M was used as an activator. 20% piperidine in DMF was used as deprotection mixture. All the amino acids required for CGNKRTR peptide were dissolved in DMF as a concentration of 0.2M separately. After coupling of all the amino acids required for CGNKRTR, resin was dried using DCM, methanol and diethyl ether. Peptide was cleaved from the resin using cleavage cocktail containing trifluoroaceticacid (TFA), thioanisole, phenol, 1, 2-ethanedithiol (EDT) and water (8.25:0.5:0.5:0.25:0.5).Then, the peptide was precipitated out in cold methyl-tert butyl ether and purified through reverse phase HPLC on a C18 column using water/acetonitrile gradient in 0.1% TFA as mobile phase and characterized by Matrix Assisted Laser Desorption Ionisation Time of Flight (MALDI-TOF) analysis. We have obtained m/z: 834, which correspond to the calculated mass of the peptide (MW = 834).

(b) Synthesis of FITC-CGNKRTR peptide and purification: CGNKRTR peptide was synthesised on Wang resin (100-200 mesh) as described before. We have attached 5(6)-Carboxyfluorescein on the N-terminal of CGNKRTR peptide following similar coupling procedure of amino acid as described before. We have removed the N-terminal Fmoc protection of the CGNKRTR peptide using 20% piperidine in DMF. Resin was washed by DMF and a solution of 5(6)-Carboxyfluorescein, HOBT and DIC in DMF was added to it. Peptide was cleaved from the resin using cleavage cocktail containing trifluoroaceticacid (TFA), thioanisole, phenol, 1,2-ethanedithiol (EDT) and water (8.25:0.5:0.5:0.25:0.5).Then, the peptide was precipitated out in cold methyl-tert butyl ether and purified through reverse phase HPLC on a C18 column using water/acetonitrile gradient in 0.1% TFA as mobile phase and characterized by MALDI-TOF. We have obtained m/z: 1193 and 1194, which exactly matched as [M+1] and [M+2] respectively with the calculated mass of the peptide (MW= 1192).

(c) Synthesis of maleimido modified docetaxel (MDX): Docetaxel (6 mg, 0.0074mmole) and dimethylaminopyridine (0.8 mg, 0.0064mmole) were added in a 10 mL round bottom flask. Then dry dichloromethanewas added and stirred on ice bath at 0 ^{0}C for 30 minutes under nitrogen atmosphere. Then 3-(maleimido) propionic acid N-hydroxysuccinimide ester (2 mg, 0.008mmole) was added to it and the reaction mixture was stirred for overnight at 0 ^{0}C. The reaction mixture was diluted by dichloromethane and it was washed by cold 1N HCL and then brine solution. The organic part was dried over anhydrous Na_2SO_4. Dichloromethane was evaporated out and we have obtained pure MDX. The mass of the resulting compound was calculated as m/z: 958. From LC-MS analysis, we have obtained m/z: 981.32 $[M+Na]^+$ and m/z: 1081 $[M+DMAP]^+$. We have also obtained m/z: 1081$[M+DMAP]^+$ from MALDI-TOF. The molecular weight of DMAP is 122. The mass at m/z: 1081 was obtained due to formation of ionic salt of the resulting compound with dimethylaminopyridine (DMAP). Maleimido modified docetaxel (MDX) was purified through HPLC and used in the next step for the preparation of PMDX.

(d) Synthesis of peptide modified docetaxel (PMDX): MDX (2 mg, 0.002 mmole) was dissolved in acetonitrile and ethanol mixture. CGNKRTR peptide (5 mg, 0.006 mmole) was dissolved in water. Now CGNKRTR peptide solution was added into the MDX solution and it was stirred for 24h at room temperature under nitrogen atmosphere[1].Solvent was evaporated out, purified through HPLC and characterized by MALDI-TOF. We have calculated the mass of the PMDX as m/z: 1792and we have obtained m/z: 1793 as [M+1] from MALDI-TOF.

(e) Synthesis of FITC-PMDX: MDX (2 mg, 0.002 mmole) was dissolved in acetonitrile and ethanol mixture. FITC-CGNKRTR peptide (5 mg, 0.006 mmole) was dissolved in acetonitrile and water mixture. Now FITC-CGNKRTR peptide solution was added into the MDX solution and it was stirred for 24h at room temperature under nitrogen atmosphere. Solvent was evaporated and purified through HPLC (purity > 95%) followed by characterization through Matrix Assisted Laser Desorption Ionisation Time of Flight (MALDI-TOF) analysis. Mass of the FITC-PMDX 1118 (m/2, 100%) (MW =2236$[M+2H^++Na^++K^+]$ and FITC-PMDX contains two positive charges).

^{1}H-NMR Spectra in 300 MHz: Formation of MDX from DX was confirmed by ^{1}H-NMR.[2] In DX chemical shift of C2′-OH was observed at 3.3 to 3.4 ppm which was absent in MDX. Conjugation of maleimide functionality was also confirmed from chemical shift value at6.72 ppm for vinylic hydrogens of maleimide. Formation of PMDX from MDX was also confirmed by chemical shift value at 8.32 ppm for amide NH- of the peptide (Figure S1, S2 and S3).

^{1}H NMR of DX ($CDCl_3$) δ: 8.11 (d, 2H, $H_{2,6\text{-}OBz}$), 7.61 (t, 1H, $H_{4\text{-}OBz}$), 7.5 (t, 2H, $H_{3,\ 5\text{-}OBz}$), 7.30-7.45 (m, 5H, aromatic hydrogen of side chain), 6.2 (t, 1H, H_{13}), 5.65 (d, 1H, H_2), 5.4 (d, 1H, $H_{3'}$), 5.25 (d, 1H, $H_{4'}$ N-H), 4.95 (d, 1H, H_5), 4.6 (s, 1H, $H_{2'}$), 4.15-4.35 (m, 4H, $H_{10\text{-}OH}$, H_{20} &H_7),3.91 (d, 1H, H_3), 3.3-3.4 (s, 1H, $H_{2'\text{-}OH}$), 2.5-2.6 (m, 2H, H_6), 2.35 (s, 3H, $H_{4\text{-}OAc}$), 2.25 (d,

2H, H_{14}), 1.76 (s, 3H, H_{18}), 1.58 (s, 3H, H_{19}), 1.35 (s, 9H, $H_{6'\text{-tBu}}$), 1.22 (s, 3H, H_{16}), 1.11 (s, 3H, H_{17}).

^{1}H NMR of MDX ($CDCl_3$) δ: 8.12 (d, 2H, $H_{2,\ 6\text{-OBz}}$), 7.62 (t, 1H, $H_{4\text{-OBz}}$), 7.51 (t, 2H, $H_{3,\ 5\text{-OBz}}$), 7.3-7.4 (m, 5H, aromatic hydrogen of side chain), 6.72 (s, 2H, vinylic hydrogen of maleimide), 6.22 (t, 1H, H_{13}), 5.69 (d, 1H, H_2), 5.44 (d, 1H, $H_{3'}$), 5.21 (s, 1H, $H_{4'}$ N-H), 4.95 (d, 1H, H_5), 4.63 (s, 1H, $H_{2'}$), 4.18-4.34 (m, 4H, $H_{10\text{-OH}}$, H_{20} &H_7), 3.92 (d, 2H, maleimido-N-CH_2-), 3.85 (d, 1H, H_3), 3.25 (t, 2H, 2′C-O-CO-CH_2), 3.5 (s, 6H, DMAP CH_3-N-CH_3), 2.55-2.66 (m, 2H, H_6), 2.38 (s, 3H, $H_{4\text{-OAc}}$), 2.29 (d, 2H, H_{14}), 1.86 (s, 3H, H_{18}), 1.77 (s, 3H, H_{19}), 1.26 (s, 9H, $H_{6'\text{-tBu}}$), 1.14 (s, 3H, H_{16}), 0.89 (s, 3H, H_{17}).

^{1}H NMR of PMDX (d^6-DMSO) δ: 8.32 (s, 7H, Peptide amide -NH), 7.96 (d, 2H, $H_{2,\ 6\text{-OBz}}$), 7.71 (t, 1H, $H_{4\text{-OBz}}$), 7.62 (t, 2H, $H_{3,\ 5\text{-OBz}}$), 7.30-7.45 (m, 5H, aromatic hydrogen of side chain), 6.96 (s, 2H, -NH_2 of Asn), 6.55 (s, 4H, -NH_2 of Arg), 5.89 (t, 1H, H_{13}), 5.4 (d, 1H, H_2), 5.05 (d, 1H, $H_{3'}$), 4.97 (s, 1H, $H_{4'}$ N-H), 4.89 (d, 1H, H_5), 4.51 (s, 1H, $H_{2'}$), 4.18-4.34 (m, 4H, $H_{10\text{-OH}}$, H_{20} &H_7), 4.02 (d, 2H, maleimido-N-CH_2-), 3.85 (d, 1H, H_3), 3.25 (t, 2H, 2′C-O-CO-CH_2), 2.55-2.66 (m, 2H, H_6), 2.21 (s, 3H, $H_{4\text{-OAc}}$), 1.73 (s, 2H, H_{14}), 1.52 (s, 3H, H_{18}), 1.35 (s, 3H, H_{19}), 1.23 (s, 9H, $H_{6'\text{-tBu}}$), 1.08 (q, peptide), 1.05 (s, 3H, H_{16}), 0.85 (s, 3H, H_{17}).

Cell culture: Human breast cancer cell line MCF-7 (Michigan Cancer Foundation-7), MDA-MB-231, MDA-MB-453 and human lung cancer cell line A549 were purchased from National Centre for Cell Science (NCCS), Pune, India. We have obtained HEK293T cells from Dr. D. Biswas's laboratory at Molecular and Human Genetics Division, IICB, Kolkata. Cells were cultured in our lab in a 5% CO_2 humidified sterile atmosphere at 37 ^{0}C with Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, kanamycin sulfate (110 mg/L), penicillin (50 units/mL), streptomycin (50 µg/mL) in cell culture treated flasks. After 70-80% of confluence, the cells were splitted. All the cell culture works were performed under aseptic condition of laminar hood.

Cell surface expression of neuropilin 1 in MCF-7 cell and HEK293T cell: We have analyzed the cell surface expression of NRP-1 on MCF-7 breast cancer and HEK293T normal cell line. Cells were trypsinized, collected in a micro centrifuge tube and washed with PBS. We have incubated both the cell lines with polyclonal IgG neuropilin1primary antibody (1:300) for 2 hour at 37 ^{0}C followed by washing with serum free media. For staining, cells were incubatedwith Cy3.5 pre adsorbed polyclonal goat anti-rabbit (IgG) secondary antibody (1:600) for 1 hour at 37 ^{0}C in serum free media. Both the cell lines were checked for neuropilin 1 expression with respect to untreated cells (cells were not treated with neuropilin 1 primary antibody) using flow cytometer. We have selected the excitation and emission wavelengths as 581 and 596 nm respectively during flow cytometric analysis.

Cell surface binding of FITC-PMDX: MCF-7 cells were seeded in a cover glass bottom dish before 24 hours of treatment. Cells were incubated with 10 µM solution of FITC-PMDX for 1h.

Then, cells were washed by serum free media and fixed with 4% paraformaldehyde solution for 30 minutes. Nucleus was stained with Hoechst 33258 (1 µg/mL) for 30 minutes before imaging.Fixed cells were imaged through an Andor spinning disc confocal microscope with a 60X objective (Olympus) and an Andor iXon3 897 EMCCD camera in bright field, 405 and 488 nm wavelength laser lights.

Docking: Autodock-Vina version 1.1.2 was used for docking study. The PMDX peptide was docked with 2ORZ receptor.[3]The carboxyl end group of PMDX interacts with Thr349, Ser346, Tyr353, Glu348 and Trp301 of NRP-1 through H-bonding (Figure S6b). The carboxyl end of tuftsin peptide also shows the same binding pocket of Asp320, Thr349, Ser346, Tyr353 in 2ORZ pdb structure (Figure S6 c, d). The Gly2 of PMDX interacts with Glu348 of NRP-1 while side chain of Arg7 and Thr6 of PMDX interacts with Asp320 and Tyr353 of NRP-1 respectively through H-bonding (Figure S6b). In addition, the DX moiety of PMDX interacts with Tyr310, Asn300, Arg305 and Ser302 residues of NRP-1.

Cell viability assay: Cellular viability study was conducted with well-known method using 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT). Percentage viability was analyzed to understand the effect of PMDX on MCF-7 cells in comparison to DX. MCF-7 cells were seeded at a density of 10000 cells per well in 96-well plate before 24 hours of treatment. We have prepared solutions of PMDX and DX in cell culture media. We have also maintained the concentration of DMSO less than 0.01% in all experiments. Cells were incubated with these solutions for 4 hours at 37 ^{0}C. Media was changed and cells were incubated in fresh media for another 44h at 37 ^{0}C. Then 50 µL of MTT solution (5 mg/mL in PBS) was added all the wells excluding blank. Cells were incubated with MTT solution for 4 hours at 37 ^{0}C. In principle if the cells are live, the cellular reductase enzymes will act on soluble yellow color MTT solution and convert it into an insoluble purple color product formazan. Purple colored formazan was dissolved in 1:1 (v/v) DMSO/MeOH and absorbance of each well was measured at 550 nm by microplate ELISA reader. Result was plotted as concentrations (nM) in X-axis and percentage of viable cells in Y-axis (n=6). In similar way, we have also checked the cell viability of the CGNKRTR peptide.

%Viability = [(A_{550}Treated Cells - A_{550} Backgrounds)/ (A_{550}Untreated Cells - A_{550} Backgrounds)]*100.

Blocking experiment and Cell viability of CGNKRTR peptide: MCF-7 cells were seeded at a density of 10000 cells per well in 96-well plate before 24 hours of treatment. In this experiment we have incubated MCF-7 cells with the CGNKRTR peptide for 2 hours. Media was changed and cells were treated with different concentrations of PMDX (5 nM to 40 nM). Cells were incubated for another 24 hours at 37 ^{0}C. In another row of the 96-well plate we have treated the cells with different concentrations of PMDX (5 nM to 40 nM), where cells were not incubated with CGNKRTR peptide previously. We have compared percentage cell viability using MTT

assay as described previously. We have also checked the cell viability of different concentrations of the CGNKRTR peptide (5 nM to 40 nM).

Cell viability assay in HEK293T cells and toxicity study of PMDX compared to DX: HEK293T cells were seeded at a density of 10000 cells per well in 96-well plate before 24 hours of treatment. We have prepared different concentrations of solutions of PMDX and DX in cell culture media. We have also maintained the concentration of DMSO less than 0.01% in all experiments. Cells were incubated with these solutions for 48 hours at 37 ^{0}C. We have compared percentage cell viability using MTT assay as described previously.

Cellular uptake study of FITC-PMDX in MCF7 and HEK293T Cells: MCF-7 and HEK293T cells were seeded in different 6-well plate at a density of 5 X 10^5 cells per well before 24 hours of treatment. Cell suspension was treated with 10 μM solutions of FITC-PMDX and incubated in cell culture incubator for 1h. Cells were washed with serum free media. Uptake of FITC-PMDX in MCF7 and HEK293T cells was quantified using flow cytometer in FITC channel.

Immunofluorescence microscopy for microtubule cytoskeleton study: MCF-7 cells were seeded in a cover glass bottom dish before 24 hours of treatment. Cells were incubated with solutions of DX and PMDX having concentration of drugs at 50 nM for 24 hours. Then, cells were washed by PBS and fixed with 4% paraformaldehyde solution for 30 minutes. We have treated the cells with0.1% Triton X-100 in PBS for 10 minutes. Cells were washed with PBS and incubated with 5% BSA in PBS for 30 minutes. Antibody solutions were prepared in PBS. After washing with PBS, cells were incubated with monoclonal anti-α-tubulin IgG [EP1332Y] antibody with dilution 1:300 for 2 hours. After that cells were washed with PBS and incubated with secondary antibody (Cy3.5 pre-absorbed goat anti-rabbit IgG) with dilution 1:600 for 2 hours. Cells were washed with PBS and incubated with Hoechst 33258 (1 μg/mL) for 30 minutes before imaging. Fixed cells were imaged through Nikon Ti-U eclipse fluorescence microscope equipped with AndoriXon3 897 EMCCD camera in bright field, 405 and 561nm wavelength laser lights.

Immunofluorescence microscopy for microtubule cytoskeleton with FITC-PMDX: MCF-7 cells were seeded in a cover glass bottom dish before 24 hours of treatment. Cells were incubated for 12h with 10 μM solutions of FITC-PMDX. Then, cells were washed by PBS and fixed with 4% paraformaldehyde solution for 30 minutes. We have treated the cells with 0.1% Triton X-100 in PBS for 10 minutes. Cells were washed with PBS and incubated with 5% BSA in PBS for 30 minutes. Antibody solutions were prepared in PBS. After washing with PBS, cells were incubated with monoclonal anti-α-tubulin IgG [EP1332Y] antibody with dilution 1:300 for 2 hours. After that cells were washed with PBS and incubated with secondary antibody (Cy3.5 pre-absorbed goat anti-rabbit IgG) with dilution 1:600 for 2 hours. Cells were washed with PBS and incubated with Hoechst 33258 (1 μg/mL) for 30 minutes before imaging. Fixed cells were imaged through an Andor spinning disc confocal microscope with a 60X objective (Olympus)

and an Andor iXon3 897 EMCCD camera in bright field, 405, 488 and 561 nm wavelength laser lights.

Cell cycle analysis by flow cytometer: MCF-7 cells were seeded at a density of 5 X 10^5 cells per well in a 6-well plate before 24 hours of treatment. Cells were treated with solutions of DX and PMDX having concentration of drugs 50 nM for 24 hours. After incubation, the media containing free cell suspension were taken in micro centrifuge tube and cells were collected by centrifugation at 3000 rpm for 3 minutes. Then the cells in 6-well plates were trypsinized and collected by centrifugation at 3000 rpm for 3 minutes. All cell pellets were accumulated in phosphate buffer saline (PBS, pH 7.4) and fixed by adding cold ethanol slowly. The final concentration of ethanol was 70% (v/v). Cells were incubated for overnight at 4 ^{0}C. Cells were washed with PBS (pH 7.4) through centrifugation to remove ethanol. Then cell suspensions in PBS were incubated with PI and RNase A for 45 minutes at room temperature. The working concentration of PI and RNase A was 100 µg/mL and 10 µg/mL respectively. Cell suspensions were transferred into FACS tube followed by analysis through FACS.

Immunoblotting of p53 and p21: MCF-7 cells were seeded at a density of 5 X 10^5 cells per well in a 6-well plate before 24 hours of treatment. 50 nM of DX and PMDX treated MCF-7 cells were lysed and protein isolation carried out using RIPA lysis buffer. Similarly, protein isolation has been carried out from un-treated control MCF-7 cells. Then concentration of the protein has been calculated using Bradford assay. 20-30 µg of proteins were electrophoresed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% gels followed by electrotransferred to Immobilon-P Polyvinylidene fluoride membranes (Millipore Corporation, Bedford, MA, USA). Then membrane was blocked with skimmed milk and incubated overnight with different primary antibodies, such as anti-p53 (Sigma), anti-p21 (Santa Cruz Biotechnology, Heidelberg, Germany) and anti-α-tubulin (Merck Millipore, Darmstadt, Germany) at 4 ^{0}C temperature. Then protein immobilized membrane was washed and incubated with secondary antibody conjugated with peroxidase. To detect protein expression HRP substrate (Luminata Forte Western HRP substrate, Merck Millipore, Darmstadt, Germany) has been applied on the membrane and analysed under chemiluminescence detection system. The protein band intensity has been normalized with loading control and relative expression has been quantified.

Immunofluorescence microscopy of p53 and p21: MCF-7 cells were seeded in a cover glass bottom dish before 24 hours of treatment. Cells were incubated with solutions of DX and PMDX having concentration of drug 50 nM for 24 hours. Then, cells were washed by PBS and fixed with 4% paraformaldehyde solution for 30 minutes. We have treated the cells with 0.1% Triton X-100 in PBS for 10 minutes. Cells were washed with PBS and incubated with 5% BSA in PBS for 30 minutes. Antibody solutions were prepared in PBS. After washing with PBS, cells were incubated with mouse monoclonal IgG P53 (F-8) antibody with dilution 1:300 for 2 hours. After that cells were washed with PBS and incubated with secondary antibody (R-phycoerythrin conjugated goat anti-mouse IgG) with dilution 1:600 for 2 hours. Cells were washed with PBS and incubated with Hoechst 33258 (1 µg/mL) for 30 minutes before imaging. Fixed cells were

imaged through NikonTi-U eclipse fluorescence microscope equipped with AndoriXon3 897 EMCCD camera in bright field, 405 and 561nmwavelength laser lights.

In case of p21, cells were incubated with mouse monoclonal IgG P21 (F-5) antibody with dilution 1:300 for 2 hours. After that cells were washed with PBS and incubated with secondary antibody (R-phycoerythrin conjugated goat anti-mouse IgG) with dilution 1:600 for 2 hours. Cells were washed with PBS and incubated with Hoechst 33258 (1 µg/mL) for 30 minutes before imaging. Fixed cells were imaged through Nikon Ti-U eclipse fluorescence microscope equipped with Andor iXon3 897 EMCCD camera in bright field, 405 and 561nm wavelength laser lights.

AnnexinV/PropidiumIodide (PI) assay: MCF-7 cells were plated in a cover glass bottom dish before 24 hours of treatment. Cells were incubated with solutions of DX and PMDX having concentration of drugs at 50 nM for 24 hours. Cells were washed with phosphate buffer (PBS, pH 7.4) and then with assay buffer. Assay buffer was supplied along with the Annexin V apoptosis detection kit (Santa Cruz Biotechnology). We have added 2.5 µL of Propidium iodide (PI) and 2.5 µL of annexin V into the 200µL of assay buffer and live cells were incubated with this solution for 15 minutes at 37 ^{o}C inside the cell culture incubator. All those reagents were supplied along with the apoptosis detection kit (Santa Cruz Biotechnology). The stock concentration of PropidiumIodide and annexin V was 50 µg/mL and 200 µg/mL respectively. Cells were washed with the assay buffer and immediately live cell images were captured through Nikon Ti-U eclipse fluorescence microscope equipped with AndoriXon3 897 EMCCD camerain bright field, 488 and 561nm wavelength laser lights.

FACS analysis for apoptosis: Fluorescence activated cell sorting (FACS) experiment was performed for studying the type of cell death. MCF-7 cells were seeded at a density of ~5 X 10^5 cells per well in a 6-well plate before 24 hours of treatment. Cells were incubated with solutions of DX and PMDX having concentration of drug 1000nM for 4 hours. After 4h media was changed and cells were incubated in the fresh media for another 44h. Cells were trypsinized and washed with cold PBS through centrifugation. Cells were incubated in dark at 37^{o}C for 15 minutes with a 100 µL solution of assay buffer containing 2.5 µL of PropidiumIodide (PI) and 2.5 µL of annexin V. All those reagents were supplied along with the apoptosis detection kit (Santa Cruz Biotechnology). The stock concentration of PropidiumIodide and annexin V was 50 µg/mL and 200 µg/mL respectively. After that another 400 µL of assay buffer was added to the cells and these total 500 µL of cell solution was analyzed by FACS. We have detected the emission of Annexin V and PI in the FITC and PI channels of BD LSRFORTESA flow cytometer using emission filters at 530 and 610 nm respectively. In the represented data, cells in the Q1, Q2 and Q4 quadrants are regarded as necrotic, late apoptotic and early apoptotic cells respectively. Cells in the Q3 quadrant are regarded as normal cells or healthy cells. Data was analyzed using FACS DIVA software.

Spheroid Growth Experiment: Multicellular Tumor Spheroidal (MCTS) cultures has been developed using liquid overlay method.[4] Briefly, 3000-5000 MCF-7 cells/mL cells were

collected, transferred to the 1% agarose (wt:vol) coated 35 mm dishes and incubated in a humidified atmosphere with 5% CO_2 for 4 days. After development of spheroid, growth inhibition experiment has been performed following previously standardized protocol.[4] Growth of the spheroid has been examined by change in spheroid morphological structures that has been captured in bright field mode using inverted Olympus IX83 microscope equipped with EMCCD camera. Firstly, all the spheroids were untreated and represented as Day1. Then the spheroids have been divided into untreated, DX (100 nM) treated and PMDX (100 nM) treated groups and spheroid morphology was assessed for up to day 10 days.

Volume of the sphere was analyzed using following formula as described before.[5]

$$V = 0.5 \times \text{Length} \times \text{Width}^2$$

Here, length and width was evaluated using cellSence software.

Data Analysis: Microscopic images were analyzed using Image J software.

Reference:

1. Translocation of non-natural chemical entities through anthrax protective antigen pore. 2013, Publication number: WO 2013177231 A1
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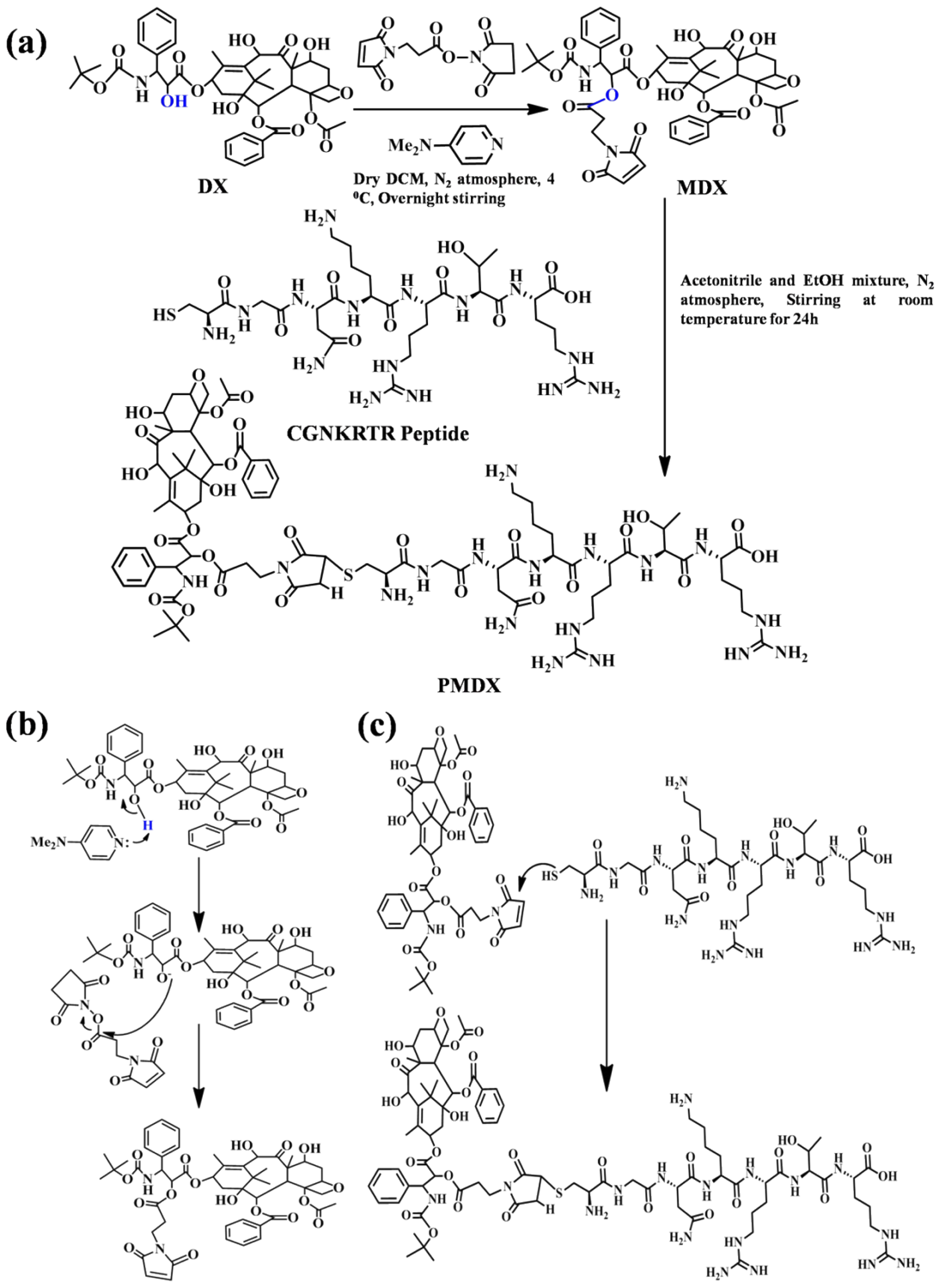
Supplementary Figures



Scheme 1. Synthetic route of PMDX.

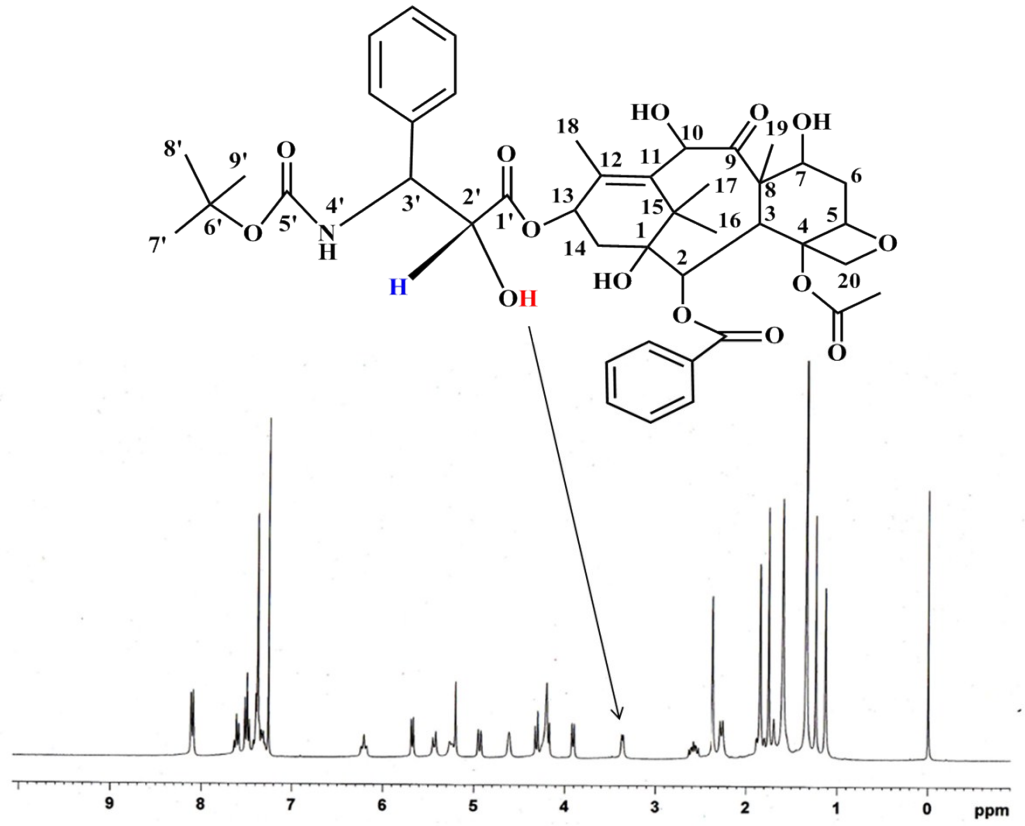


Figure S1.[1]H-NMR spectrum of docetaxel (DX).

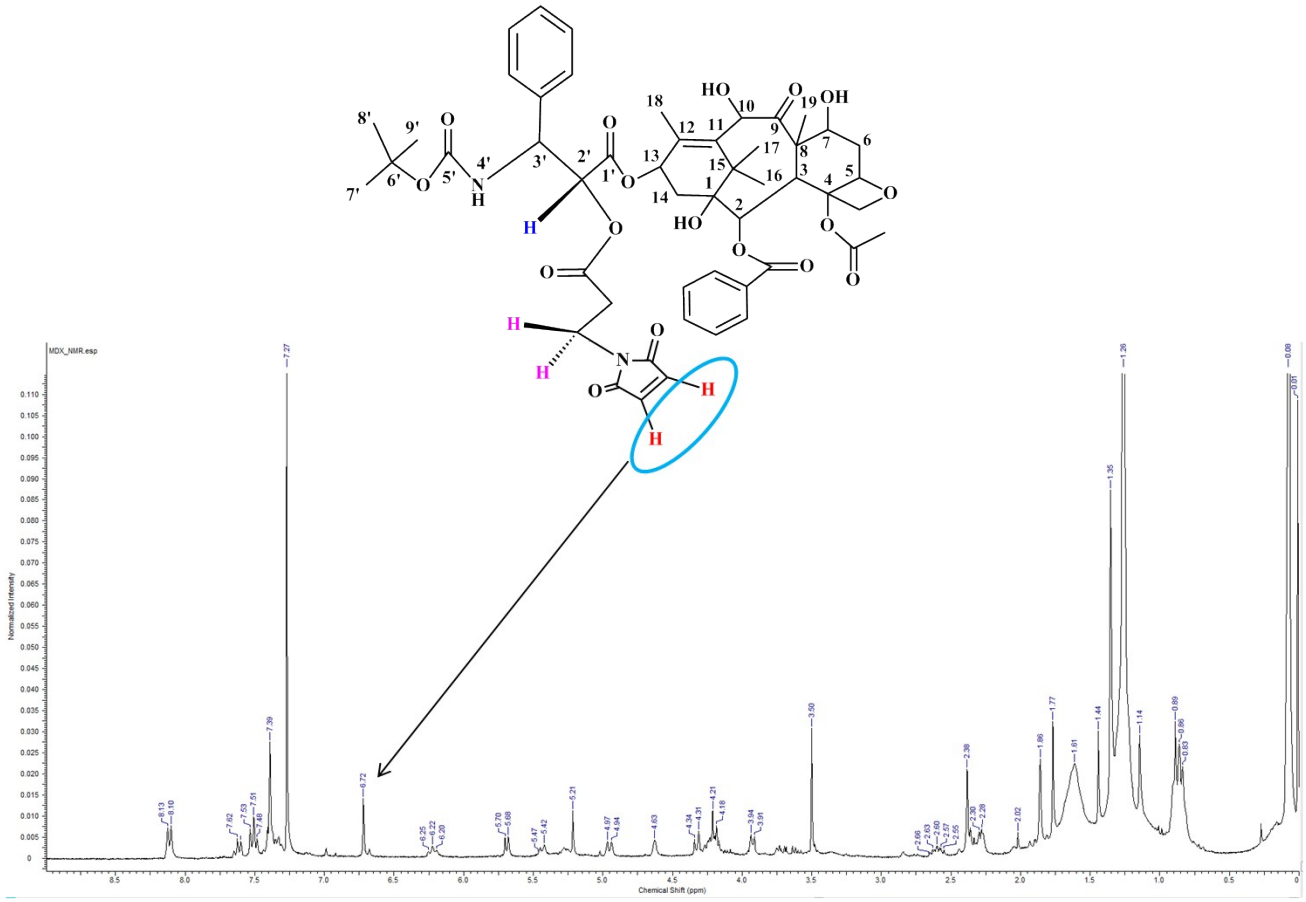


Figure S2.[1]H-NMR spectrum of maleimide modified docetaxel (MDX).

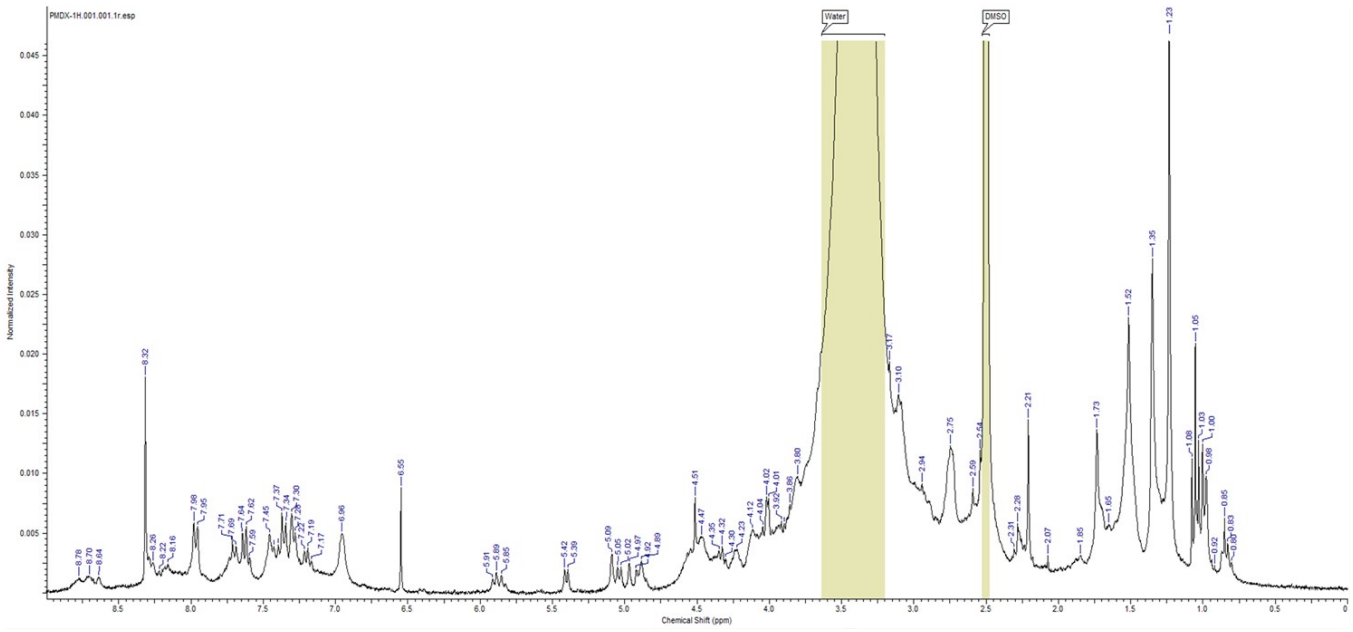
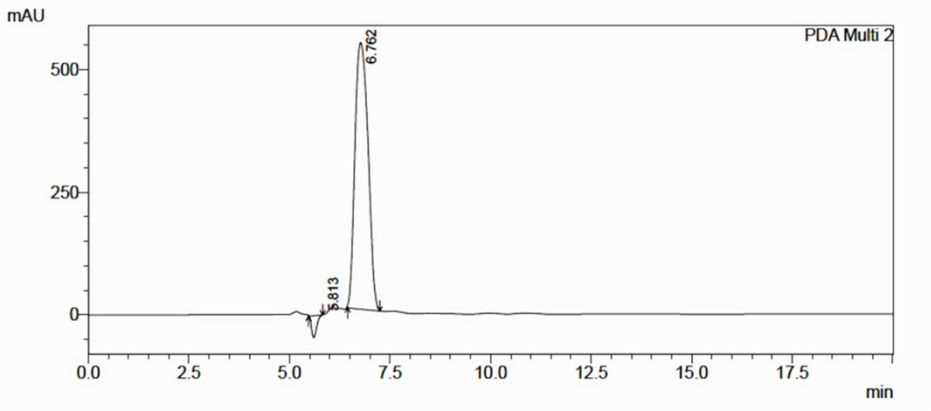


Figure S3. ^{1}H-NMR spectrum of peptide modified docetaxel (PMDX).



PeakTable

PDA Ch2 210nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	5.813	-382305	-220	-3.198	-0.040
2	6.762	12337814	543939	103.198	100.040
Total		11955509	543719	100.000	100.000

Figure S4. Analytical HPLC chromatogram of PMDX.

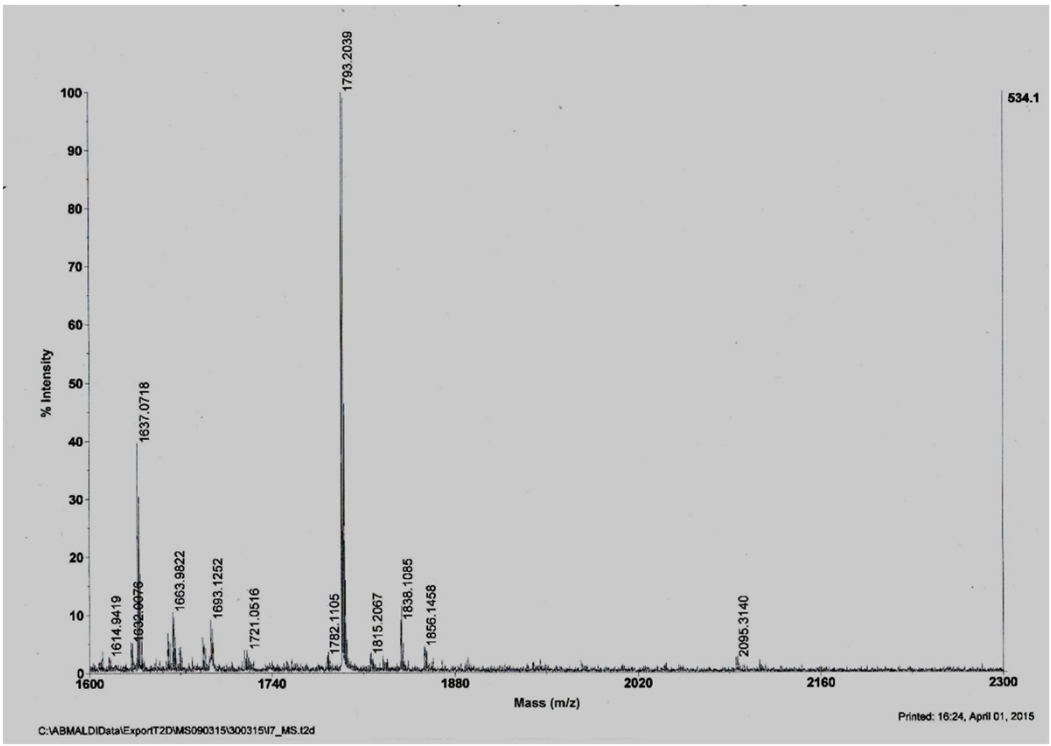


Figure S5. Mass spectrum of PMDX.

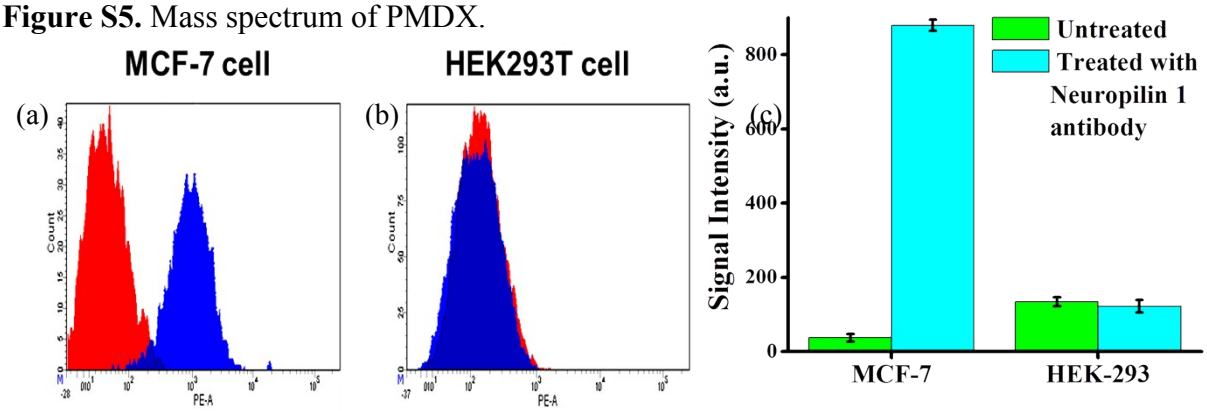


Figure S6. FACS data represents high expression of NRP-1 receptor in cell surface of MCF-7 cell (a) compared to HEK293T cells (b). Bar diagram shows higher expression of Neuropilin 1 receptor in MCF-7 cells compared to HEK293T cells (c).

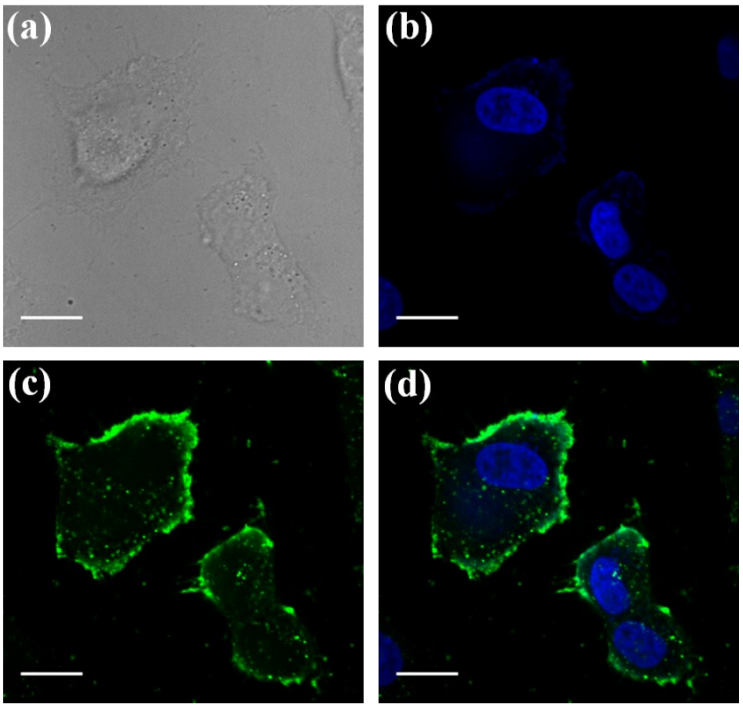


Figure S7. Fluorescence images in (a) bright field, (b) 405 channel, (c) 488 channel and (d) Merged image of 405 and 488 channels show that FITC-PMDX binds on the cell surface of MCF-7 cells. Scale bar corresponds to 20 µm.

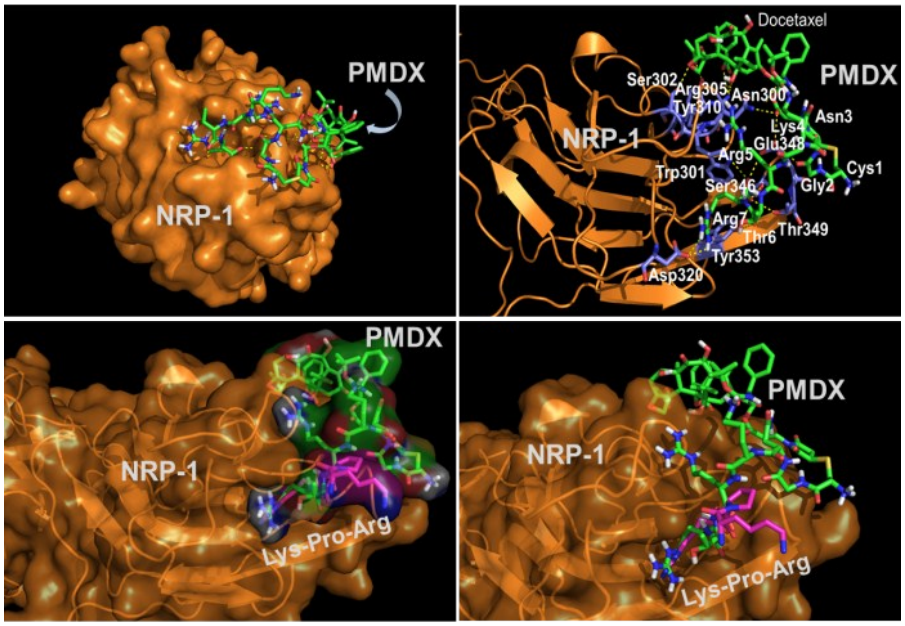


Figure S8. Docking images show interaction of PMDX (green stick structure) with neuropilin-1 (blue stick structure of amino acids on brown tape receptor protein) receptor.

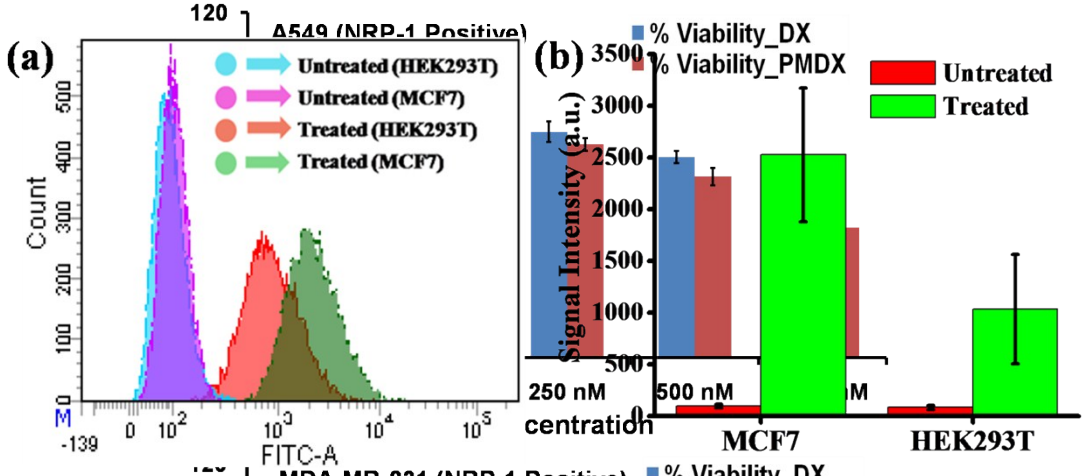
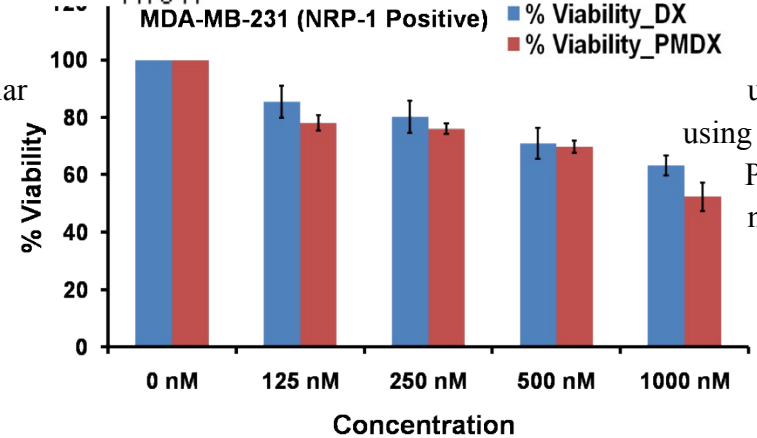


Figure S9. Cellular uptake study of FITC-PMDX using FACS shows that uptake of FITC-PMDX in MCF7 cells was much more than the HEK293T cells.



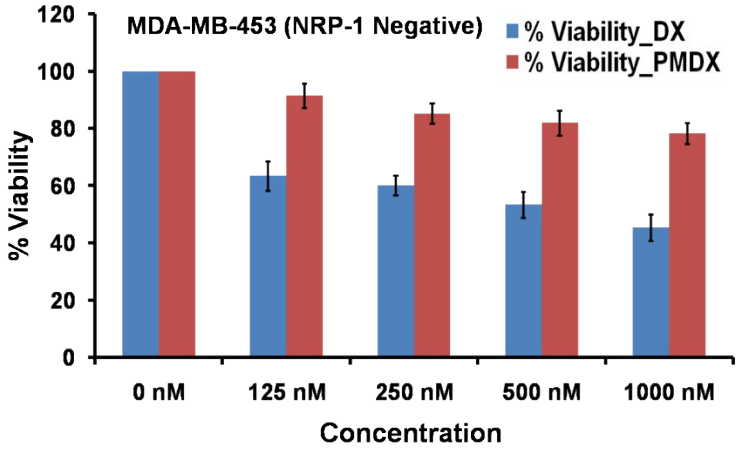


Figure S10. Cell viability using MTT assay in A549 ($NRP1^+$), MDA-MB-231 ($NRP1^+$, ER^-, $HER2^-$) and MDA-MB-453 ($NRP1^-$, ER^-, $HER2^+$) cell lines shows that PMDX causes more cytotoxicity compared to the DX in case of NRP-1 positive ($NRP1^+$) cell lines whereas it shows less cytotoxicity compared to the DX in case of NRP-1 negative ($NRP1^-$) cell line ($p<0.05$; N=6; N = number of experiments and p = statistical significance).

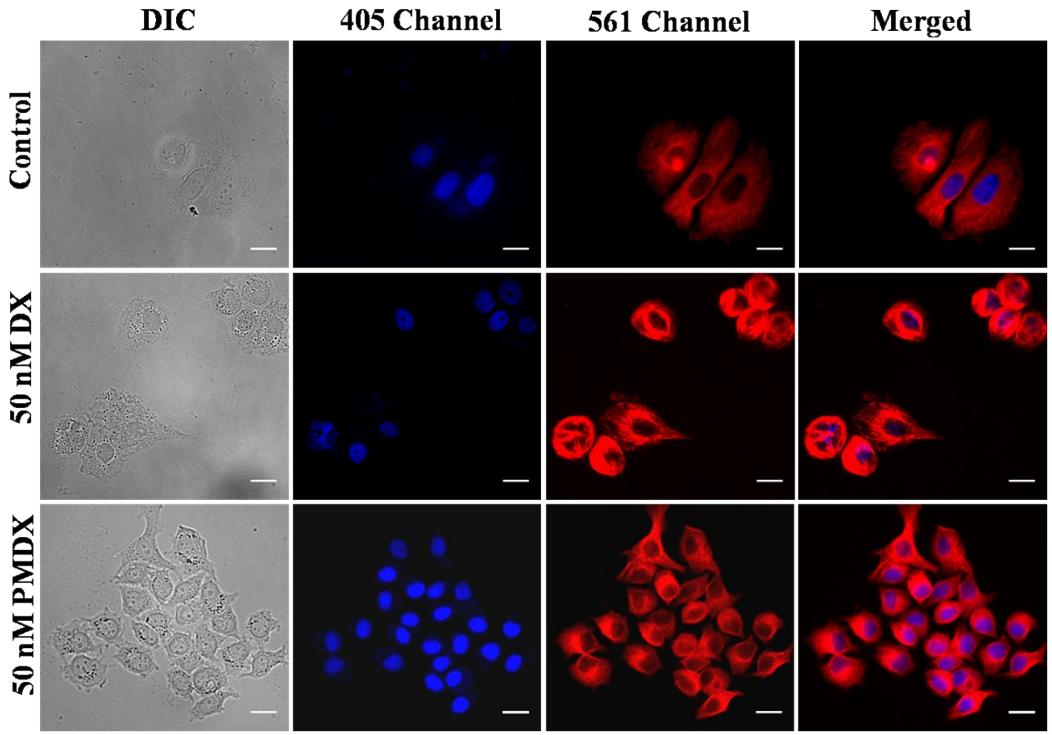


Figure S11. Microtubule network of MCF-7 cells after treatment with DX and PMDX. Scale bar corresponds to 20 μm.

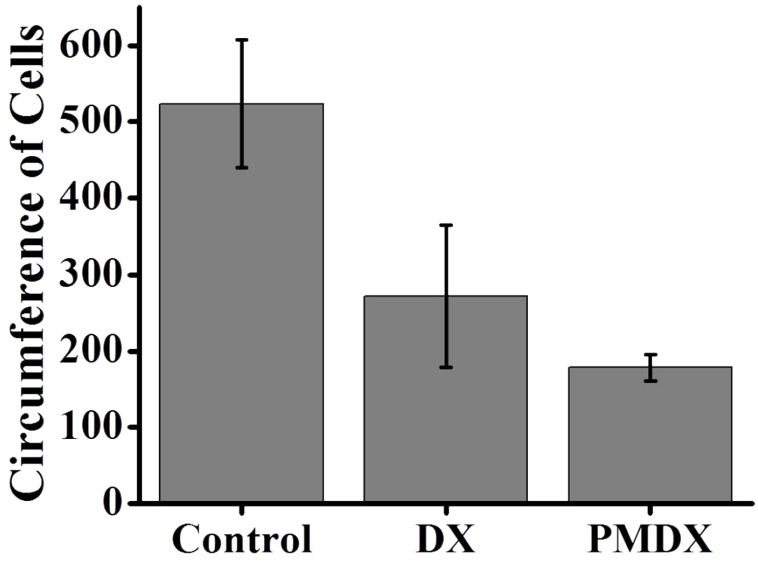


Figure S12. Circumference of MCF-7 cells after treatment with DX and PMDX.

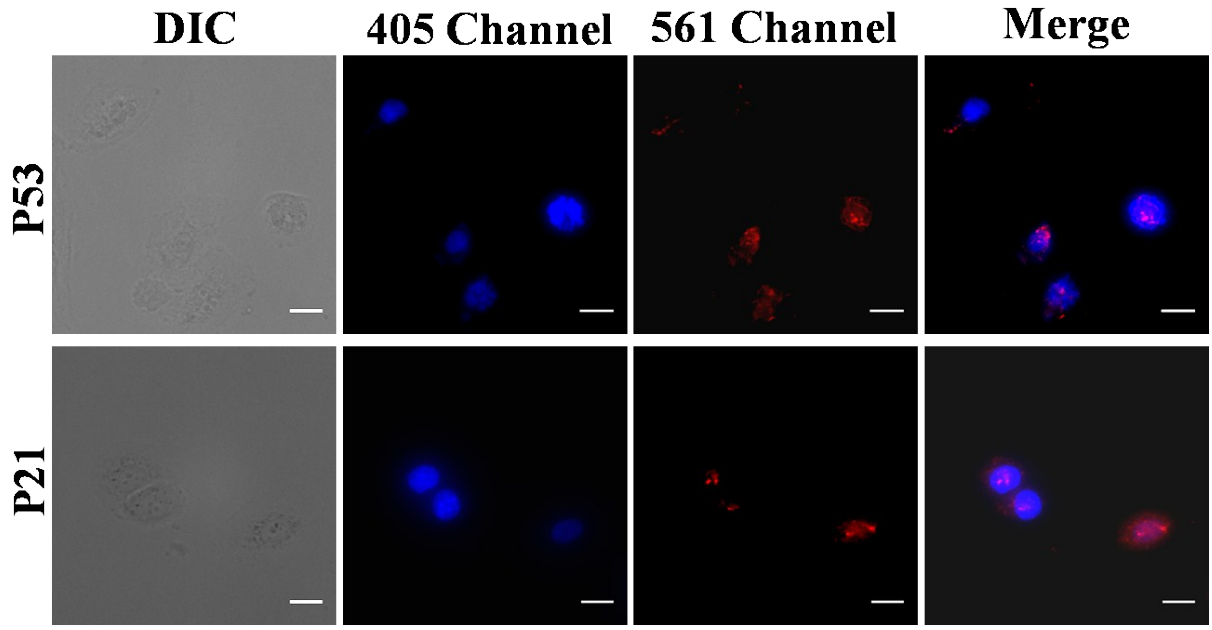


Figure S13. Activation of p53, p21 proteins after treatment with 50 nM DX. Scale bar corresponds to 20 μm.

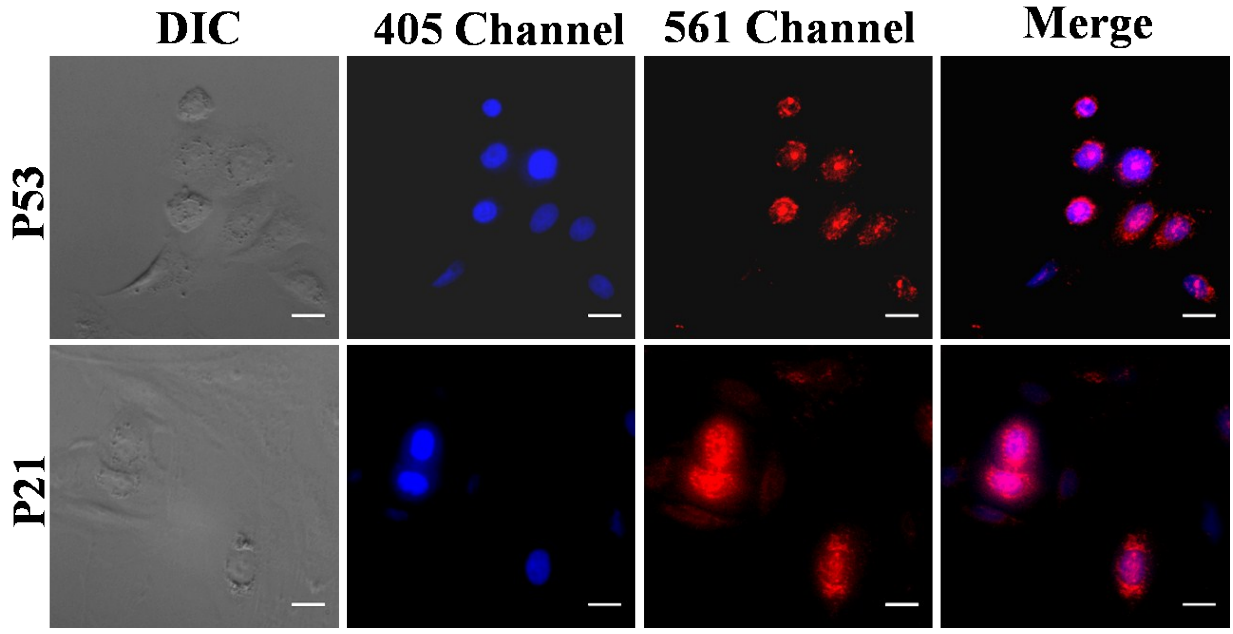


Figure S14. Activation of p53, p21 after treatment with 50 nM PMDX. Scale bar corresponds to 20 µm.

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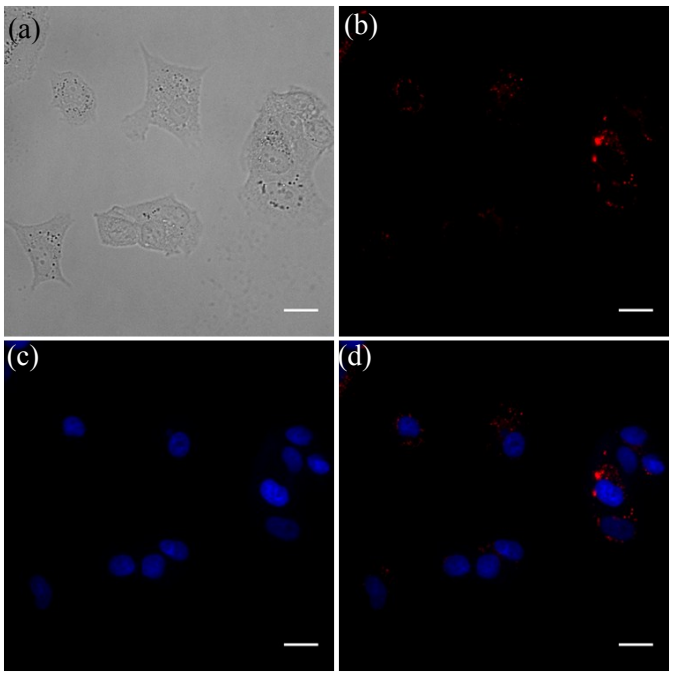

Figure S15. Fluorescence microscopic images of control cells for p53 assay. Nucleus was stained with Hoechst (blue). Fluorescence microscopic images in bright field (a); 561 nm channel (b); 405 nm channel (c) and merged image (d). Scale bar corresponds to 20 µm.

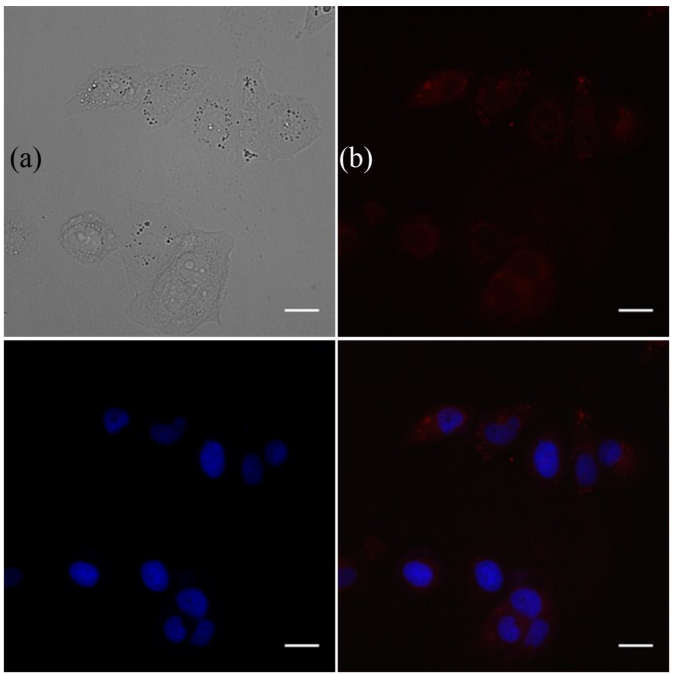

Figure S16. Fluorescence microscopic images of control cells for p21 assay. Nucleus was stained with Hoechst (blue). Fluorescence microscopic images in bright field (a); 561 nm channel (b); 405 nm channel (c) and merged image (d). Scale bar corresponds to 20 µm.

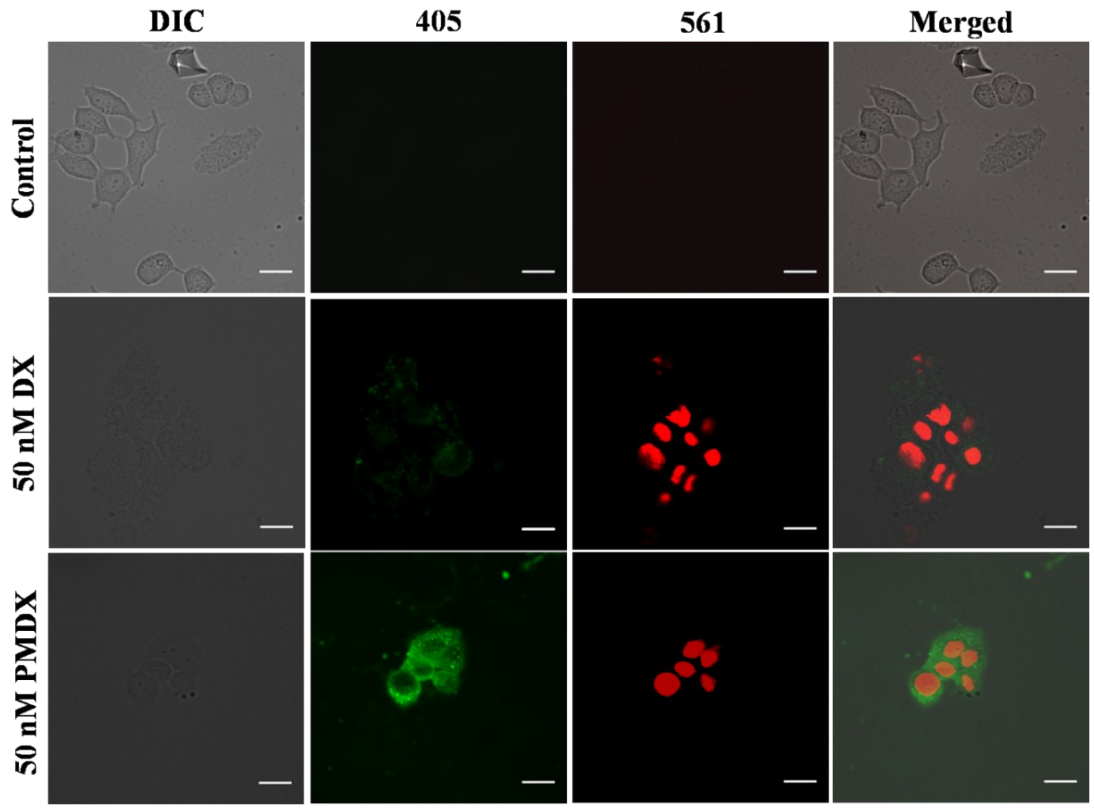

Figure S17. Qualitative determination of apoptosis and necrosis using annexinV/propidiun iodide by fluorescence microscopy. Scale bar corresponds to 20 µm.