

Abstract

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The antitumor agents camptothecin (CPT) and its derivative topotecan (TPT) are DNA topoisomerase-1 (TOP1) inhibitors. By stabilizing the otherwise transient "cleavable complexes" of TOP1-DNA, they prevent re-ligation of DNA strand breaks during DNA replication. It has been recently shown that in DNA-replicating cells, TPT induces dsDNA breaks (DSBs) which can be immunocytochemically detected; their frequency, as well as the subsequent caspase-3 activation, was assessed by cytometry (Huang *et al.*). It was also reported that exposure of some cells to CPT arrests them in G₁ and leads to reversible and irreversible states of senescence or to necrotic death (Poele, *et al.*). The aim of the present study was to examine a relationship between induction of DSBs, cell-cycle phase and possible induction of senescence.

Chinese hamster ovary (CHO) and human melanoma (M14) cells seeded in multiwell plates were treated with varying concentrations of CPT for the approximate duration of the cell cycle, and then assayed. Fixed-cell formats involved cell fixation in ethanol, followed by DNA staining with propidium iodide (PI) and immunocytochemical detection of either phosphorylated histone H2AX (marker of DSBs) or activated caspase-3. Live-cell analysis involved DNA staining with Hoechst 33342; Mitoshift staining was used to assess mitochondrial potential; and plasma membrane integrity was assessed by YoPro1 or PI exclusion. Cytometric analysis by laser scanning cytometry (LSC) obtained quantitative fluorescence and morphometric data.

In the assays on fixed cells, apoptotic cells (defined by the presence of active caspase-3) detached from plates, leaving predominantly live cells for analysis. The most notable effects were cell arrest in S-phase at low (2-37nM) concentrations of CPT and the presence of DSBs in all cells. At higher concentrations (75-6uM), the population of S-phase cells was decreased; a number of cells had a G_{0/1}-DNA content and showed no evidence of H2AX phosphorylation (DSBs). These cells were either arrested in G_{0/1} (or at the entrance to S) or were S-phase cells that showed some DNA loss but without typical apoptotic phenotype. In live-cell assays, the apoptotic cell loss by detachment was less apparent. The pattern of DNA changes in CHO cells was similar to that in the fixed-cell assay. The significant loss of DNA in M14 cells was apparent at CTP concentrations above 18nM, leading to the appearance of the sub-G₁ subpopulation typical of apoptotic cells.

The data show differential response of CHO vs. M14 cells to CPT. While CHO cells were blocked in S phase at low CTP concentration and in G_{0/1} (or in S but having lost part of their DNA) at higher concentration, M14 cells were also blocked in S at low CPT concentration but were undergoing typical apoptosis with significant loss of DNA at higher concentration. Further studies are needed to assess long-term viability of the CHO cells arrested with a G_{0/1} content and their possible senescent phenotype or propensity to undergo necrosis. The high-content analysis offered by LSC makes it possible to discriminate different responses of the target cells to anti-tumor drugs, depending on drug concentration. This could be of special prognostic value in assessing an individual patient's response to various drugs in different dosages, utilizing tumor cells from primary cultures.

I. Materials and Methods

CHO cells were obtained from ATCC and kept in continuous culture. M14 primary human melanoma cells and LNCaP prostate carcinoma cells were provided by Dr. Shazib Pervais at the National University of Singapore. Cell lines were kept in continuous culture and tested prior to experiments for DNA cycle and proper growth.

Cells were seeded into 96-well optical glass microtiter plates (Whatman) at empirically determined densities and allowed to adhere to the growing surface for about 7 hours. They were exposed to test substances overnight to allow cells to traverse through approximately one cell cycle.

Test substances: Camptothecin (Sigma) was diluted in PBS to a concentration of 6uM and stored as a stock solution. Nine twofold dilutions were used in experiments starting at a 1:1000 dilution of the stock solution.

Fixed-cell formats: Plates were washed three times with phosphate-buffered saline, fixed with ethanol fixative, rehydrated, and stained with antibodies to phosphorylated histone γ H2AX (Trevigen) or cleaved Caspase-3 (Cell Signaling). Antibody staining was developed with Alexa Fluor 488 goat anti-rabbit immunoglobulins (Molecular Probes). Cells were counterstained with 5 ug/ml propidium iodide (Molecular Probes) and 200 ug/ml RNase (Sigma).

Live-cell format: Live cells were stained with a homogenous cocktail consisting of Hoechst 33342 (Molecular Probes) at 10 ug/ml, Mitoshift (Trevigen) at 100 nM and propidium iodide at 1 ug/ml.

	1	2	3	4	5	6	7	8	9	10	11	12
CPT	0.6 <u>uM</u>	0.3 <u>uM</u>	0.15 <u>uM</u>	75 <u>nM</u>	37.5 <u>nM</u>	18.75 <u>nM</u>	9.37 <u>nM</u>	4.68 <u>nM</u>	2.3 <u>nM</u>	0	0	0
A												
B												
C												
D												
E												
F			C	H	O							
G			L	N	C	A	P					
H			M	I	4							

Microtiter plate layout for drug titration experiments: Columns 10 - 12 serve as no-drug control wells and columns 1 to 9 have decreasing amounts of CPT.

II. Plate analysis

Plates were analyzed on an **iCyte™ Automated Imaging Cytometer (CompuCyte Corporation)** using a 488 nm argon laser to excite propidium iodide (long red), Alexa 488 (green) and Mitoshift (orange). A 405 nm diode laser was employed to excite Hoechst 33342 (blue). Photomultiplier tubes with appropriate dichroic filters were utilized to obtain fluorescence information as the laser scanned the surfaces of the wells. Nuclear fluorescence was used for cellular segmentation. Quantitative and morphometric feature information was calculated, including DNA content per cell and others as indicated in the table below. Population and image data were stored and analyzed using the **iBrowser™ Data Analysis software**.

Features Measured in Analysis

<u>Dye</u>	<u>Laser</u>	<u>Detector</u>	<u>Feature</u>	<u>Cell State</u>
Hoechst 33342	405 nm diode	Blue	<ul style="list-style-type: none"> • DNA content • Nuclear area • Chromatin density 	Live
Propidium iodide	488 nm Argon	Long Red	<ul style="list-style-type: none"> • DNA content • Nuclear area • Chromatin density 	Fixed
Propidium iodide	488 nm Argon	Long Red	<ul style="list-style-type: none"> • Nuclear membrane Integrity 	Live
H2AX Alexa 488	488 nm Argon	Green	<ul style="list-style-type: none"> • dsDNA strand breaks 	Fixed
Caspase 3 Alexa	488 nm Argon	Green	<ul style="list-style-type: none"> • Apoptosis induction 	Fixed
Mitoshift	488 nm Argon	Orange	<ul style="list-style-type: none"> • Mito. Potential • Mito. Density 	Live
Laser scatter	488 nm Argon	Photodiode	<ul style="list-style-type: none"> • Cell Morphology 	Live

III. Results

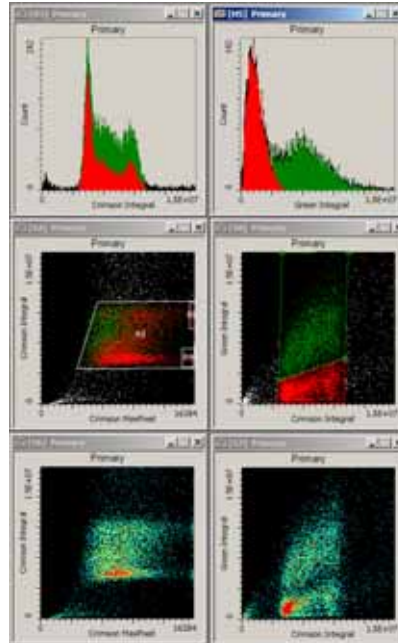
Fixed-Cell Analysis, CHO Cells

In the adherent CHO cell line model, the induction of apoptosis by CPT occurs at a much slower rate than was reported for HL60 cells.

PANEL A

At 36 hours, dose-response studies show effects on the cell cycle and the induction of DNA strand breaks.

H2AX positive (color-coded RED) and negative (color-coded GREEN) cells are clearly demarcated.



PANEL B

A normal cell cycle distribution for exponentially growing cells is evident in untreated cells.

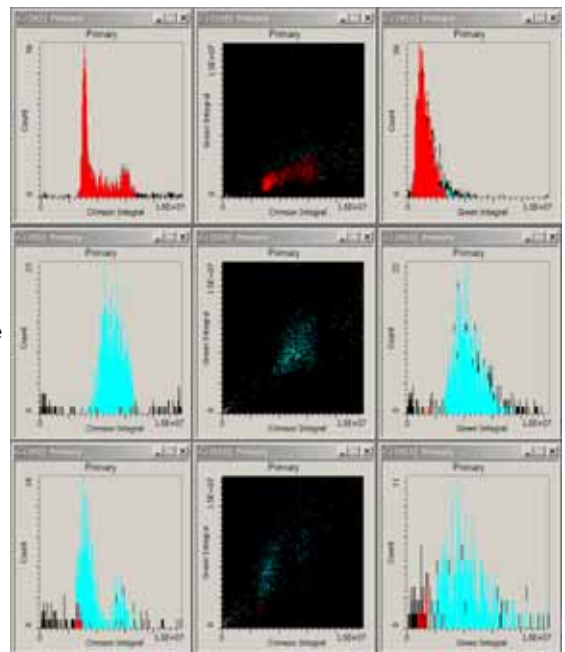
The cells are blocked in the S-phase of the cell cycle at a low dosage of CPT. DNA strand breaks are also evident in S-phase cells.

The cells are blocked at the G1/S and S/G2 checkpoints of the cell cycle at a high dosage.

**NO
CPT**

**Low
Dosage
CPT**

**High
Dosage
CPT**



DNA

**DNA
vs
H2AX**

H2AX

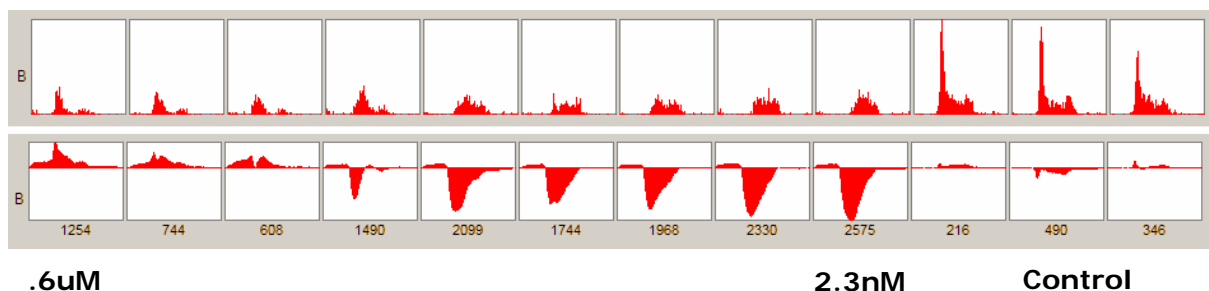
III. Results, continued

Relevant histograms from a camptothecin dose-response study are shown along with the derived Kolmogorov-Smirnoff differential plots below them.

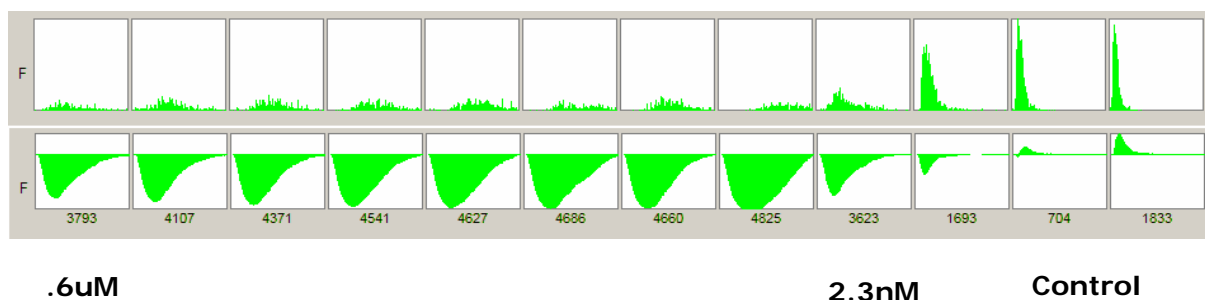
In the DNA histograms, even at the lowest dosage of CPT, there is a decided difference from the control samples – a reflection of the blockage of cells in S-phase. At the higher dosages, the differences change from being a down-going difference to an up-going difference. The K-S analysis implies that these are not normally cycling cells, and that they have been affected by the drug treatment.

All of the CPT-treated samples show a large K-S difference in the H2AX staining analysis, with a maximal response at the lower dosages.

DNA Content Corresponding to Panel A



Phosphorylated H2AX Corresponding to Panel B

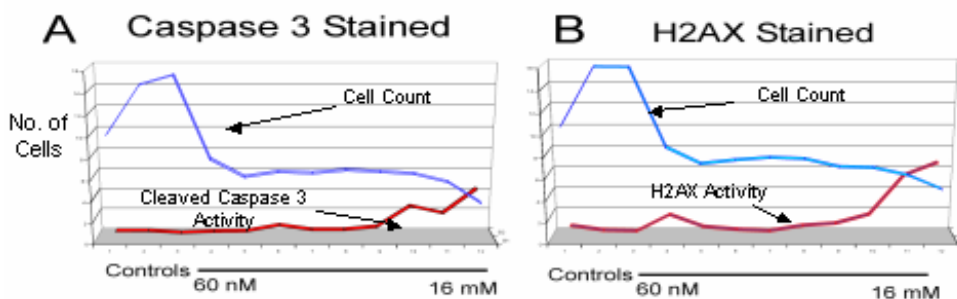


III. Results, continued

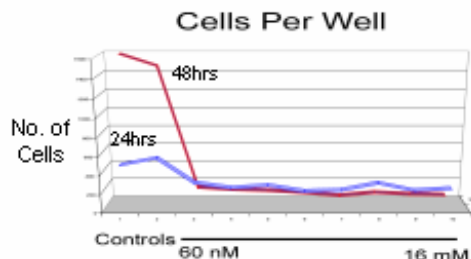
Differentiating between the Effects of Loss of Proliferation and Cell Loss to Apoptosis

A) Cells exhibiting the apoptotic morphology characterized by intense caspase staining around the nucleus, rounded morphology, and fragmented nuclei were induced by highest CPT dosages.

B) In an H2AX-stained titration, apoptotic bodies were quantified with similar results.



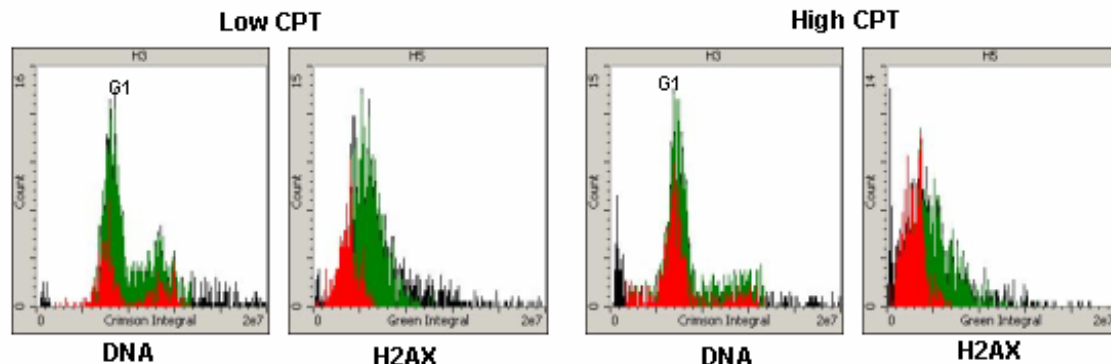
Duplicate wells of cells were treated with camptothecin and allowed to incubate for 24 hours and 48 hours. The control wells showed an increase in the number of cells, indicating that the cells were proliferating. There were no changes in the cell counts in the treated wells, indicating that the cells were in a senescent-like state.



IIIa. Fixed- and Live-Cell Results

Fixed-cell analysis of M14 tumor cells stained for DNA content and DNA strand breaks

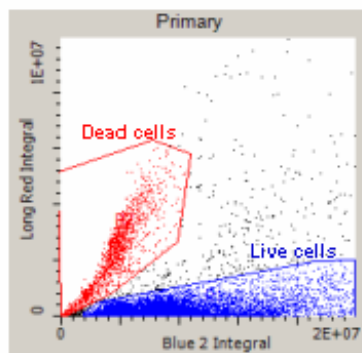
There is no apparent blockage of cells in the S-phase of the cell cycle at either high or low dosages of CPT. At the higher dosages, there are some sub-G1 DNA content cells present. Also at the higher dosages, there is less H2AX staining than at the lower dosage.



Live-Cell Analysis

An alternative approach to analyzing the effects of CPT and other compounds is to use a homogenous live-cell staining method where there is no washing procedure, so that apoptotic and other cells are not lost from the analysis. Aggregate data for a tumor cell line experiment stained according to the second format is displayed in the following scattergrams.

Two distinct populations consisting of live cells and dead cells are shown in the Hoechst DNA staining vs. the propidium iodide scattergram. DNA staining with propidium iodide is a commonly used cell viability indicator. One region was drawn around the live cells and color-coded blue. A second region was drawn around the dead cells and color-coded red.

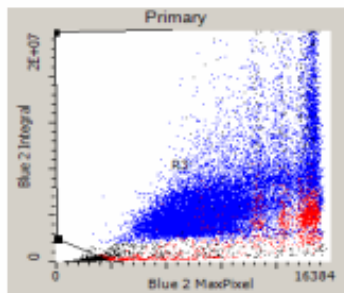


Hoechst 33342 DNA Staining vs. Propidium iodide DNA Staining

IIIa. Fixed- and Live-Cell Results, continued

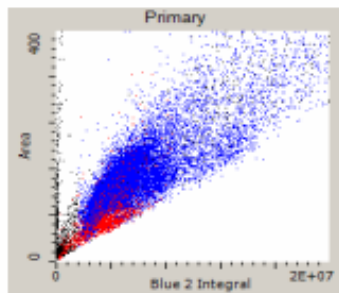
The same color-gating scheme is used for other scattergrams, so that further characterization of the dead cell population can be carried out.

Hoechst DNA Condensation vs. DNA Content



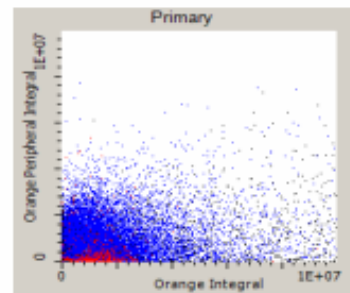
Hoechst Max. Pixel, an indicator of the chromatin density
The red cells have a very high max pixel value.

Hoechst DNA Content vs. Nuclear Area

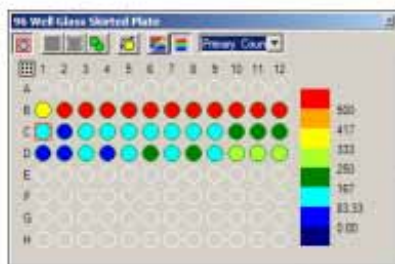


The dead cell population has smaller areas than the main cell population.

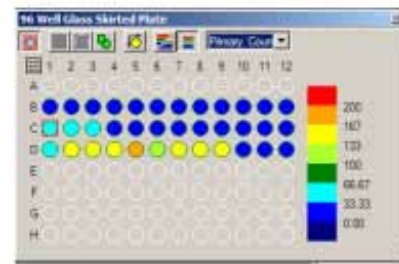
Mitoshift Nuclear Area vs. Mitoshift Cytoplasmic Integral



Mitochondrial membrane potential has been lost in the dead cell population.



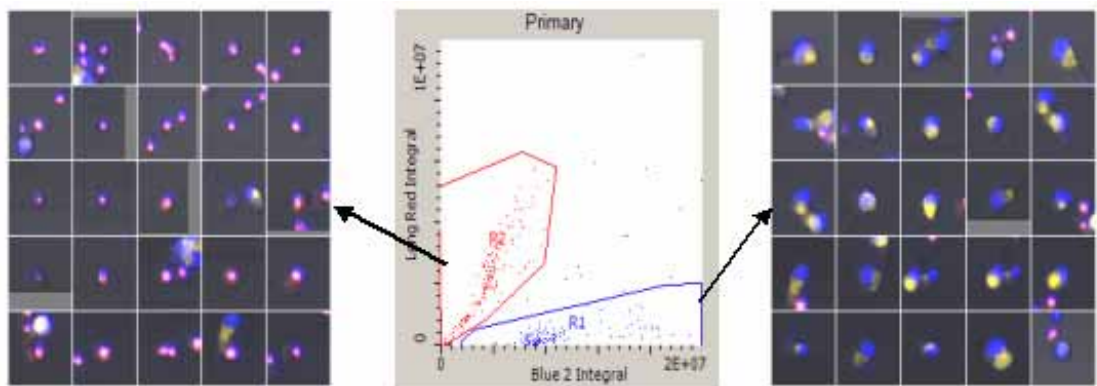
Live-cell counts color-coded per well for CHO (row B), LNCaP (row C) and IM4 (row D).



Dead-cell counts color-coded per well for CHO (row B), LNCaP (row C) and IM4 (row D).

IIIb. Live-Cell Galleries

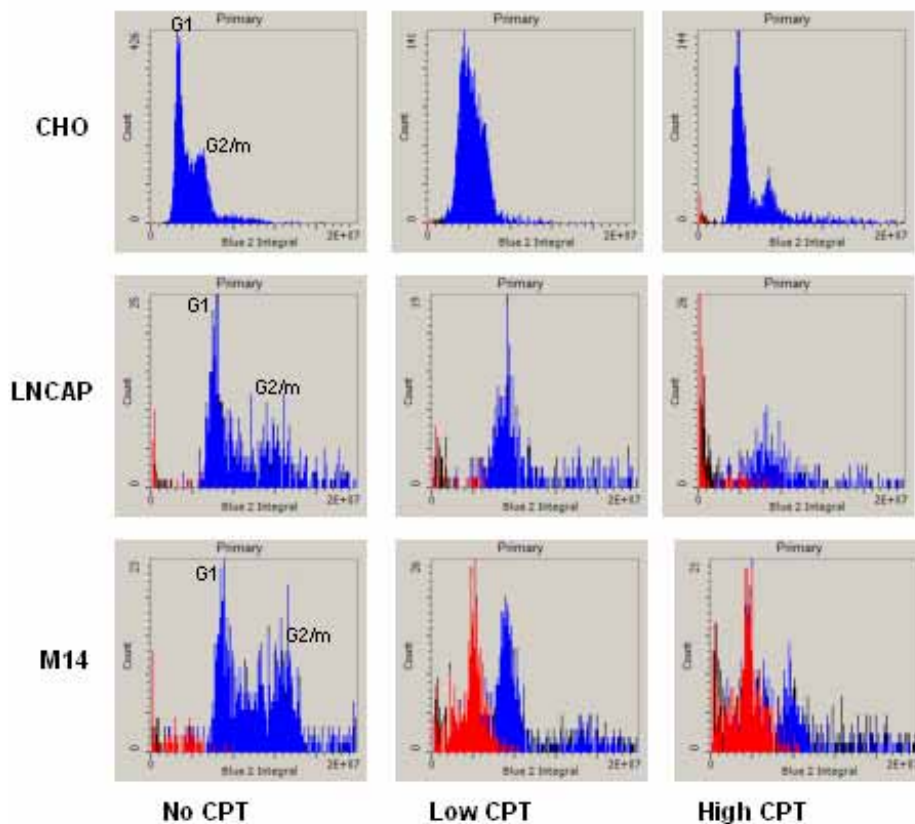
The morphometric features of the cell populations can be verified by observing images of the cells that correspond to the points on the scattergram. Below is an example of the M14 cell line treated with a moderate dosage of CPT. The cells from the red region have small, pyknotic nuclear staining for both Hoechst and propidium iodide, findings consistent with apoptotic cell characterization. The cells from the blue region have large nuclei, and also exhibit mitochondrial staining.



Galleries of dead and live cells relocated from a CPT treated M14 well

IIIc. Live-Cell Histogram Results

DNA histograms from an analysis of CHO, LNCaP and M14 cells are shown below. Coloration of the histograms is based on propidium iodide permeability, with dead cells colored red.



The CHO cells exhibit the same cell cycle patterns as seen in the fixed cells, with a blockage in S-phase at low CPT concentrations and cells predominately in G1 and G2 at the high dosages. Interestingly, the CHO samples show very few dead cells.

The LNCaP cell line shows blockage of cells in S-phase at the lower dosage of CPT, and a small number of cells undergoing apoptosis.

The M14 cell line exhibits strong response to the CPT. At the low dosage, there appears to be a slight blockage in early S-phase, but the predominant effect is a large number of cells that have become apoptotic. The process is more notable at the higher dosages.

IV. Conclusions

DNA content analysis of CPT-treated CHO cells showed blockage of the cells in S-phase of the cell cycle at low dosages. At mid- to higher dosages of CPT, the cell cycle distribution showed fewer cells in S-phase. The reduction of S-phase cells in the CHO cell line could have been due to loss of the CHO cells to apoptosis and detachment from the plates during the staining process.

Analysis of caspase staining, apoptotic bodies and cell numbers in time-course experiments indicated that the treated cell populations were in a static state, possibly senescence. Homogenous live-cell analysis of CHO cells did not reveal a significant population of apoptotic cells.

In contrast, live-cell analysis of the tumor lines LNCaP and M14 showed that a significant portion of the cells treated with CPT were undergoing cell death as measured by propidium iodide uptake. Analysis of morphometric features of the dead cells showed that they have small size, condensed chromatin, sub-G1 DNA content and loss of mitochondrial membrane potential—hallmarks of apoptotic cells. Morphology of the dead cells confirms their apoptotic nature.

In fixed-cell studies of the tumor cell lines, the apoptotic populations were substantially reduced due to the loss of the cells in the washing process; live-cell staining is therefore better for analyzing apoptotic cells.

Further studies are needed to assess long-term viability of the CHO cells arrested with a $G_{0/1}$ content and their possible senescent phenotype or propensity to undergo necrosis.

The high-content analysis offered by LSC makes it possible to discriminate different responses of the target cells to anti-tumor drugs, depending on drug concentration. This could be of special prognostic value in assessing an individual patient's response to various drugs in different dosages, utilizing tumor cells from primary cultures.

References

1. Huang, Xuan, Okafuji, Masaki, Traganos, Frank, Luther, Ed, Holden, Elena and Darzynkiewicz, Zbigniew, Assesment of Histone H2AX Phosphorylation Induced by DNA Topoisomerase I and II Inhibitors Topotecan and Mitoxantrone and by the DNA Cross-Linking Agent Cisplatin. *Cytometry, Part A* 58A:99-110 (2004).
2. te Poele, Robert H, Okorokov, Andrei L., Jardine, Lesley, Cummings, Jeffrey and Joel, Simon P., DNA Damage Is Able to Induce Senescence In Tumor Cells *in Vitro* and *in Vivo*, *Cancer Research* 62, 1876-1883, March 15, 2002.