Figure legends

Figure 1. ~~2D chemical s~~Structure of cerberin (CR) ~~with numbering of atoms shown~~.

Commented [MB1]: It's actually a 1D representation of a 3D structure…

Figure 2. Growth inhibitory effects and cell count of CR.

(**A**) Representative graph showing growth inhibitory effects of CR on PANC-1 and A549 cells. Cells were seeded at a density of 3×10^3 cells/well in 96-well plates and incubated for 72 h (n = 6; ≥3 trials). Effects of CR on PANC-1 (**B**) and A549 (**C**) cell numbers. Cells (2×10^4 cells/well) were seeded in 6-well plates and incubated overnight before treatment with CR (10 – 200 nM; 72 h). Cells were harvested and counted by haemocytometer (n = 2; ≥3 trials).

Figure 3. Mean survival fraction (%) of CR treated cells.

(**A** and **B**) Mean ± SEM survival fraction (%) of treated cells as a percentage of the control population for A549, MDA-MB-468, MDA-MB-231, PANC-1, HK-1, HCT-116, HT-29, MCF-7 and MIA PaCa-2 cells. CR caused a significant reduction in colony formation (****p < 0.0001, n = 2 for each of the 3 trials). (**C**) Representative photograph showing the effect of CR on PANC-1 colony formation at control, 1 × GI_{50} and 2 × GI_{50}.

Figure 4. Effects of CR on cell migration in PANC-1, MDA-MB-468 and A549 cells.

(**A-C**) CR inhibited migration of PANC-1, MDA-MB-468 and A549 cells; wounds were evaluated after 24 ~~h~~ and 48 h exposure to CR at 1 ~~x~~ and 2 ~~x~~ GI_{50} values. CR induced significant (****p <0.0001) inhibition of cell migration compared to the control with medium only. Mean ± SD ≥3 independent trials (n=2). (**D-F**) Representative microscopic images from one of the experiments demonstrating potent inhibition of cell migration of PANC-1, MDA-MB-468 and A549 cells by CR (1 × GI_{50} and 2 × GI_{50}). Image J was used to measure the surface area of the wound.

Figure 5. CR-induced DNA DSBs in PANC-1, MDA-MB-468 and A549 cells.

(**A**) CR (2 x GI_{50} value) induced DNA DSBs in PANC-1, MDA-MB-468 and A549 cells after 24 h exposure. Etoposide (2 µM) was used as a positive control as it is a known DNA damaging agent. CR caused a significant increase in γ-H2A.X formation (**p < 0.01, ***p < 0.001 and ****p < 0.0001 vs untreated control). (**B**) Representative γH2A.X dot plots from an independent trial illustrating induction of DNA DSBs in PANC-1, MDA-MB-468 and A549 after 24 h of CR treatment. Experiments were repeated at least 3 times (n=2).

Figure 6. Effect of CR on cell cycle in PANC-1, MDA-MB-468 and A549 cells.

PANC-1 (**A**), MDA -MB-468 (**B**) and A549 (**C**) cells were treated with 1 ×~~x~~ GI_{50} and 2 ×~~x~~ GI_{50} CR for 24, 48 and 72 h. CR evoked significant arrest in the G2/M phase (*p < 0.01, ***p<0.001 and ****p<0.0001; experiments were repeated ≥3 times, n = 2).

Figure 7. Effects of CR on PANC-1 (A), MDA-MB-468 (B) and A549 (C) apoptosis.

Cells were treated with 1x and 2x GI_{50} CR for 24, 48 and 72 h. Annexin-V/PI apoptosis assays were performed to determine the percentage of apoptotic cells. Total apoptosis comprises early apoptotic (annexin V-positive and PI negative) and late apoptotic (annexin V-positive and PI positive) populations. Mean ± SEM ≥3 independent trials (n = 2 per trial; 10,000 events were analysed per sample).
(**D**) Effect of CR on caspase 3/7 activity in PANC-1, MDA-MB-468 and A549 cells after 24 h exposure to 1 ×~~x~~ GI_{50} and 2 ×~~x~~ GI_{50}.
Mean ± SEM ≥3 independent trials (n = 2 per trial). CR caused significant increase in caspase 3/7 activity (*p < 0.05, **p<0.01, ***p<0.001 and ****p<0.0001; experiments were repeated ≥3 times, n = 2).

Figure 8. CR induced significant levels of ROS production in PANC-1, MDA-MB-468 and A549 cells.

Cells were treated with CR (1 xGI_{50} or 2 xGI_{50}) or vincristine (10 nM). Mean ±SD ROS generation is depicted as a percentage of untreated control (***p<0.001 and ****p<0.0001, n = 2 for each of 3 independent trials).

Figure 9. (**A**) Representative Western blots of lysates prepared from PANC-1, MDA-468 and A549 cells exposed to 2 x GI_{50} CR for 24 ~~h~~ or 72 h; extracted proteins were separated by SDS-PAGE and analysed using antibodies to detect whole and cleaved PARP, Mcl-1, Bcl-2, PLK-1 and housekeeping gene GAPDH. (**B-F**) Collated densitometric measurement of protein expression levels; PARP cleavage was observed at 2 ×~~x~~ GI_{50} and was accompanied by a time-dependent decrease in Mcl-1 and Bcl-2. PLK-1 downregulation was observed in all three cell lines. GAPDH was used as an internal loading control. Data represent the mean ± SD of 3 independent experiments (ANOVA followed by Dunnett's test). Significance is reported as: *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 compared to controls.

Figure 10. (A) Representative Western blots of lysates prepared from PANC-1, MDA-468 and A549 cells treated with 2 ×~~x~~ GI_{50} CR for 24 or 72 h; extracted proteins were separated by SDS-PAGE and analysed using antibodies to detect total and phosphorylated PI3K (p-PI3K), AKT (p-AKT), 4EBP1 (p-4EBP1), STAT3 (p-STAT3) and housekeeping gene GAPDH.

E) Collated densitometric analyses of protein expression levels; time-dependent downregulation of p-PI3K, p-AKT, p-4eBP1 and p-STAT3 were observed at 2 ×~~x~~ GI_{50} CR after 24 ~~h~~ and 72 h treatment; no significant changes occurred in total AKT, 4eBP1, PI3K and STAT3 protein expression. GAPDH was used as an internal loading control. Data represent the mean ± SD of 3 independent experiments (ANOVA followed by Dunnett's test). Significance is reported as: *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 compared to controls.

Figure 11. Confocal microscopic illustrations of disruption in cytoskeletal architecture.

Confocal microscopy illustrates the effects of CR and vincristine (24 h exposure) on MDA-MB-468 cell morphology. 1st row (**A**–**D**): untreated with vehicle only, arrowhead indicates typical morphology of nuclei, cell division and microtubule alignment; 2nd row (**E**–**H**): vincristine (10 nM); 3rd& 4th row (**I**–**P**): CR (1 ×~~x~~ GI_{50} = 73.7 nM). CR caused multinucleation (1), nuclear fragmentation (2), membrane blebbing (3), the lunate morphology of chromatin and chromatin condensation (4), membrane disruption of nuclei (5), tubulin network disruption (6) and uneven cell division (7). CR and vincristine both showing similarity in induced morphological changes. The experiments were repeated 3 times. Cells were immunostained with an antibody specific for α-Tubulin (green) and counterstained with DRAQ5 (purple).

Figure 12. *In silico* simulation of pharmacokinetics of CR in mice

(**A**) Predicted plasma concentration of cerberin following intravenous, oral and subcutaneous administration at 10 mg/kg. (**B**) Predicted unbound concentration of cerberin in plasma following oral administration at a range of doses. The orange dotted lines are the GI_{50} values of CR obtained in this study for MDA-MB-468, A549 and PANC-1 cell lines, respectively, from top to bottom. (**C**) Amount of cerberin predicted to be absorbed in each compartment of the gastrointestinal tract following oral administration of cerberin at 10 mg/kg in fasted or fed state.