Electronic Supporting Information

A potent tumoricidal co-drug 'Bet-CA' - an ester derivative of betulinic acid and dichloroacetate selectively and synergistically kills cancer cells

Suchandrima Saha[§], Monisankar Ghosh[§], Samir Kumar Dutta*

Drug Development Diagnostic and Biotechnology, CSIR- Indian Institute of Chemical Biology (IICB) 4, Raja S.C. Mullick Road, Kolkata-700032, West Bengal, India.

([§]S.S. and M.G. contributed equally to this work.)

* Author for correspondence: Tel No.: - 91-033-24995711/ 5792, Fax: - 91-033-24735197, 033-24723967, Email: - samirkdutta@iicb.res.in, somir_india@yahoo.com

Methods

Cell viability assay. MTT assay was employed to estimate cell viability. 10^4 cells of MCF7, MDA-MB-231, MDA-MB-468, DU 145, PC-3, B16-F10, WI-38 and NIH/3T3 were seeded onto flat bottom 96-well plates and incubated at 37°C in 5% CO_2. Cells were treated with 5, 10, 15, 20, 30, 50 and 70 µM of Bet-CA and incubated for 72 h. 20 µl of 5 mg/ml MTT labelling reagent was added and absorbance of the samples were measured at 570 nm using Thermo MULTISKAN FC plate reader.

Chromosomal aberration and micronuclei formation. For cytotoxicity study venous blood from five individual donors was collected in EDTA coated tubes and informed consent was obtained from all the respective individuals. The experimentations were approved by the Institutional Human Ethics Committee and performed in concordance to the institutional ethics committee's guidelines. Lymphocytes from blood were separated using ficoll-gradient centrifugation (Histopaque-1077) and cultured in RPMI-1640 for 72 h followed by incubation with 100 µM of Bet-CA for another 72 h. As positive controls, standard dose of Mitomycin C and Cyclophosphamide were used for chromosomal aberration and micronuclei (MN) formation assay respectively. For chromosomal aberration study, 0.04% of Colchicine was added to the culture during the

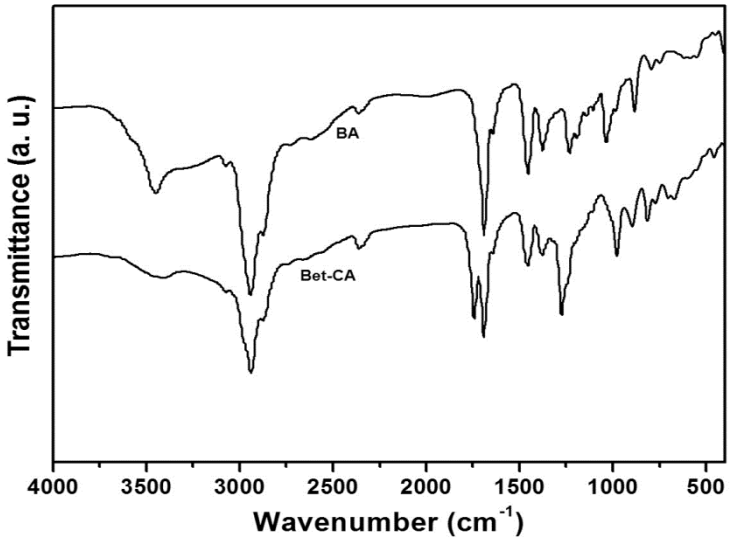
last 2 h of incubation and in case of MN formation 6 µg/ml of Cytochalasin B was added 4 h before harvesting the cells. Slides were prepared, stained with giemsa and observed randomly for deducing the mitotic index and scored for chromosomal aberrations at 100X. Data derived is expressed in percentage. For MN detection 1000 cells from each slide was scored at 60X magnification.

Western blot. Cell pellets were resuspended in NP-40 cell lysis buffer (1% NP40, 50 mM Tris (pH 8.0), 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, protease inhibitors containing 10 µg/ml aprotinin, 5 µg/ml pepstatin, 10 µg/ml leupeptin, and 50 µg/ml PMSF, 50 mM NaF, 0.1 mM $NaVO_4$), left on ice for 30 minutes and centrifuged. The protein concentrations of the supernatant were determined using Bradford protein estimation assay kit. A total of 50 µg protein was separated using SDS-PAGE and subjected to immunoblotting using primary antibodies against Bax and β-actin and the later was used as loading control. Bands were detected using Gel Logic imaging systems (Carestream, Kodak).

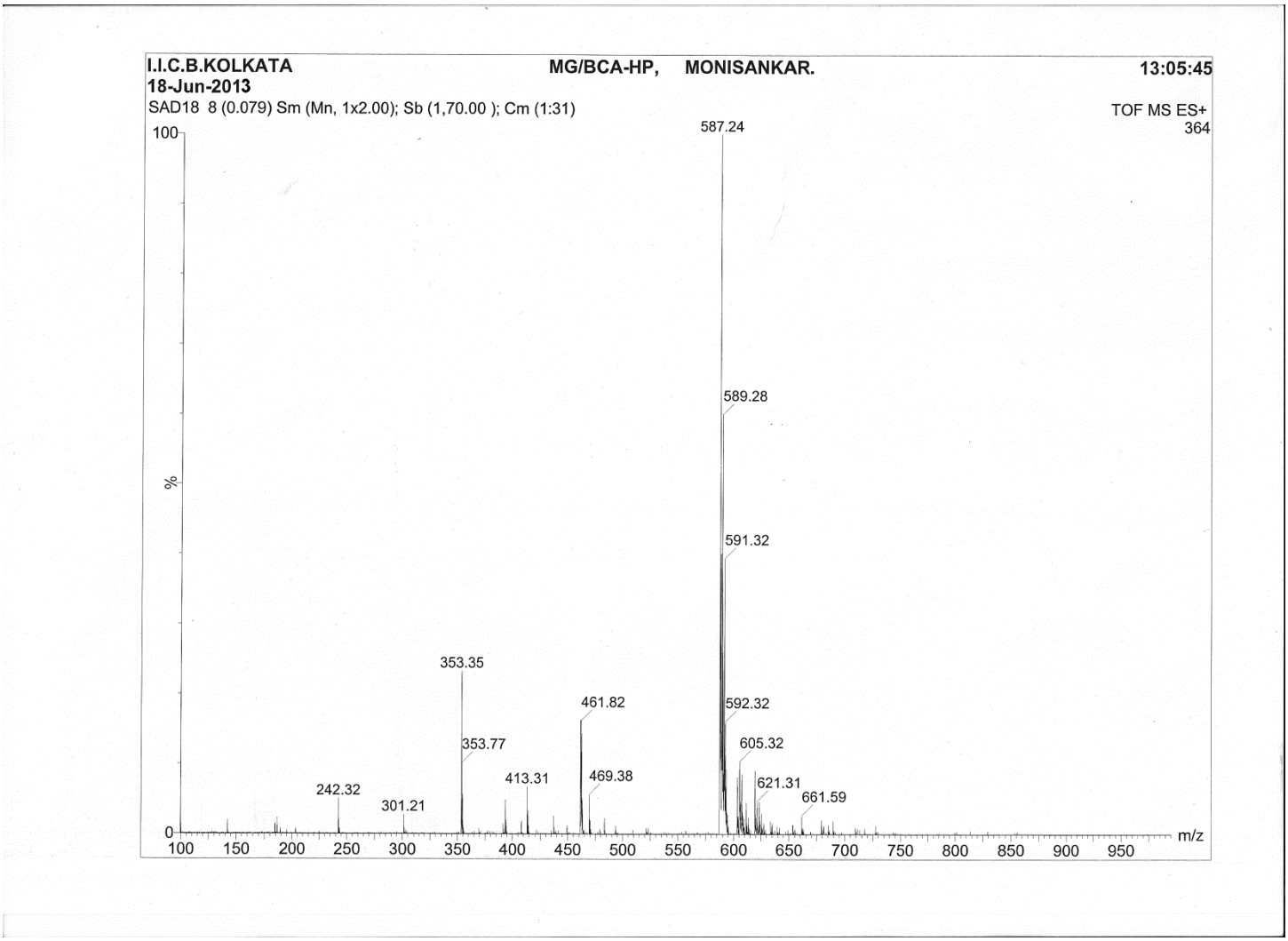
Apo-Brdu TUNEL assay for apoptosis. Fragmentation of DNA in apoptotic cells was detected using TUNEL assay. Control and treated cells were fixed with 4% formaldehyde for 15 min at 4 °C, permeabilized using 70% ethanol and stored at -20°C overnight. Post washing, control and experimental sets were labelled using 5-bromo-2′-deoxyuridine 5′-triphosphate (BrdUTP) and incubated for 1 h in DNA-labelling solution at 37°C by swirling gently. Subsequently, alexa fluor-488 labelled anti-BrdU antibody was added and incubated for 30 min and finally PI/RNase A staining buffer was added and incubated for 30 min at room temperature. Cells were deposited on slides and visualized using CLSM. Images were captured at 20X randomly from 10 different fields and the percentage of TUNEL positive cells was calculated.

Histology. Tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin. 5 µm thin sections were prepared from lung, liver, spleen and kidney paraffin embedded blocks. The slides were subjected to staining using standard haematoxylin/ eosin staining protocol for histology.

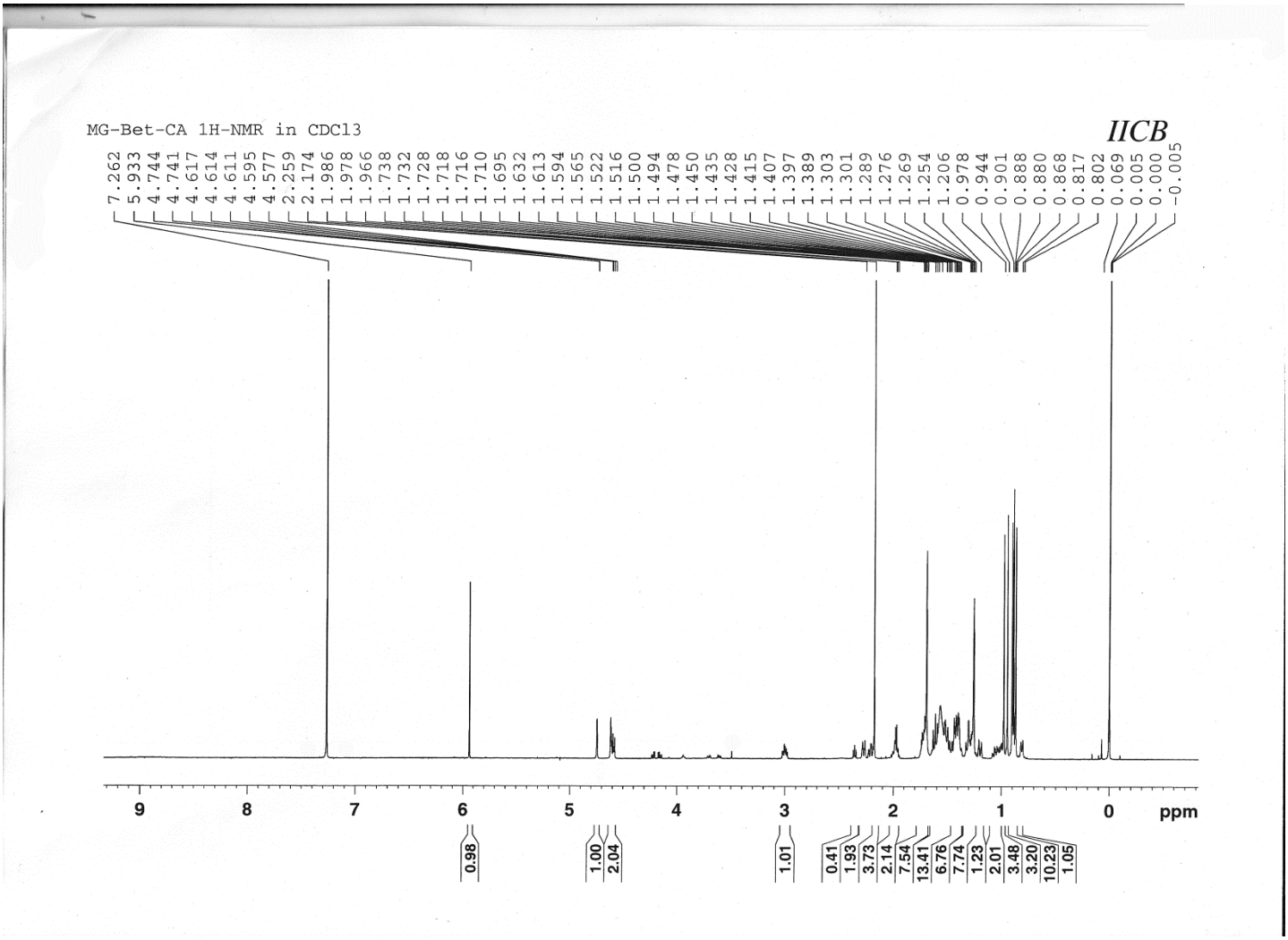
Supplementary Figures



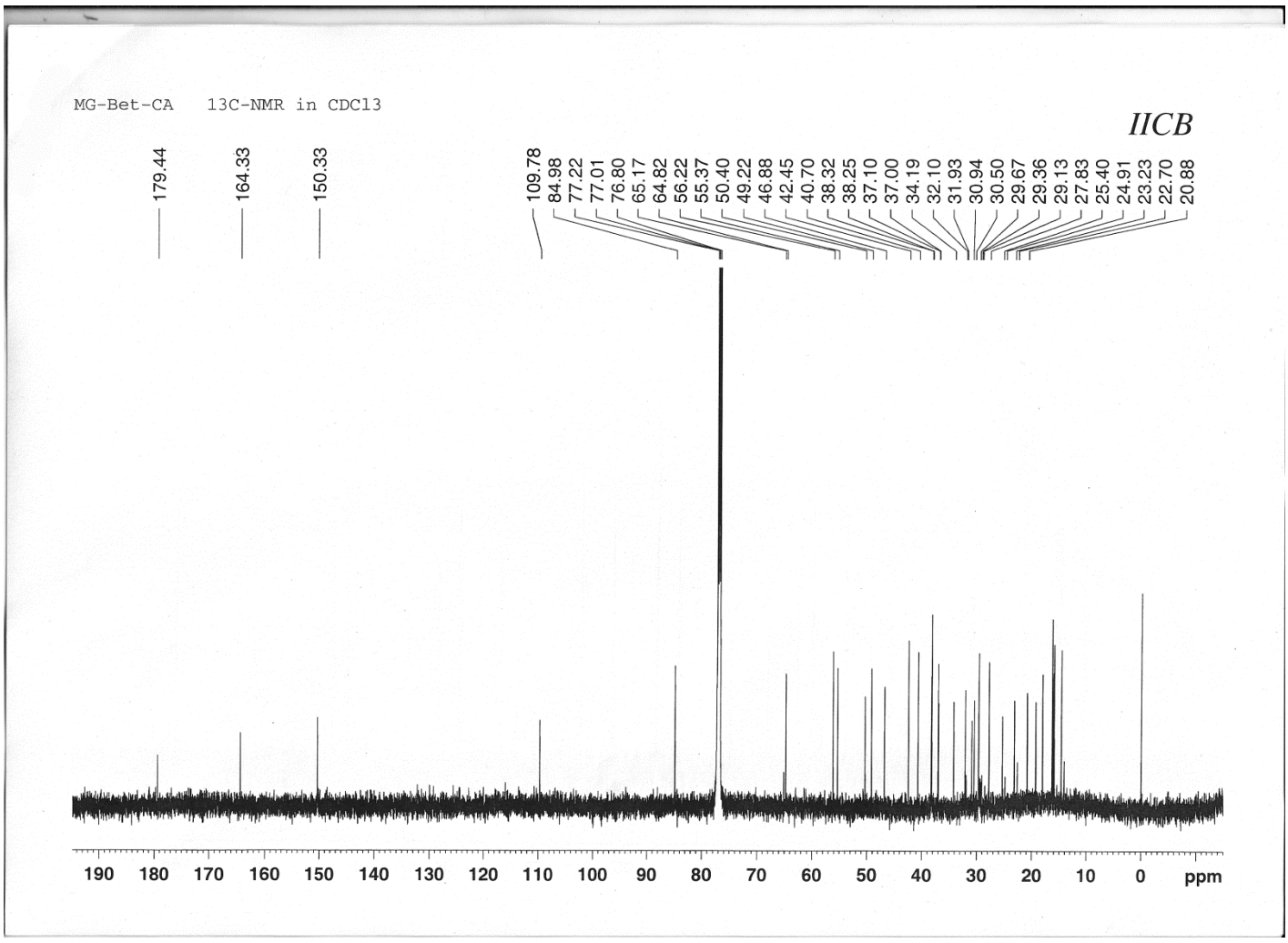
Supplementary Figure S1| IR spectra of BA and Bet-CA.



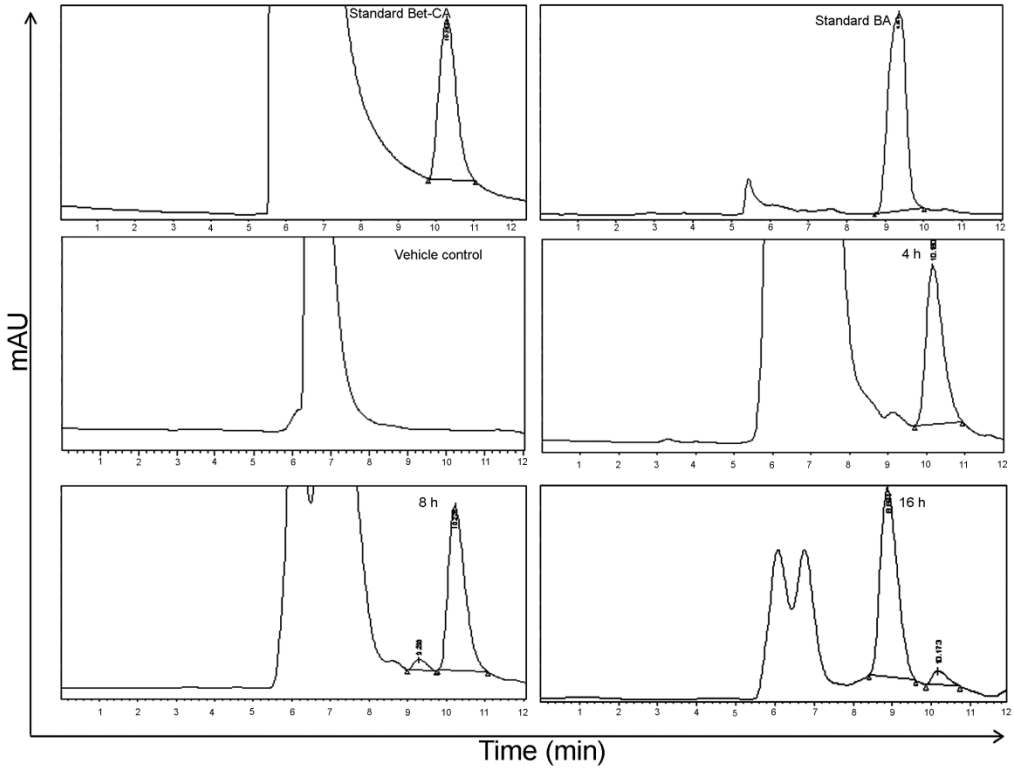
Supplementary Figure S2 | Mass spectra of Bet-CA.



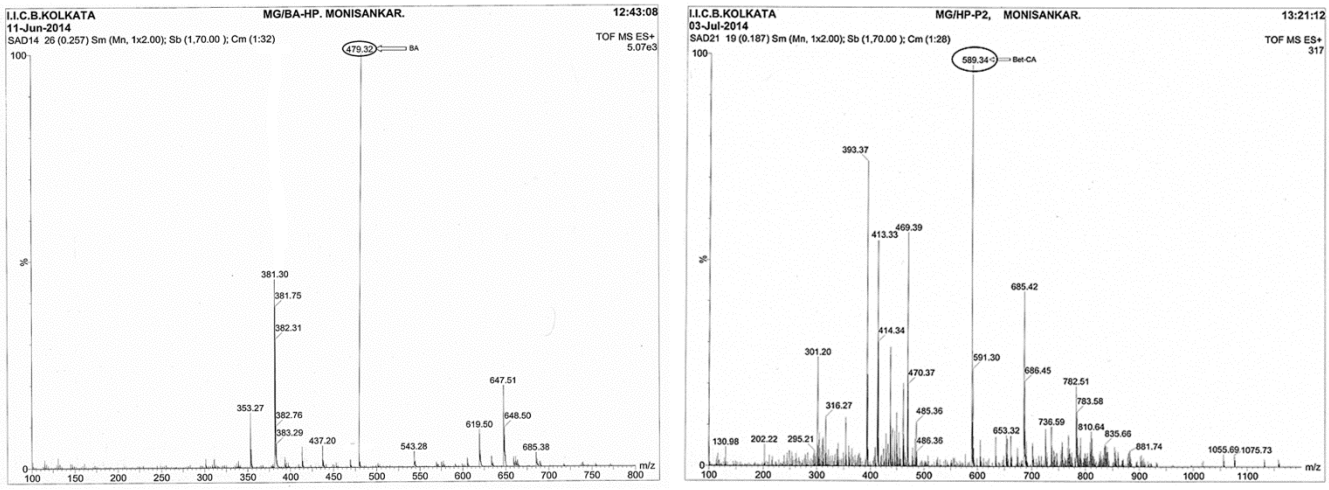
Supplementary Figure S3 | ^{1}H-NMR spectra (600MHz, $CDCl_3$) of Bet-CA.



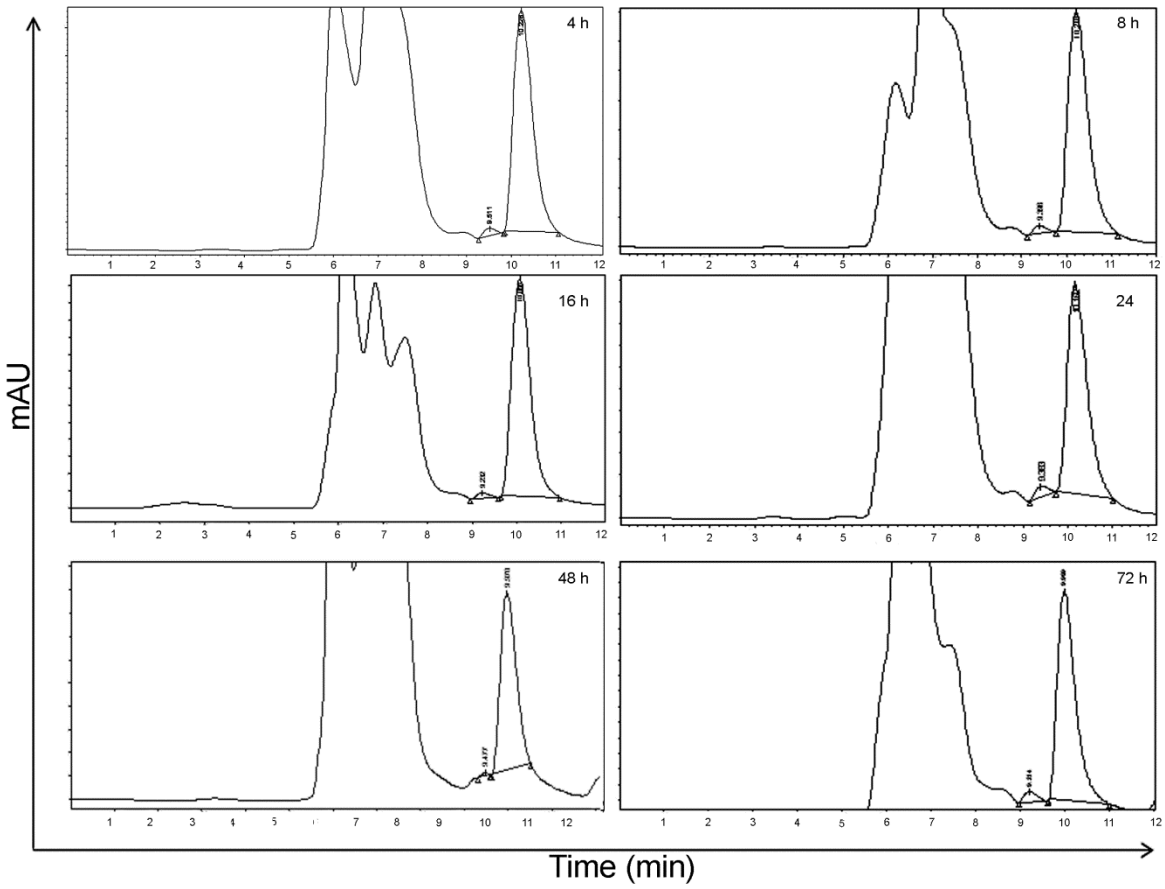
Supplementary Figure S4 | 13C-NMR spectra (600MHz, CDCl3) of Bet-CA.



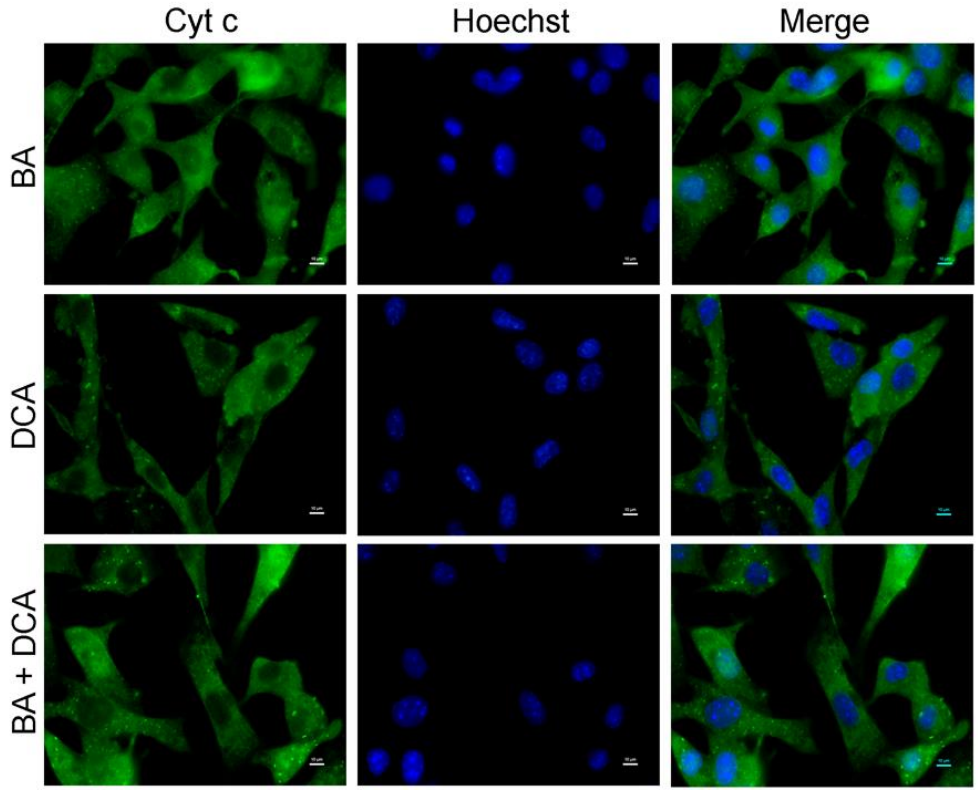
Supplementary Figure S5 | Representative HPLC chromatograms depicting intracellular accumulation and subsequent cleavage of Bet-CA at 4, 8 and 16 h.



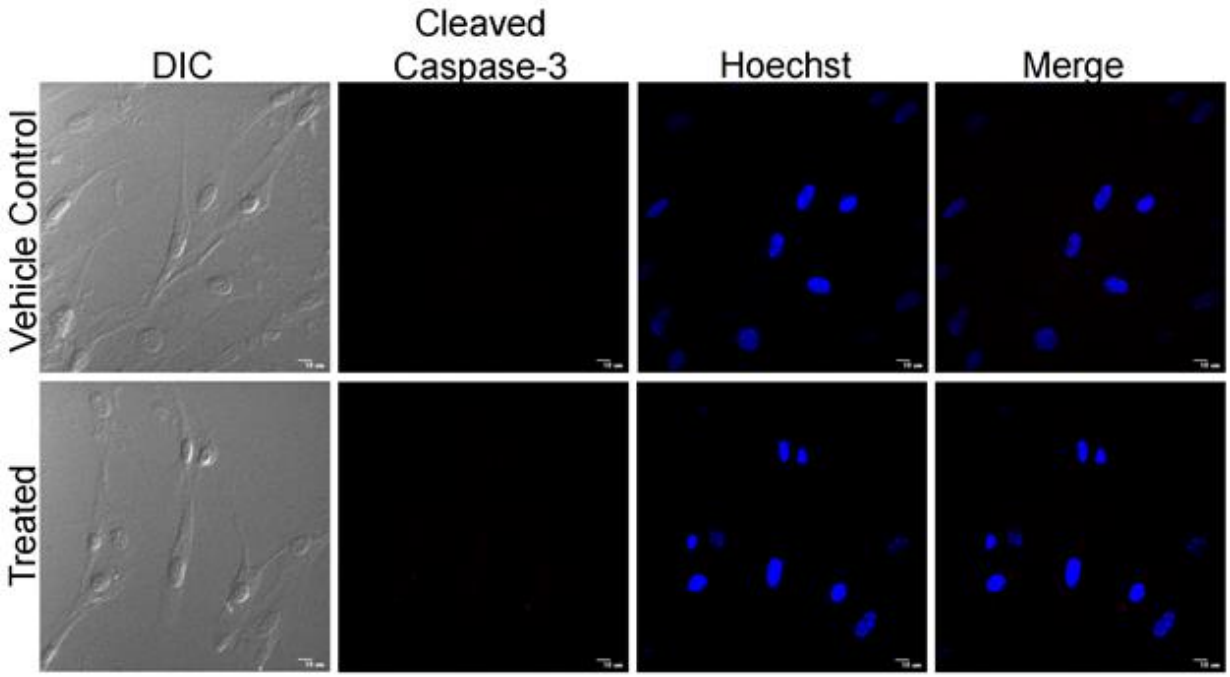
Supplementary Figure S6 | Representative ESI-MS analysis of BA eluted at 9.2 min and Bet-CA eluted at 10.2 min from the experimental sets.



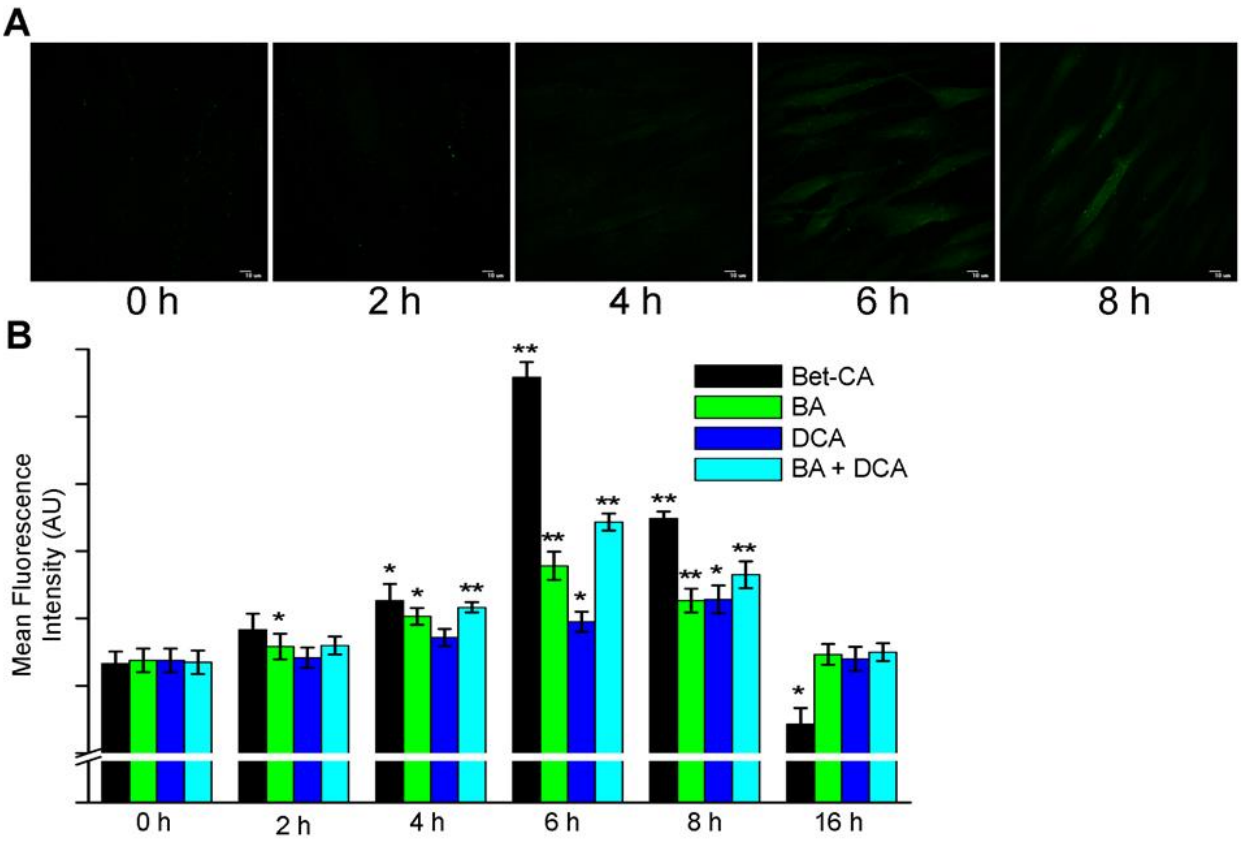
Supplementary Figure S7 | Representative HPLC chromatograms depicting extracellular cleavage of Bet-CA at 4, 8, 16, 24, 48 and 72 h.



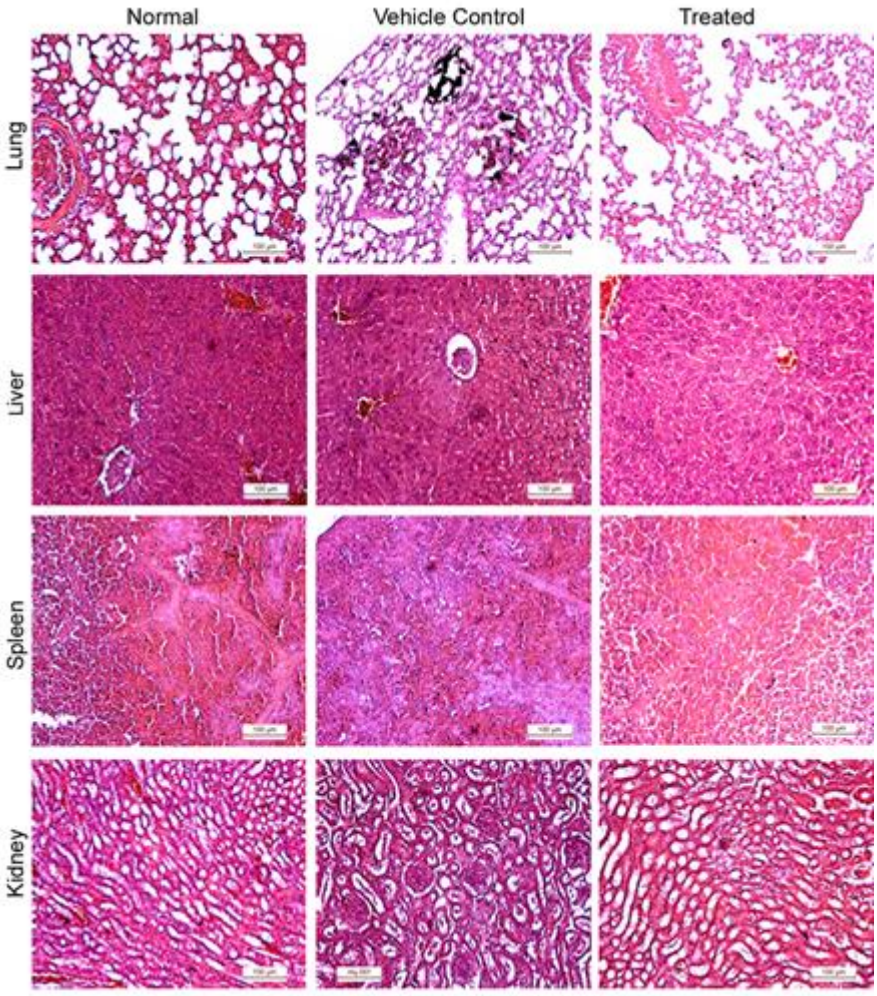
Supplementary Figure S8 | Representative confocal micrographs demonstrating Cyt c release in MCF7 cells upon treatment with 50 μM each of BA, DCA and BA+DCA (1:1).



Supplementary Figure S9 | WI-38 cells were exposed to 50 μM of Bet-CA for 16 h and release of cleaved caspase-3 was studied employing CLSM. Images show absence of cleaved caspase-3 emphasising the selective killing potential of Bet-CA.



Supplementary Figure S10 | (A) Representative confocal images depicting the generation of ROS after treatment with 50 μM of Bet-CA on WI-38 cells for varying time points. There was no significant generation of green DCF fluorescence as observed. (B) Graphs demonstrating the accumulation of ROS for varying time points in MCF7 cells after treatment with 50 μM of Bet-CA, BA, DCA and BA+DCA (1:1) respectively. (* $p < 0.05$, ** $p < 0.01$, compared to the vehicle control). In all panels error bars represent mean ± SD



Supplementary Figure S11 | Representative micrographs of normal, vehicle control and Bet-CA treated organs (lung, liver, spleen and kidney) of tumor bearing mice. The panels show similar staining patterns and morphological characteristics on virtually all control and treated organ sections affirming lack of Bet-CA's cytotoxic potential.