

Abstract**Sanjiv K Yadav***, **Ed Luther****, **Elena Holden****, and **Shazib Pervaiz***

*Department of Physiology and Oncology Research Institute, National University of Singapore

** CompuCyte Corporation, Cambridge, MA, USA

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It is now well established that one of the major factors that endows tumor cells with the ability to evade death signals is a defect or deficiency in the cell death circuitry. Therefore, an enormous amount of effort and funds are being dedicated to identify cellular targets and to design compounds for the selective engagement of these targets. Results of some of these efforts have unravelled the effector components of the death signaling machinery of the cell, and highlighted the critical interplay between intracellular caspase proteases and mitochondrial-derived factors in efficient induction of apoptosis in tumor cells.

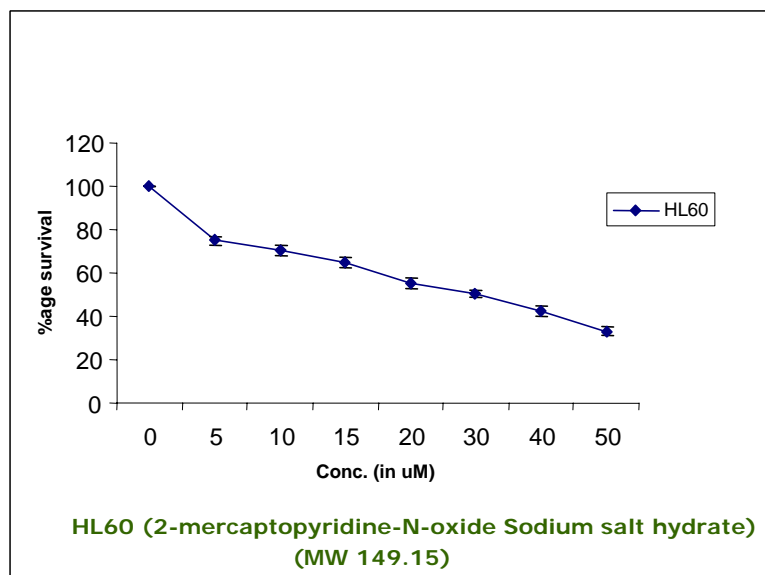
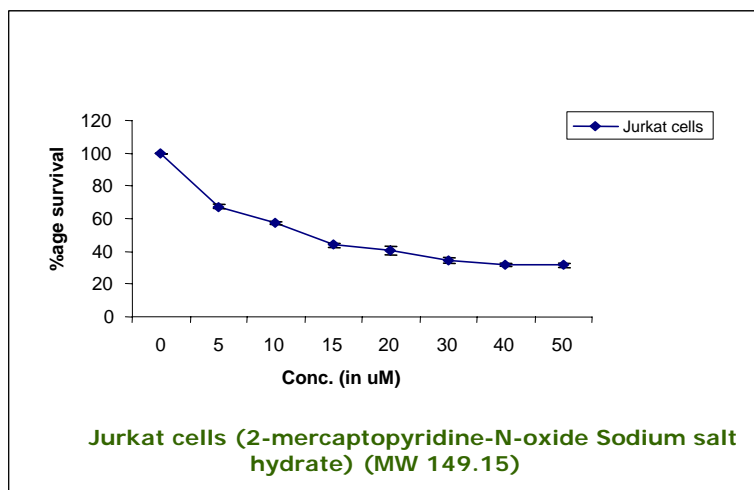
Over the years, our group has been active in the identification of small biologically active molecules with special predilection for the mitochondria and the role of a permissive intracellular milieu in overcoming the problem of drug resistance in tumor cells. Here we report the apoptosis-inducing activity of mercaptopyridine-*N*-oxide MPO (MW149.15), a compound previously investigated for its anti-bacterial activity, and compare it to four novel compounds and two known chemotherapeutic agents. The compound elicited a dose-dependent inhibition of cell survival (IC₅₀=12.5mM), induced robust activation of caspases 8, 2, 9 and 3, DNA fragmentation, and Cytochrome C release in human leukemia and lymphoma cells. In addition, our results indicate that an early trigger in the activation of the apoptotic pathway could be the significant increase in intracellular reactive oxygen species (ROS) production and the drop in cytosolic pH, observed as early as four hours after treatment.

In order to gain insight into the pathways controlling apoptotic signaling in live cells, we also analyzed cells by automated laser scanning cytometry to monitor apoptosis induction, cell cycle events, and mitochondrial targeting. Automation of the analysis process allows simultaneous screenings in a single assay, at multiple dosage levels, and at multiple end points. Results of these experiments strongly corroborate findings of our biochemical analysis and underscore the versatility of the automated platform for simultaneous analyses of multiple variables as a means for high-content screening.

I. MPO Induces Cell Death in Tumor Cells and Inhibits Tumor Cluster-Forming Ability

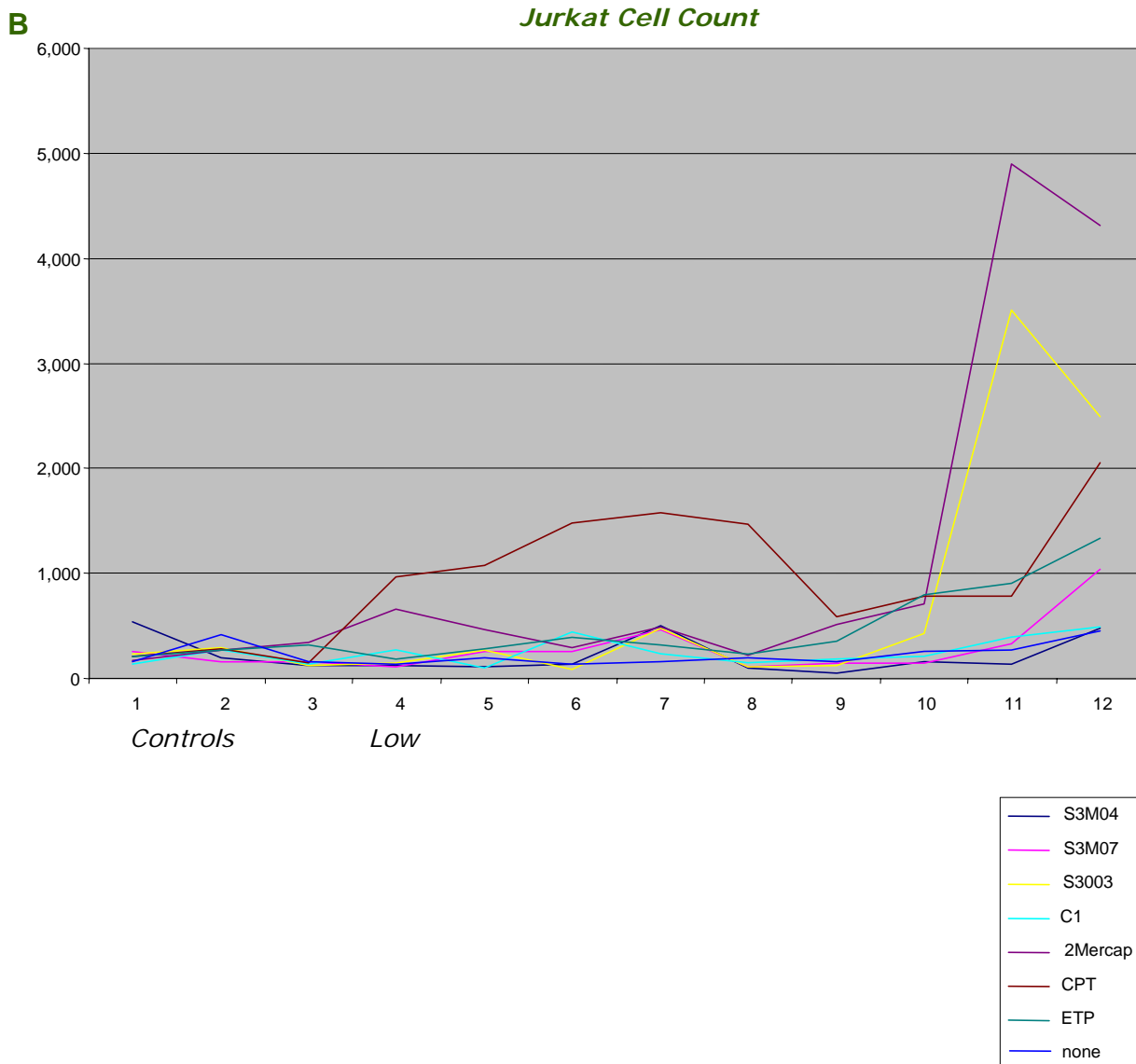
A) Human leukemia (HL60) and lymphoma (Jurkat) cells were exposed to increasing concentrations of 2MPO, and cell survival was assessed by the MTT assay. Jurkat cells were significantly more sensitive to the effect of MPO, as indicated by the LD50 dose of ~15uM as opposed to HL60 cells (LD50 of ~30uM).

A



I. MPO Induces Cell Death in Tumor Cells, continued

B) Using the iCyte system, a panel of drugs was used in a 96-well format to assess the effect on cell numbers at various concentrations. This analysis allows for the differentiation of cell clusters or single segmented cells. Interestingly, exposure to a known anti-cancer agent camptothecin, used as a positive control in most of the analyses, resulted in a moderate increase in the segmented cell population, whereas a very pronounced effect was observed with two novel compounds S3003 and 2MPO.

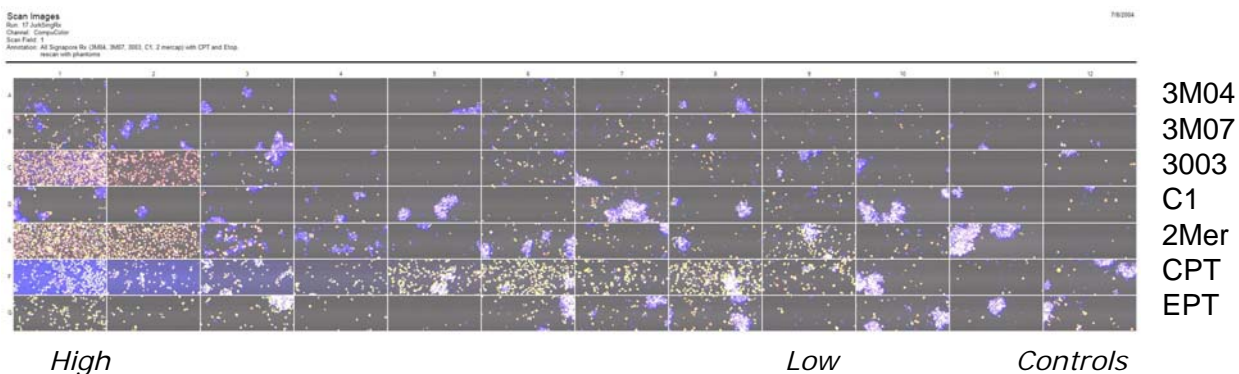


I. MPO Induces Cell Death in Tumor Cells, continued

C) Actual appearance of cells when analyzed for cluster formation or segmented populations. These data represent cells in each well in untreated (control) samples or upon exposure to low or high concentrations of the compounds. Tumor cell clusters are circled (red) for comparison with the segmented (single cell) populations obtained upon exposure to S-3003, 2MPO, and two commonly used agents, etoposide (ETP) and camptothecin (CPT). These data strongly suggest that compounds such as 2MPO and S3003 could have a strong potential for inhibiting tumor colony formation ability.

C

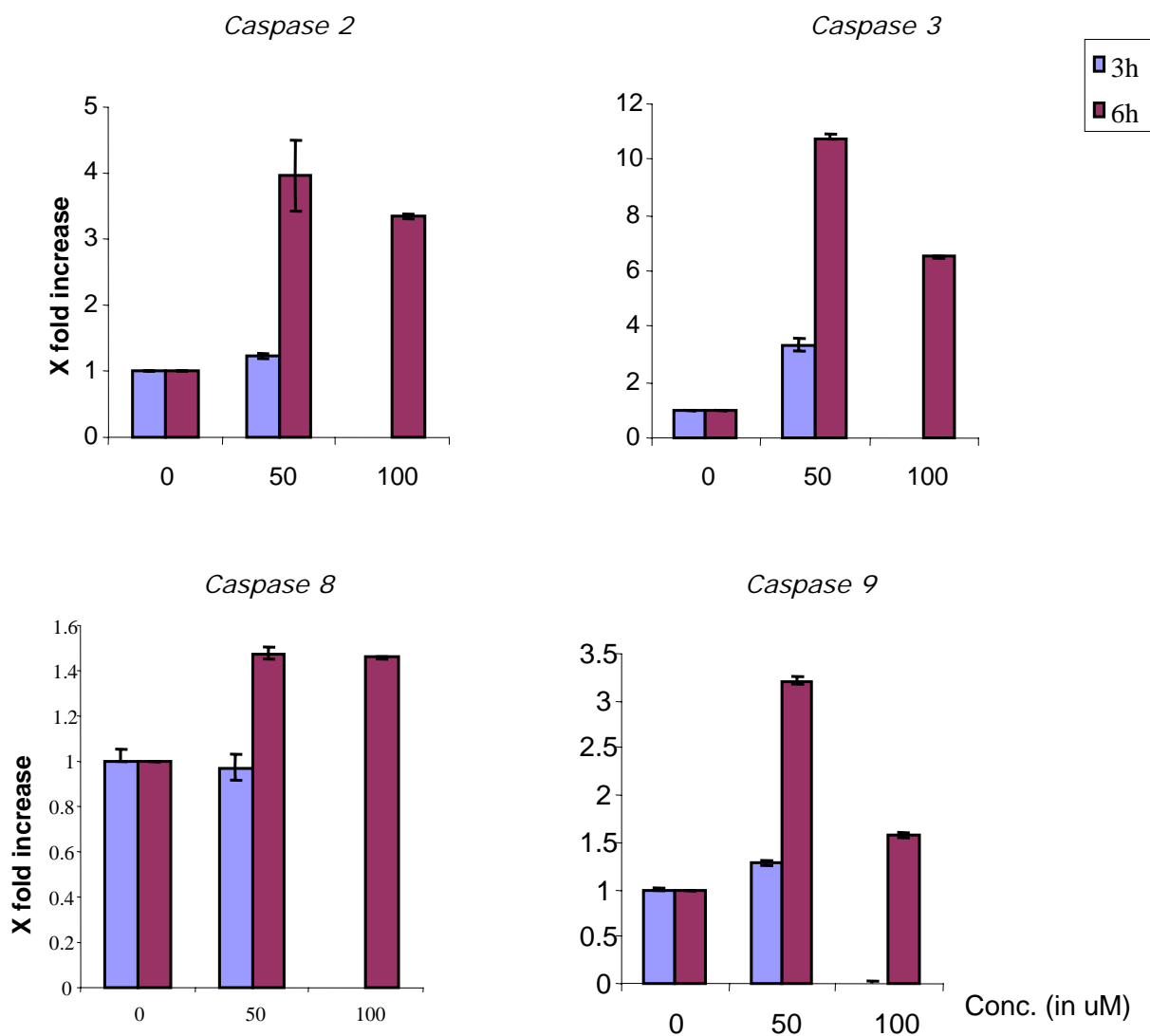
Jurkat Scan Images



II. MPO Induces Caspase 3, 8, 2, and 9 in Tumor Cells

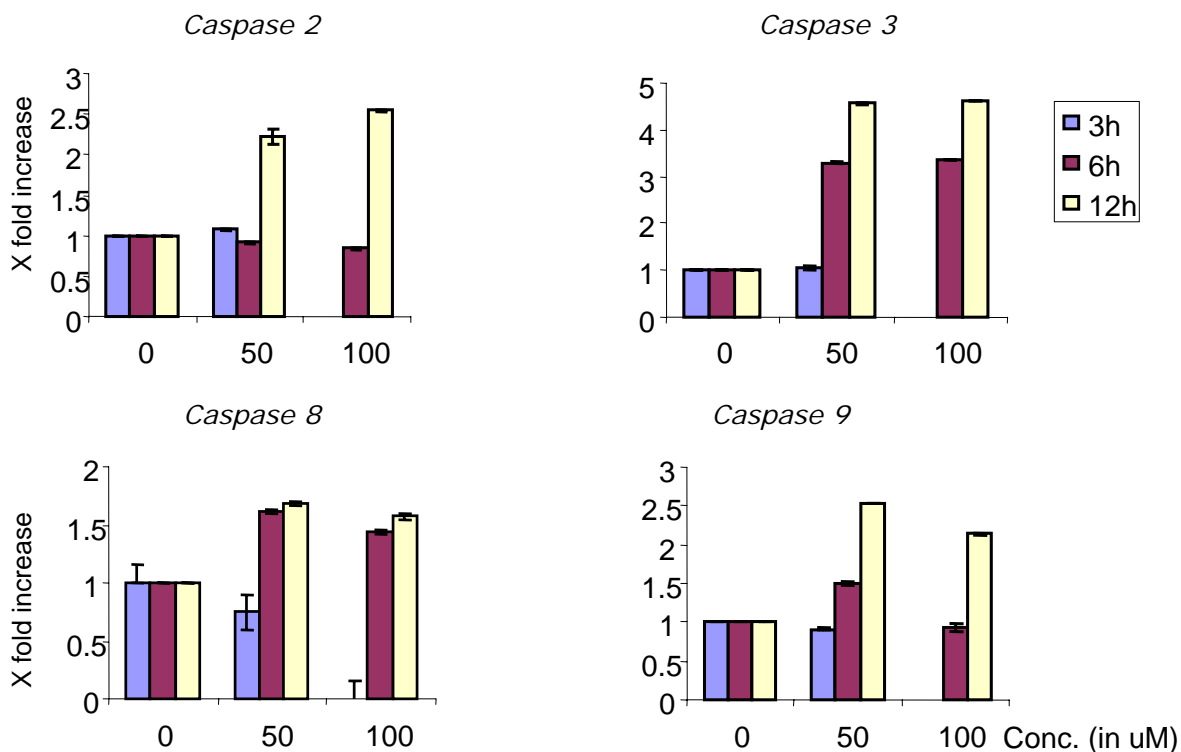
In order to assess the involvement of the apoptotic pathway in 2MPO-induced cell death, Jurkat and HL60 cells were exposed to 0, 50, or 100uM 2MPO for 3-12 hours and protease activity of caspase 2, 3, 8, and 9 was assessed by fluorimetric assays, using the respective tetrapeptides tagged to a fluorophore. Release of the free fluorophore was then measured with a spectrofluorimeter as an indication of protease activation. As indicated in the figure, 2MPO treatment induced a robust increase in caspases 2 and 3 and a moderate increase in caspase 9 activity in both Jurkat and HL60 cells. Consistent with the survival data, Jurkat cells appeared relatively more sensitive to 2MPO-induced apoptosis.

Activity in Jurkat cells at 3 and 6 Hours



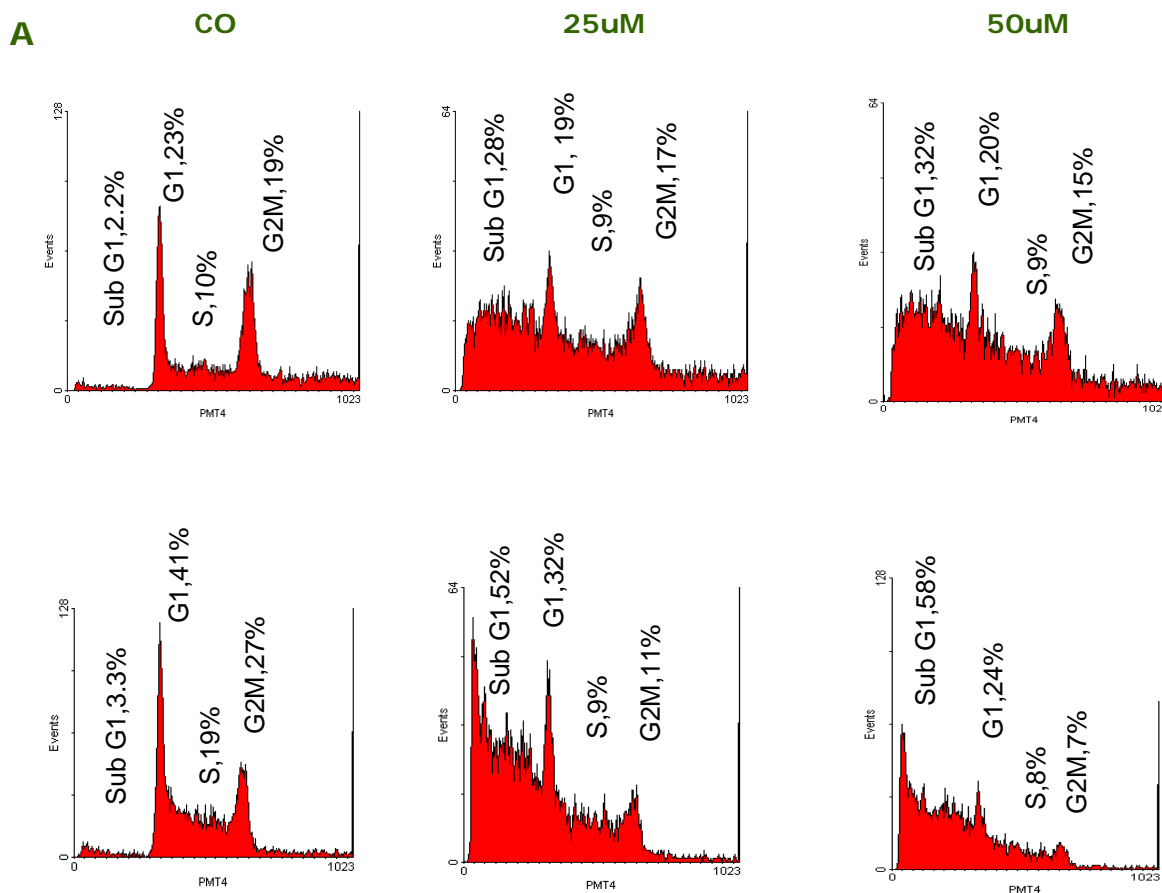
II. MPO Induces Caspase 3, 8, 2, and 9 in Tumor Cells, continued

Activity in HL60 cells at 3, 6, and 12 Hours



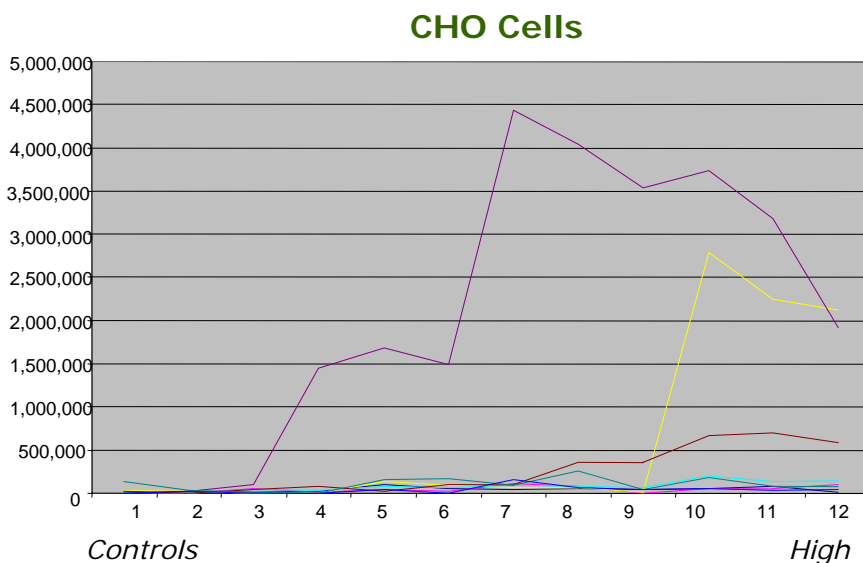
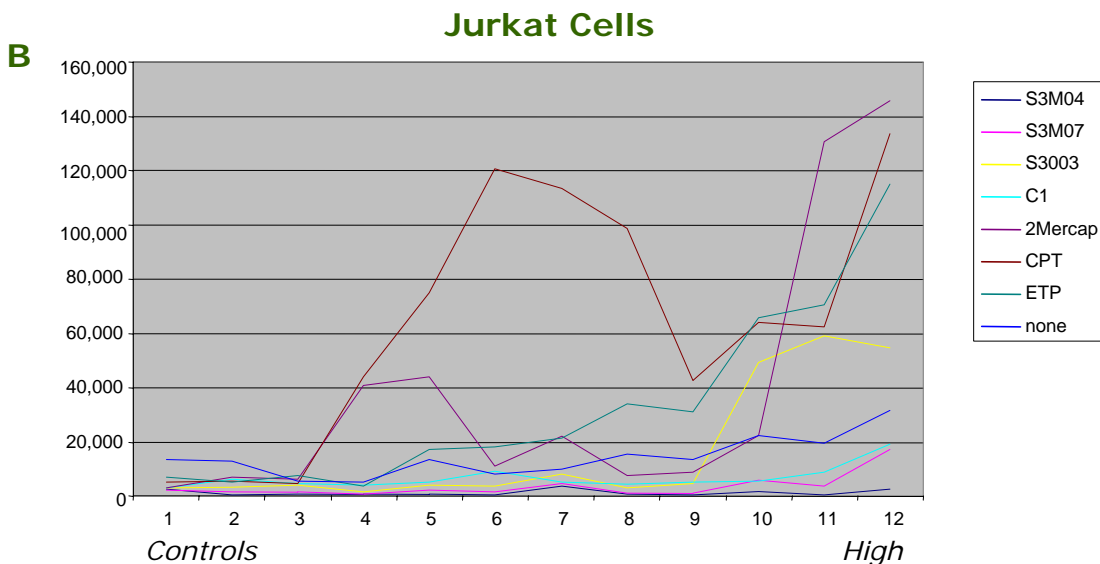
III. Reduction in DNA Content and Appearance of Sub-G1 Population Confirms Apoptosis Induction in Tumor Cells

A) A classic hallmark of apoptosis is the fragmentation of DNA, analyzed by cell cycle analysis and the appearance of sub-diploid DNA (sub-G1). We assessed DNA damage using the classic propidium iodide staining of cells and cell cycle analysis or with the iCyte system in a 96-well format. Cell cycle analysis shows the ability of 2MPO to trigger DNA fragmentation in both cell lines, with the Jurkat cells being consistently more sensitive (~50% sub-G1).



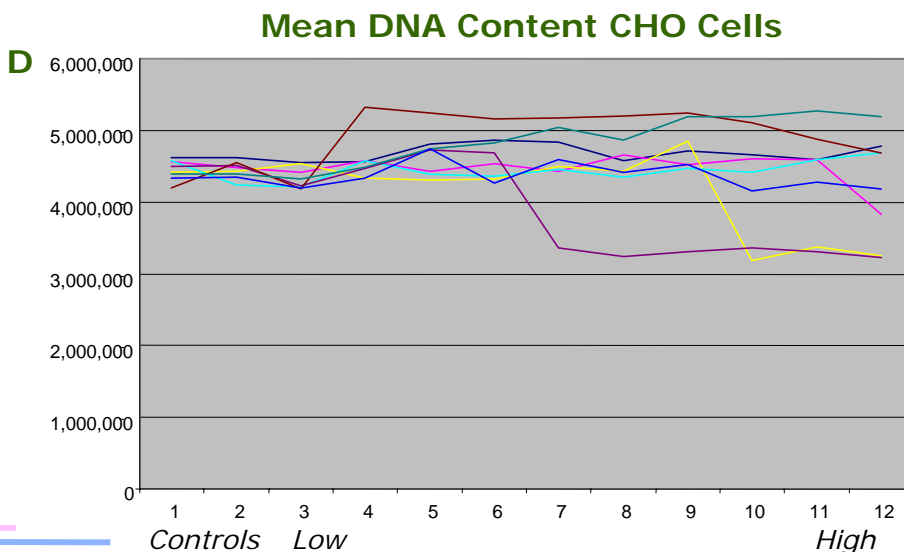
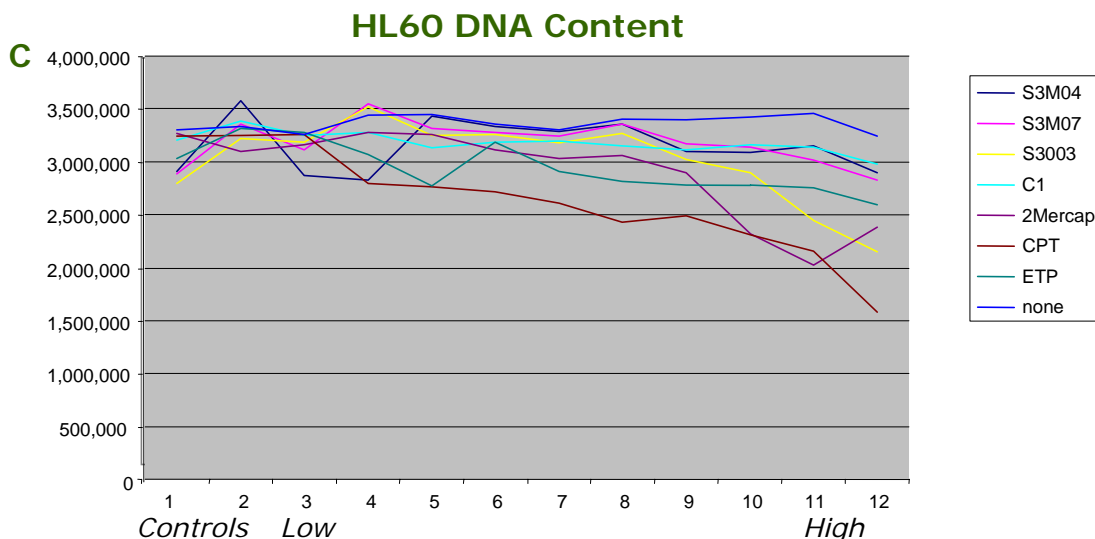
III. Reduction in DNA Content, continued

B) Plots of the cellular plasma membrane permeability measured by YoPro-1 show early apoptosis related effects. In Jurkat cells, camptothecin had the strongest effect on cells, followed by etoposide, and then the mitochondrial agents MPO and 3003. In adherent CHO cells, the mitochondrial agent MPO and 3003 had the greatest effect, with only a slight response seen by the camptothecin in the dosages used.



III. Reduction in DNA Content, continued

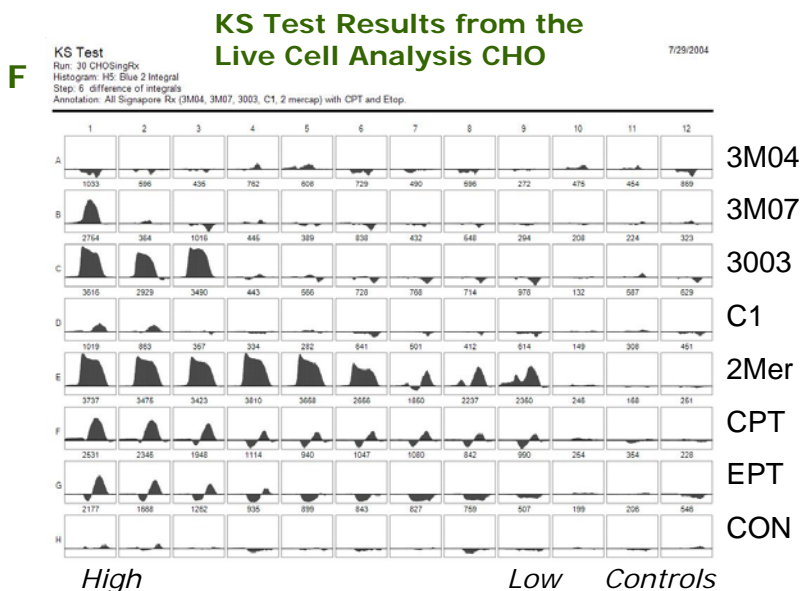
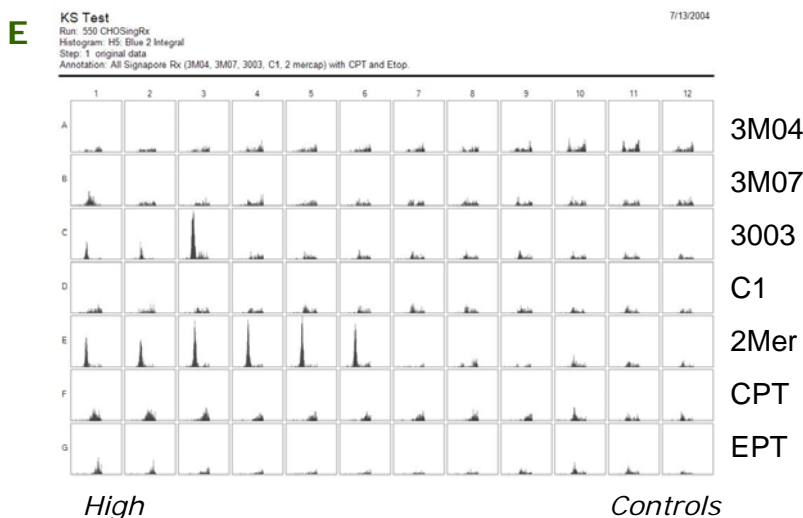
- C) Using the iCyte technology we also used a 96-well format to assess cell cycle changes upon exposure to the anti-cancer compounds. Interestingly, this technology provides additional information on the effect of each compound at various concentrations on the cell cycle profile. The damage to DNA was also verified by the loss of DNA content by iCyte technology. This appears to be the case with most of the compounds tested, especially the two novel compounds S-3003 and 2MPO.
- D) Adherent cells (CHO) cells have a slower response to apoptotic signals, and thus pre-apoptotic effects on the cells, such as cell cycle blockage, can be monitored. As evident, 2MPO treatment induced an increase in the G1 population of cells, indicating arrest in that phase of the cell cycle. As a positive control, cells exposed to CPT and EPT appear to accumulate in the G2/M phase.



III. Reduction in DNA Content, continued

- E) The DNA content histograms of live cells treated with the drug panel show the blockage of cells in various phases of the cell cycle. The G1 block of 3003- and 2Mer-treated cells is prominent.
- F) The Kolmogorov-Smirnov test is a method for displaying the differences between a histogram and its controls. The difference histograms for the DNA staining of live CHO cells is shown. Effects on the cell cycle by many of the drugs are evident, with the largest effects being from the 3003- and 2MER-treated cells.

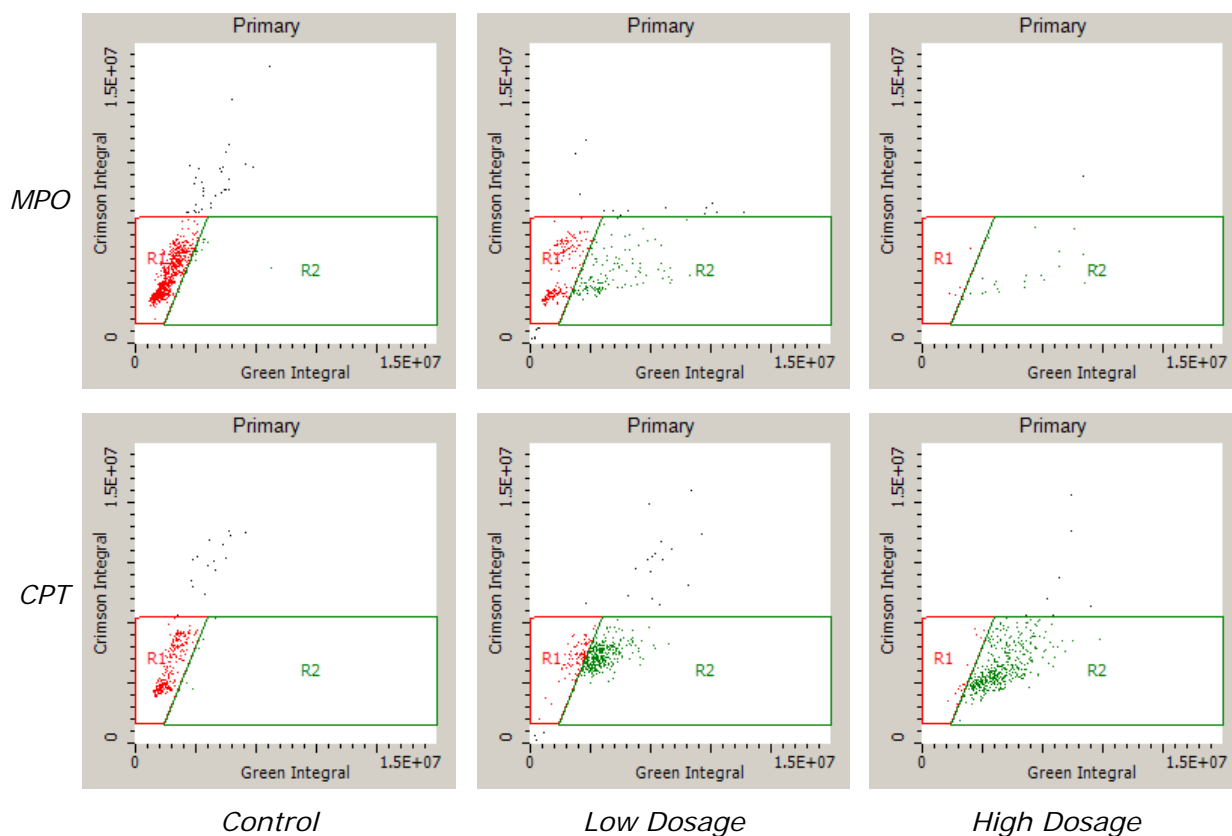
CHO Live DNA Histograms



IV. Phosphorylated Histone H2AX Labeling of DNA Strand Breaks

Phosphorylated H2AX antibody (Trevigen) was used to label DNA strand breaks in MPO- and CPT-treated CHO cells (X axis) vs. the DNA content. At low dosages of MPO, only S-phase cells label with the H2AX; and at high dosages most of the cells have been lost from the analysis due to apoptosis. At low dosages, CPT blocks all of the cells in S-phase of the cell cycle, and they all stain with the H2AX. Unlike the MPO-treated cells, at higher dosages, the cell loss is not evident. Other evidence indicates that these are pre-apoptotic cells.

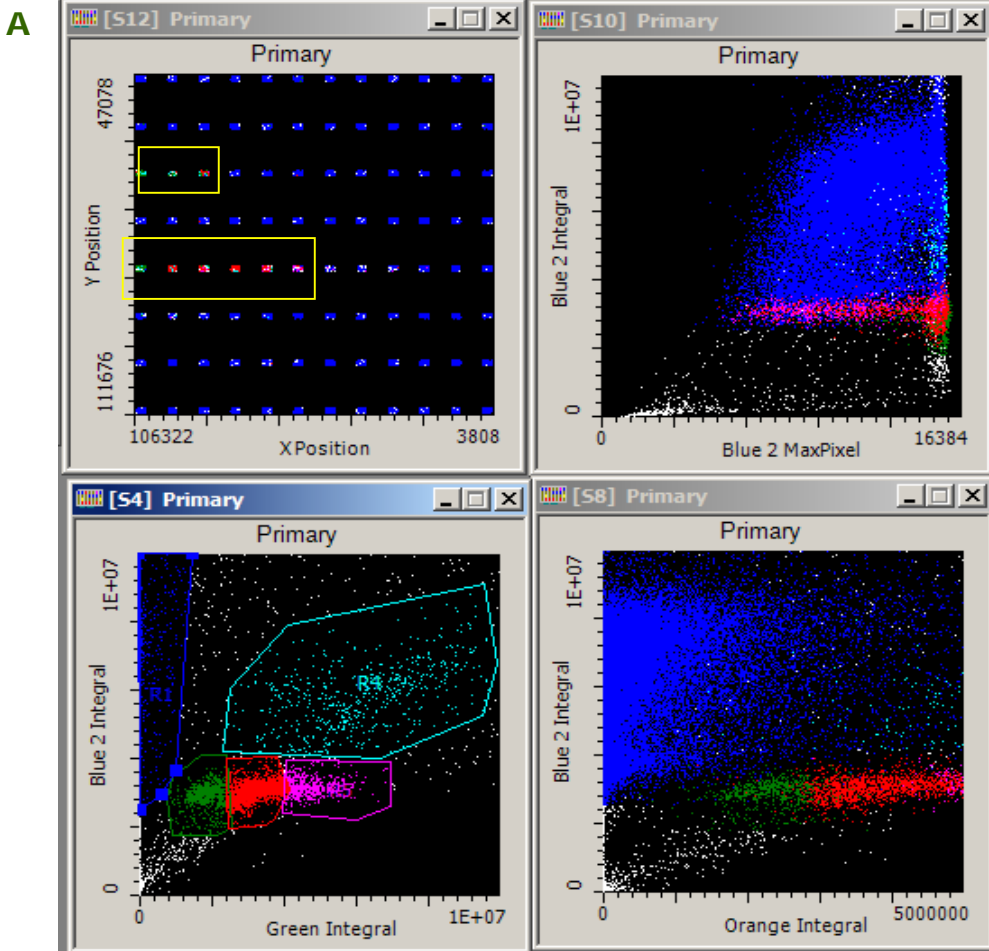
Phosphorylated H2AX



V. Characterization of Apoptotic Cells Induced by MPO

A) iCyte analysis of a test panel of drugs and controls in a live-cell assay combining Hoechst 33342 DNA staining with the apoptotic marker YOPRO (Molecular Probes), Mitoshift (Trevigen) and propidium iodide. Color-coding is based on the DNA vs. YOPRO staining (live vs. apoptotic), according to the following dosage related sequence Blue (live cells), Cyan, Magenta and then Green. Apoptotic cells appear primarily in rows treated with MPO and 3003, inscribed in the yellow boxes.

4-Color Homogenous Live-Cell Assay

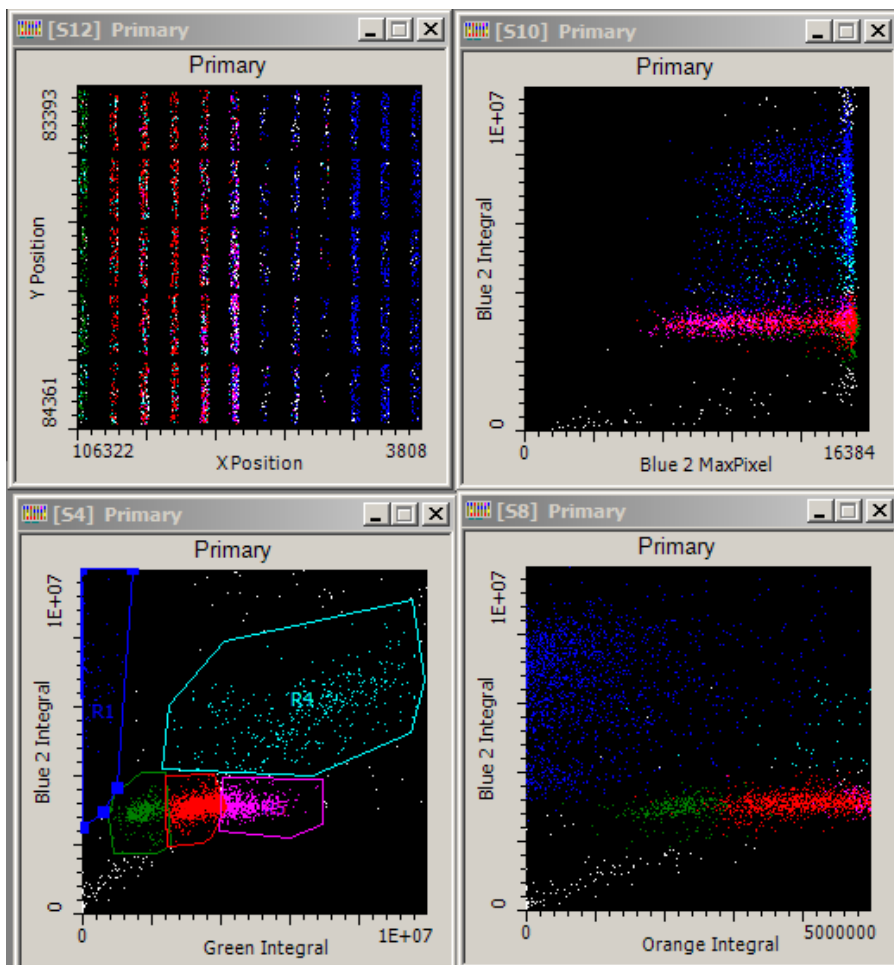


V. Characterization of Apoptotic Cells, continued

B) Detailed analysis of the MPO staining showing the dose response of the cells to the drug. In the upper left scattergram, each column represents a well of the 96-well plate with 3 control wells on the right and increasing concentrations of the drug from wells 4 to 12.

Apoptosis Induced by MPO

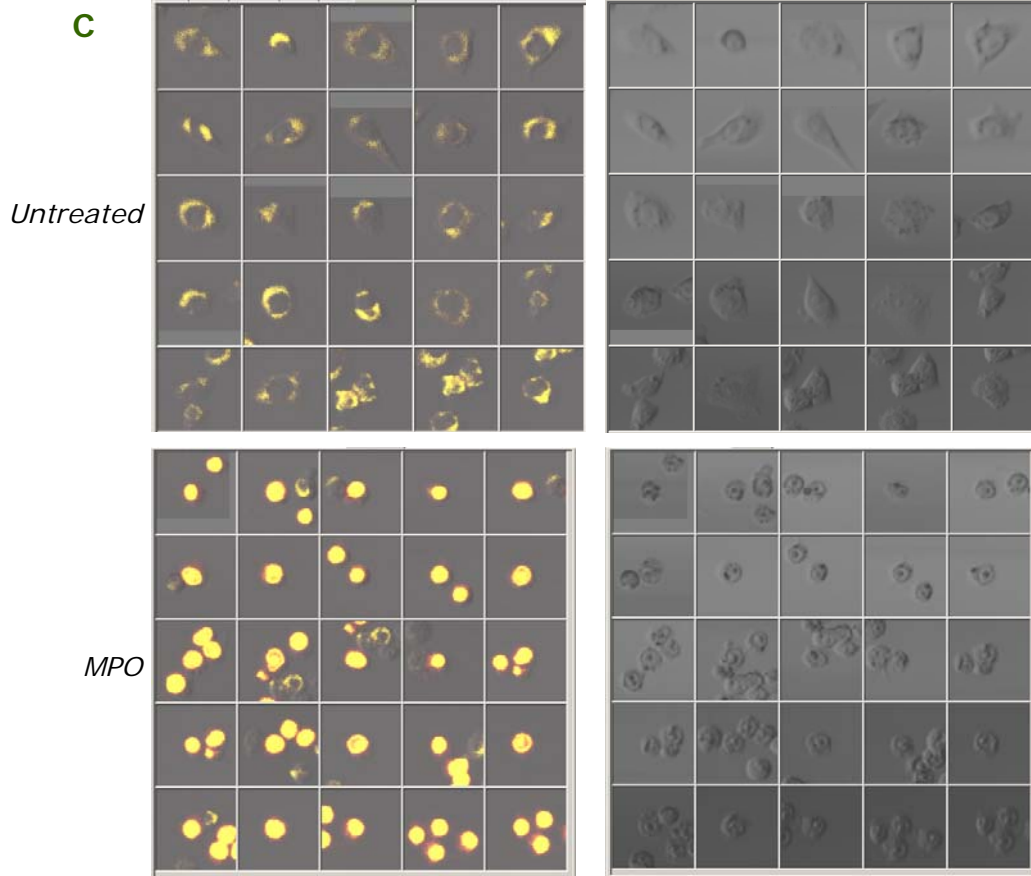
B



V. Characterization of Apoptotic Cells, continued

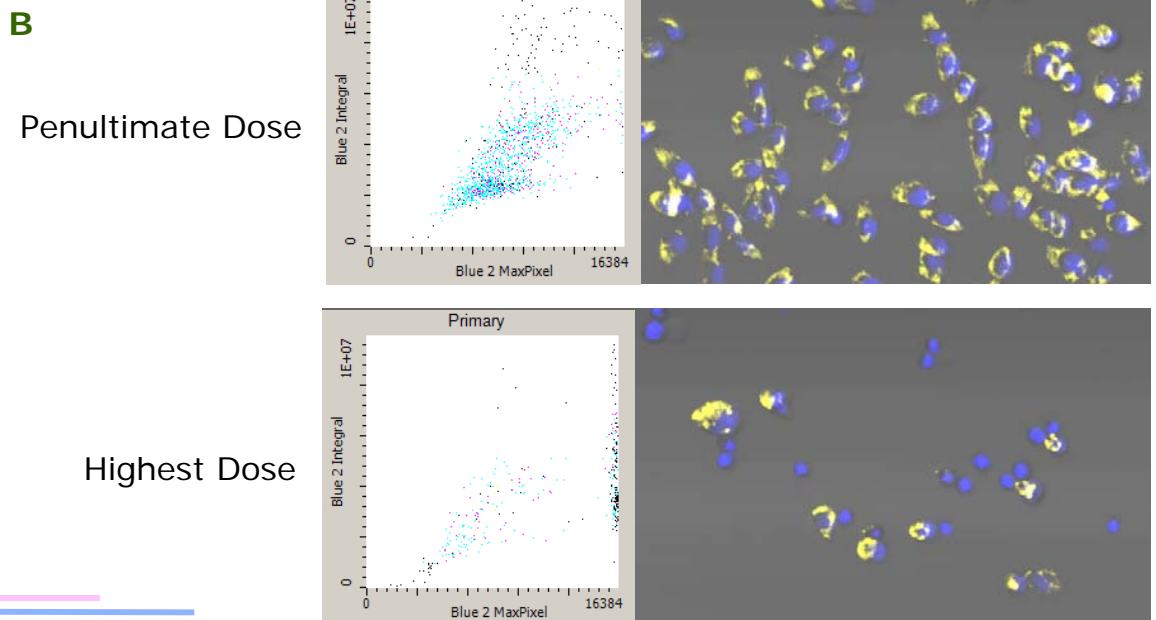
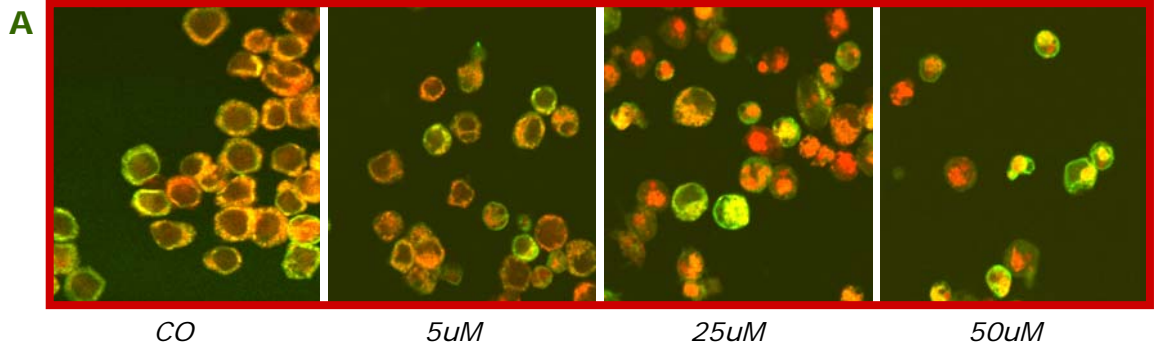
C) Galleries of images of CHO cells from the live cell experiment. MPO induces dramatic changes in the cell morphology, including cell rounding and loss of mitochondrial integrity.

Morphology Changes Induced by MPO



VI. MPO Effects on Mitochondrial Function

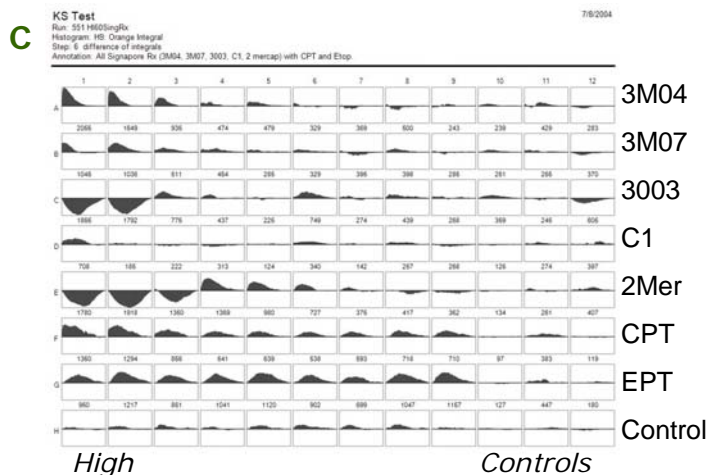
- A) In order to assess the effect of MPO exposure on mitochondrial death signaling pathways, HL60 cells were incubated with increasing concentrations of 2MPO for 12 hours, stained with Mito Tracker Red (Molecular Probes) followed by anti-Cytochrome C for one hour. Cells were then exposed to the secondary IgG conjugated to FITC and analyzed by confocal microscopy. Results show co-localization of Cytochrome C with the Mito tracker Red (yellow to orange staining); however, with increasing concentrations of 2MPO, cells tend to show separate green and red fluorescence indicating egress of Cytochrome C to the cytosol (green).
- B) The staining patterns of mitochondria in CHO cells for different twofold dosages of compound 3003 are shown. Similar to the previous experiments, there is a dramatic shift in the DNA chromatin condensation. Concurrently, there are similar changes to the mitochondria as seen before. At the highest dosage, the mitochondria can be seen to be polar and fragmented, and in many cases, the mitochondria are absent from the cells.



VI. MPO Effects on Mitochondrial Function, continued

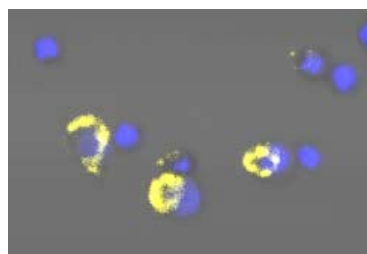
- C) In a parallel set of experiments, mitochondrial changes were also assessed upon exposure to a panel of drugs using theiCyte platform. Data obtained from each well was corrected using the Kolmogorov Smirnov analysis and reported as a qualitative change in the mitochondrial morphology. As indicated in the data, control cells do not show any significant “up” or “down” response, suggesting no change in the mitochondrial morphology. However, results clearly indicate that most drugs induced some effect on the mitochondrial morphology, with 2MPO being the most active of the new compounds tested.
- D) Visual examination of scan images from the high dosage treated wells show many examples of pairs of cells that appear to be undergoing unequal cell division. We postulate that after the mitochondria polarize, cell mitosis continues. The nuclei on the side without the mitochondria are unable to survive, and thus become pyknotic as evidenced by the bright staining of the chromatin. Studies are ongoing to verify cytoplasmic connections between the pairs of cells.

HL60 Mitochondrial Changes

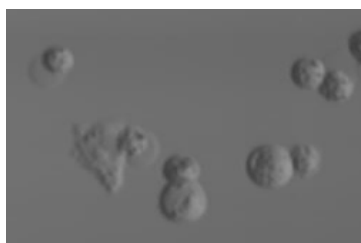


D

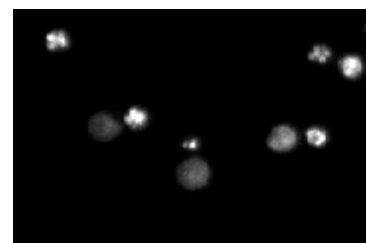
Unequal Cell Division



Mitoshift



Scatter



DNA

Conclusions

The novel compounds 2MPO and S3003 induce apoptotic cell death in human leukemia and lymphoma cells and in adherent CHO cells. 2MPO-induced apoptosis involves the mitochondrial death pathway as indicated by the changes in mitochondrial morphology and the release of Cytochrome C. Our results also point to an effect on the colony formation ability of tumor cells. In addition, death signaling triggered by 2MPO is accompanied by early ROS production and acidification of the cytosol.

A major focus of this study was to compare the biochemical data obtained with 5 novel compounds to that obtained with the iCyte technology as a means to gauge the potential of the iCyte platform for high-content screening. Our results indicate the versatility and strength of the iCyte™ Automated Imaging Cytometer, whereby it enabled analyses of parameters that usually require both flow cytometry and fluorescence imaging. In addition, the iCyte system provides a high data content analysis in a multiple-well format with the added strength for monitoring a variety of intra- and/or extra-cellular parameters.

References

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