Synergistic effect of curcumin in combination with proapoptotic drugs in human cell models for Chronic (CLL) Lymphocytic Leukaemia

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INTRODUCTION

Curcumin is a phenolic compound abundantly present in the rhizome of *Curcuma longa* that has been extensively studied in cancer for its proapoptotic, immunomodulatory and antiproliferative properties. It has activity as a metabolic enzyme inhibitor, affecting a wide variety of cancer survival mechanisms and pathways, enhancing the effect of some drugs and reversing cancer chemoresistance to others. The molecular mechanisms are varied, which can result in low rates of drug resistance and very low toxicity at active concentrations. CLL is a hematological disease originated by a pathologic expansion of B cells due to a reduced sensitivity to apoptosis. CLL cells can mature partly but not completely, have relatively lower division rates, and increased resistance to apoptosis, so it is very difficult to achieve complete remission. New therapies aimed at the eradication of refractory cells have become a priority to prevent the recurrence of cancer.

MATERIALS AND METHODS

Curcumin used in this study was obtained from Naturex (95% purity). Anticancer etoposide, camptothecin and colchicine drugs were obtained from Sigma Aldrich (stock solutions 1mg/mL, 1mM and 1mg/mL respectively). CLL cell models: three cell lines generated from CLL (I83, Mec1 and EHEB) were used to study curcumin effects in combination with anticancer drugs. CLL cells were incubated in a cell incubator at 37ºC and 5%CO_2 with different concentrations of curcumin (1nM-10µM) in combination with drugs (1nM-0.5µM), and analyzed at different incubation times (0, 24, and 72h). Cell membrane integrity and cell cycle phases were evaluated using the Attune™ NxT Flow Cytometer (Thermo Fisher), equipped with acoustic focusing, a special feature only available on this instrument that allows to achieve sample-throughput rates up to 10 times faster than traditional cytometers. Cell staining with PI and DAPI was performed for cell membrane integrity assessment and cell cycle analysis respectively. In order to avoid the possibility of color crosstalk with the emission of curcumin and etoposide, PI was excited with the 561nm yellow green laser. Drug toxicity results were analyzed using student's two tailed t-tests (P<0.05) with Microsoft Excel Software for cell membrane integrity and FCS Express 5 Research version (De Novo Software™) for cell cycle results. May Grünwald-Giemsa (Merck) staining was used for the morphological assessment of the cells. Cytospin microscopic slides was prepared using freshly harvested cells (0, 24, and 72h).

RESULTS AND DISCUSSION I

Cell membrane integrity analysis. Experiments using I83 cells show the synergistic effect of curcumin in combination with etoposide, camptothecin and colchicine (Figure 1). Respectively calculated LD50 values were as follows: 0.67µM, 8.9nM, and 6.6nM vs. 0.52µM, 4.8nM, and 3.6nM when combined with curcumin 2.5µM, supporting that this phenolic compound enhances drug proapoptotic effects by achieving the same cytotoxic effect at lower drug dose concentration. We obtained similar results with Mec 1 and EHEB cell lines that have been omitted for brevity (data not shown).

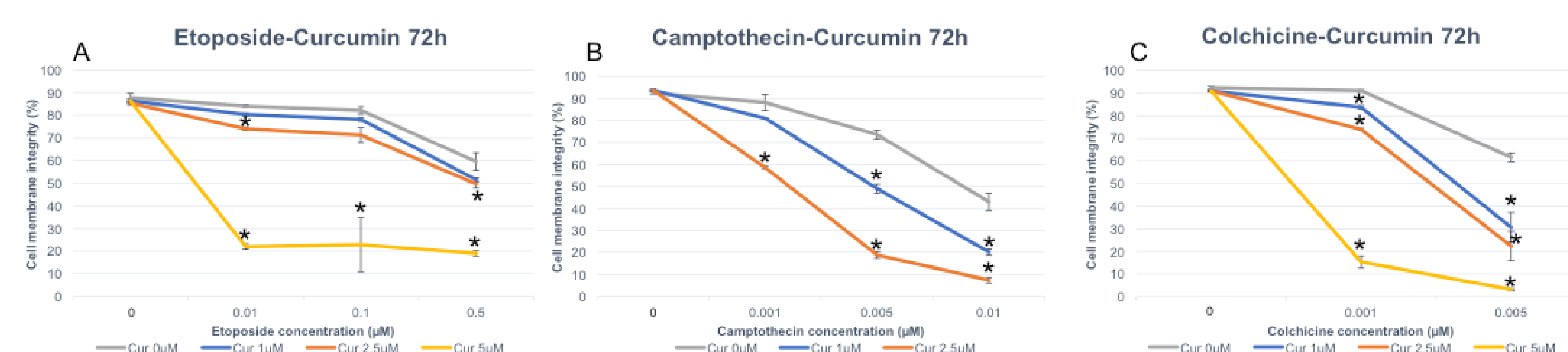

Figure 1. Cell membrane disruption and enhanced drug effect in the presence of curcumin. Etoposide, camptothecin, and colchicine induced cytotoxicity mediated by curcumin as shown in A, B and C respectively. The loss of cell membrane integrity was measured by flow cytometry on the Attune NxT, and calculated to reflect the number of propidium iodide (PI) negative cells in a FSC vs. PI fluorescence dot-plot. Significance (P < 0.05) is indicated by asterisk.

RESULTS AND DISCUSSION II

In terms of cell membrane integrity, etoposide was the less toxic drug, followed by camptothecin and colchicine. Furthermore, camptothecin and colchicine LD50 values dramatically decreased by two-fold when combined with curcumin. We next decided to explore the potential effect of these different drugs, alone and in combination with curcumin on cell cycle disruption. Cells were freshly harvested at different time points and fixed using 70% EtOH. Either PI or DAPI are able to enter fixed cells only. We used DAPI staining of fixed cells for high-resolution flow cytometry of DNA content. Cell concentration in culture also decreased when adding the extract in combination with drugs, indicating that the same effect was operating synergistically. All cell counts were obtained directly on the Attune NxT, as the fluidics are syringe-driven, making it reliable for volume measurements (data not shown).

Current ongoing synergistic experiments with curcumin are focused on the toxicity effect of fludarabine, cytarabine and rituximab. Plasma membrane potential, quantity and distribution of mitochondria, extrusion of fluorescent substrates for ATP-dependent pumps, indicators of cell proliferation, indicators of ionic homeostasis, indicators of oxidative stress, and indicators of autophagy, will provide more information about the synergistic potential of curcumin.

RESULTS AND DISCUSSION III

Cell cycle analysis. Cell cycle disruption as observed in G0/G1, S and G2M phases was strongly enhanced by 2.5µM curcumin. Cell number decreased in the presence of etoposide, colchicine and camptothecin. In the case of camptothecin and colchicine, an increased cell cycle disruption was observed when curcumin was added. Note that the dose of 2.5µM curcumin alone, showed no significant effect on cell cycle phases. These results suggest that curcumin have an important impact on cell cycle machinery.

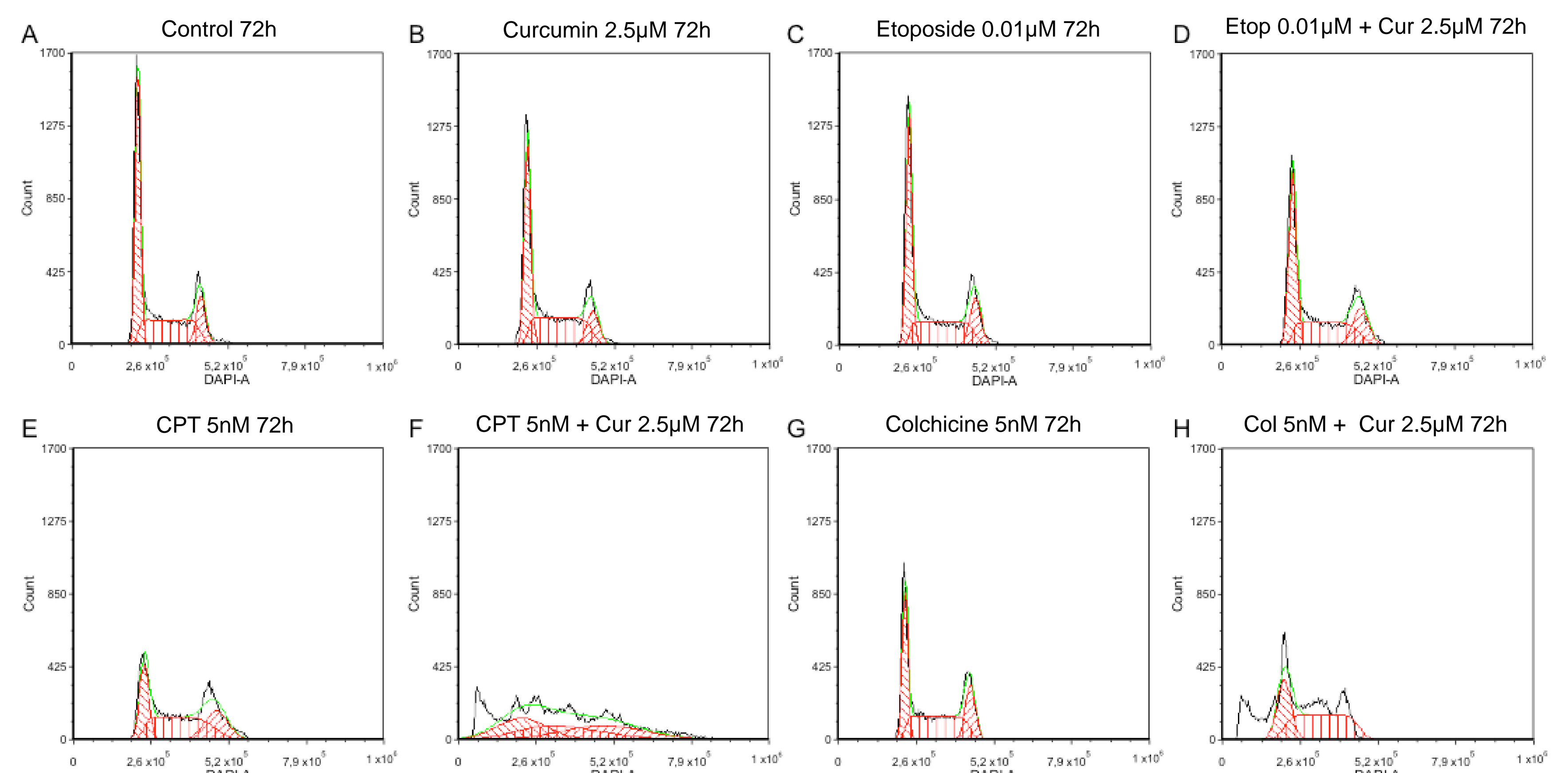

Figure 2. Curcumin enhances cell cycle disruption and the proliferation of cells. A) Control cells; B) curcumin 2.5µM caused a decrease of cells in the G0/G1 phase; C) etoposide 0.01µM causes only slight diminution of cells in G0/G1 phase; D) etoposide 0.01µM+curcumin 2.5µM cause a higher diminution of cells in G0/G1 phase than etoposide alone; E) camptothecin 0.005µM blocks cell proliferation and arrests cells on S and G2/M phases; F) camptothecin 0.005µM+curcumin 2.5µM cause a more drastic and generalized disruption of cell cycle than camptothecin alone; G) colchicine 0.005µM decreased cell proliferation and increases percentage of cells in S and G2/M phases; H, colchicine 0.005µM+curcumin 2.5µM cause high cell cycle disruption in addition of cell arrest in S and G2/M phases.

RESULTS AND DISCUSSION IV

Drug effects on cytomorphology. Microscope image observation after drug treatment showed an increase of the nuclei size and nuclear lobulation, an increase of basophilia and vesicle formation in the cytoplasm. In the case of etoposide, the number of vesicles was clearly increased in the presence of curcumin. Moreover, irregular shape of the nuclei characterized by several protrusions and depressions was also observed in the presence of the same compound. These results suggest that curcumin plays a significant role in influencing nuclei architecture, and also in increasing sensitivity to cell metabolic disruption that leads to decreased cell growth and increased cell death when curcumin is combined with drug treatment. Addition of curcumin revealed that important changes in the nuclear shape and the chromatin organization occur when administered alone. The consequences of altered nuclear size and shape are not known at this stage of the study, and they may impact chromatin organization and gene expression in leukemic cells.

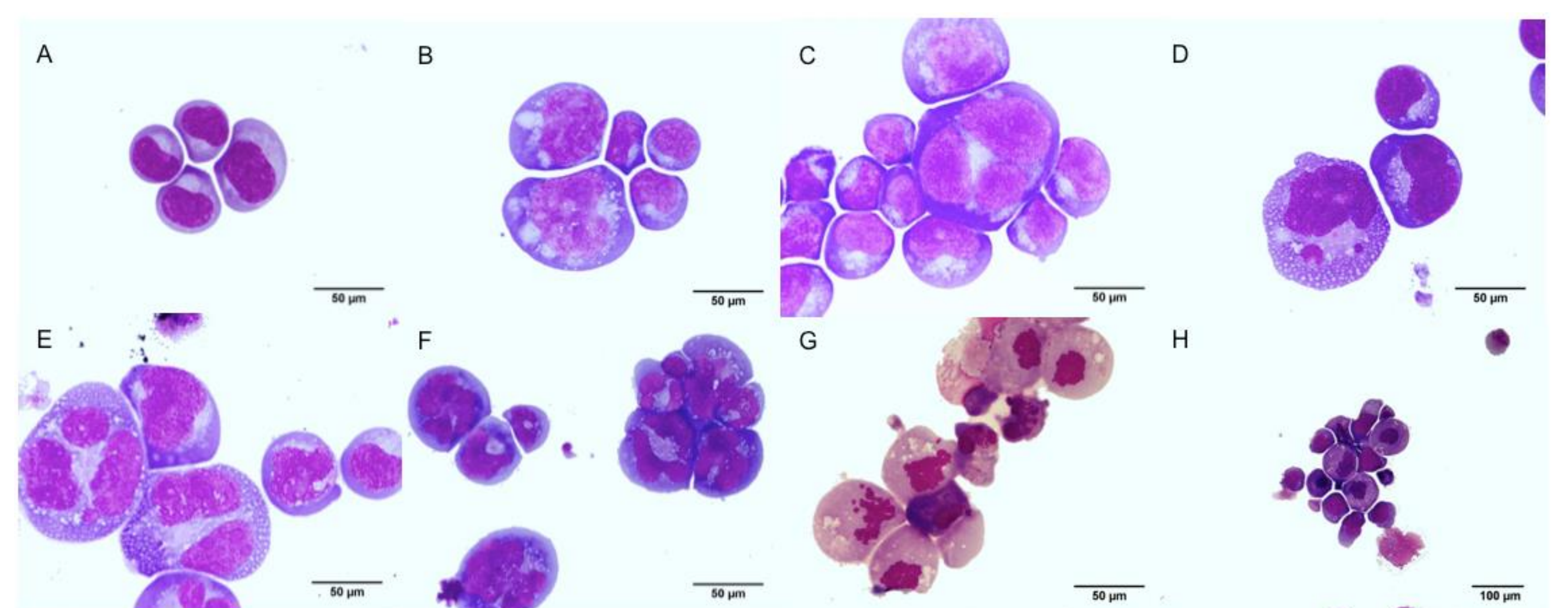

Figure 3. Drug and curcumin effects on cytomorphology. A) Untreated cells; B) 2.5µM curcumin; C) 0.05µM etoposide; D) 0.05µM etoposide combined with 2.5µM curcumin; E) 0.005µM camptothecin; F) 0.005µM camptothecin combined with 2.5µM curcumin; G) 0.01µM colchicine; H) 0.005 µMcolchicine combined with 2.5µM curcumin.

CONCLUSIONS

Curcumin significantly enhanced tested drug effects on cell membrane integrity and cell cycle disruption, and allowed to low their LD50.

Curcumin is a promising agent to decrease drug dose by 20 to 25%. Moreover, curcumin is non-toxic at the same concentrations used for simultaneous exposure to drugs, making it feasible to initiate a number of experiments to better understand how its use is safe using both normal and CLL samples.

Our results confirm previous studies demonstrating the benefits of curcumin in cancer treatment and how flow cytometry can help advance the field of drug screening and drug development.

Overall, our results confirm previous studies demonstrating the benefits of curcumin in cancer treatment and how flow cytometry can help advance the field of drug screening and drug development.

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