Reversal of Apoptosis as a Homeostatic Mechanism in Mammalian T-lymphocytes

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Abstract

Programmed cell death by apoptosis is a vital feature of multicellular life. It is characterized by a common set of morphological and biochemical changes that include chromatin condensation, caspase activation, DNA cleavage, membrane blebbing, and cellular fragmentation. Apoptosis can be reversed by a process called anastasis, which has been previously documented as a contributing mechanism in cancer recurrence following chemotherapy. The goal of our current work was to determine whether anastasis represented a more generalized cellular process independent of cancer. We used cultured mouse HT2 T-lymphocytes as a model system, a non-cancerous cell line that is dependent on the cytokine Interleukin 2 (IL2) for survival. Apoptosis was induced in HT2 cells with three different methods: IL2 deprivation (starvation), treatment with staurosporine, or camptothecin. We have previously shown that apoptosis induced by IL2 deprivation for 24 hours was reversible following IL2 re-addition, as cells were observed to gradually resume their normal physiological processes within 72 hours of rescue. Here, HT2 cells were also treated with staurosporine and camptothecin, washed free of each inducer, recovered in IL2, and analyzed by flow cytometry at 12, 24, 48, and 72 hours following the removal of the chemical inducer. Cell viability and executioner caspase 3/7 activity were determined jointly using SYTOX Red vital dye and NucView 488 substrate, respectively, whereas cell cycle distribution was monitored post-fixation using propidium iodide to determine DNA content per cell. Our findings revealed that apoptosis in HT2 cells was reversible following camptothecin treatment and IL2 deprivation and that HT2 cells gradually regained normal cell cycle distribution during the 72-hour rescue window. However, cells induced with staurosporine could not be rescued from apoptosis. Interestingly, sustained caspase activity was observed throughout the rescue period for cells treated with

camptothecin for 9 hours. Our findings suggest that anastasis may be a normal physiological process in animal cells independent of malignancy but dependent on the inducer of apoptosis.

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Introduction

Programmed cell death (PCD) is a wide-ranging set of natural homeostatic mechanisms used by multicellular organisms to manage cell proliferation and body plan during development as well as eliminate mutated or otherwise compromised cells during adult life (Fuchs and Steller, 2011). One of the most thoroughly studied and documented forms of PCD is apoptosis, an organized cellular process that cells use to induce their own dismantling in response to intracellular stress or extracellular signaling. Apoptosis was first described as a distinct form of PCD by Kerr and colleagues in 1972 (Kerr et al., 1972). They described a classic morphology that is still recognized to this day as the core features of apoptosis, namely membrane blebbing, condensation of the cytoplasm and nucleus, and fragmentation of the cell into membrane-bound structures called apoptotic bodies (Kerr et. al. 1972). The process of apoptosis can be broken down into three general phases during which these morphological changes occur. There is the initiation phase, wherein the signal to die is received by the cell. This sets off a cascade of intracellular signals and effects dubbed the effector phase, culminating in a commitment to die, followed by the degradation phase where the cell is broken down into apoptotic bodies that are subsequently engulfed by phagocytes (Green and Kroemer 1998). Apoptosis is a non-lytic form of cell death with two pathways of action, the intrinsic and extrinsic pathways. Intrinsic apoptosis is triggered when there is intracellular stress such as damage to DNA or lack of proper nutrients. As a result, the mitochondrial membrane is permeabilized leading to the release of cytochrome C, formation of the apoptosome, and activation of initiator caspase 9 (Ketelut-Carneiro and Fitzgerald, 2022). Extrinsic apoptosis is triggered by the binding of a death ligand to death receptors which activate initiator caspase 8 (Ketelut-Carneiro and Fitzgerald, 2022).

Initiator caspases cleave downstream pro-caspases 3 and 7, activating them into functional executioner caspases, which subsequently act on cellular proteins to produce the hallmarks of apoptosis such as membrane blebbing, condensation, and fragmentation (Ketelut-Carneiro and Fitzgerald, 2022). The activation of executioner caspases has been named as the 'point of no return', the activity of the effector phase which commits the cell to die (Green and Kroemer 1998).

Typically, cells induced into apoptosis initiate the aforementioned cell death pathway(s) and proceed to fragment and die; however, recent research has suggested the possibility of the reversal of apoptosis in cancer cells. Several cancer cell lines have been shown to survive and regain normal morphology and physiology after the initiation of apoptotic events, such as condensation and caspase activation, when the inducer of apoptosis is removed (Tang et. al. 2008). Apoptosis reversal is a phenomenon that has been termed anastasis, a Greek word meaning resurrection, due to the cells undergoing this process seemingly rising to life from what was thought to be inevitable death (Tang and Tang, 2012). The discovery of anastasis and subsequent study of the mechanism has primarily been in cancer cells due to implications in the success of chemotherapy treatment.

The literature regarding anastasis is generally focused on cancer cells, but we were interested in determining whether the reversibility of apoptosis is a more generalized physiological mechanism in mammalian cells. To investigate the ubiquity of anastasis in non-cancerous cells, we investigated the effects of three well-known inducers of apoptosis on the non-cancerous HT2 mouse T-lymphocyte cell line. HT2 cells are dependent on a growth factor or cytokine known as Interleukin 2 (IL 2). When in the presence of IL 2, cells grow and replicate, but previous work in this lab has shown that when deprived of IL 2, HT2 cells undergo

apoptosis and all die within 48 hours. Our lab has also previously established that anastasis consistently occurs in HT2 cells when rescued from IL 2 deprivation by re-addition of IL 2 twenty-four hours following deprivation. In this study, we tested two known chemical inducers of cell death, camptothecin and staurosporine to investigate whether reversal of apoptosis in the HT2 cell line could be induced.

Camptothecin is a topoisomerase inhibitor that blocks the action of the topoisomerase enzyme, an essential element in the DNA replication process (Liu et. al. 2006). Topoisomerases cleave, unwind and rejoin DNA strands to control supercoiling and tangling as DNA is made accessible for replication (Pommier et. al. 1998). Camptothecin forms a complex with topoisomerase and DNA that inhibits the religation of cut DNA, and when this complex comes in contact with a replication fork, DNA synthesis is irreversibly halted inducing cytotoxic effects (Liu et. al. 2006). Staurosporine is a protein kinase inhibitor affecting a wide range of processes in a cell. Rather than targeting specific pathways, the inhibition of phosphorylation halts many intracellular activities (Chae et. al. 2000). In HT2 cells, the activity of staurosporine as an inducer of apoptosis was nearly immediate. Within the first few minutes of adding staurosporine to healthy cells, they could already visually be observed to start blebbing.

In order to investigate apoptosis reversibility in HT2 cells following treatment with either camptothecin or staurosporine, I specifically investigated and quantified cell cycle distribution, cell viability, and executioner caspase activation using flow cytometry analysis of fluorescence-based assays. Previous work from this lab has shown that HT2 cells undergoing apoptosis following IL 2 deprivation can be successfully rescued from imminent death by re-addition of IL 2 to the culture medium. Additional studies have shown the reversibility of anastasis in cancer cell lines such as HeLa from inducers like staurosporine and ethanol (Sun et. al. 2017, Tang and

Tang 2018). The combination of the known possibility of anastasis in non-transformed cells with the demonstrated reversibility from various cell death inducers in transformed cells lead to the hypothesis that anastasis could represent a normal physiological antidote to apoptosis in mammalian cells. Here, I sought to address whether apoptosis reversibility (anastasis) could be induced in response to known chemical triggers of apoptosis. Our findings suggest that anastasis represents a homeostatic mechanism in normal untransformed cells that is inducer-dependent.

Methods

HT2 Cell Culture Medium

HT2 Cells were maintained in a culture medium containing 78% Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS), 10% Interleukin 2 (IL 2), 1% Glutamine Genomicine, and 1% β-Mercaptoethanol (BME). For IL2 deprivation experiments, cells were incubated in a deprivation medium (-IL 2 medium) containing 88% Dulbecco's Modified Eagle Medium, 10% Fetal Bovine Serum, 1% Glutamine Genomicine, and 1% β-Mercaptoethanol.

Cell Line Maintenance

HT2 cells were grown in a humidified incubator at 37°C with 5% CO_2, and were maintained at a maximum concentration of approximately 4×10^5 cells/ml. Stock cultures were typically maintained in 10 ml of cell culture medium. To enable a favorable growth environment, cells were passaged approximately every 3 days. When a culture reached the maximum concentration, 5×10^4 cells/ml would be transferred to a new flask of fresh culture medium.

IL2 Deprivation and Rescue

The appropriate number of cells needed for each experiment was transferred to a centrifuge tube and spun at 1200 rpm at 14°C for 8 minutes. The supernatant was decanted and the pellet was resuspended in 10 ml of -IL 2 medium and centrifuged for 8 minutes. This wash was then repeated a second time. After decanting supernatant from the second wash, the pellet was resuspended in 10 ml -IL 2 medium and then incubated at 37°C/5% CO_2 for 24 hours.

To rescue cells from IL 2 deprivation, all of the cells were transferred to a centrifuge tube and spun at 1200 rpm for 8 minutes at 14°C. The supernatant was decanted and the pellet was subsequently resuspended in 10 ml +IL 2 medium. Cells were then incubated at 37°C/5% CO_2.

Drug Treatment and Rescue

Drug treatments were performed by transferring the appropriate number of cells needed for the experiment into +IL 2 medium. For Camptothecin treatments, 0.66µM of Camptothecin was added to the cells in +IL 2 medium. For staurosporine treatment, 1µM of staurosporine was added to cells. Cells were then incubated at 37°C/5% CO_2. The length of incubation was dependent on the experiment being carried out.

To carry out cell rescue following drug treatment, HT2 cells were transferred to a centrifuge tube and spun at 1200 rpm for 8 minutes at 14°C. The supernatant was decanted and the cell pellet was subsequently washed twice with 10 ml of -IL 2 medium. After the final spin, the supernatant was decanted and the pellet was resuspended in 10 ml +IL 2 medium and incubated at 37°C/5% CO_2. Incubation time for rescue was used as an independent variable and multiple times were used.

SYTOX Red / NucView 488 Dual Staining

Cells were transferred to a centrifuge tube and spun at 1200 rpm for 8 minutes at 14°C. The supernatant was decanted and the cell pellet resuspended in 1 ml of phosphate buffered saline (pH = 7.2) supplemented with 1.5% fetal bovine serum (PBS/1.5%FBS). Cell counts and viability were determined by Trypan Blue exclusion. $5x10^5$ cells were transferred into 2 ml of PBS/1.5%FBS and centrifuged for 5 minutes. The supernatant was decanted and the pellet

resuspended in the remaining volume. The remaining volume was then measured with a micropipettor and transferred to a new microcentrifuge tube. The volume in the microcentrifuge tube was then brought up to 200µl. 1µl of NucView 488 (Biotium, Freemont,CA) and 0.2µl of SYTOX Red (Thermo Fisher, Waltham, MA) were added to each cell preparation and then incubated at room temperature for 30 minutes in the dark. After incubation, the final volume of the microcentrifuge tube was brought up to 500µl. Flow cytometry analysis was subsequently carried out using a Guava EasyCyte 6HT flow cytometer equipped with a blue (488 nm) 150mW laser and a red (642 nm) laser (MilliporeSigma, Burlington, MA).

Cell Cycle Analysis

Cell cycle analysis was conducted in two parts. First, cells were fixed in 3 ml of cold methanol by adding them dropwise into absolute methanol maintained at -20C as the tube was vortexed. These tubes were then stored at 4°C until needed for analysis. Fixed cells were centrifuged at 1200 rpm for 5 minutes at 4°C. The supernatant was decanted and the pellet resuspended in 3 ml of sterile PBS. Cells were centrifuged again and the supernatant decanted. The pellet was resuspended in the remaining liquid and its volume was then measured with a micropipettor. The remaining volume was transferred to a microcentrifuge tube, and 400µl of cell cycle reagent/propidium iodide (MilliporeSigma, Burlington, MA) was added. Cells were incubated at room temperature for 30 minutes in the dark, prior to flow cytometry analysis.

Cell Viability

Cell viability was determined by trypan blue exclusion. A 1:1 ratio of Trypan Blue and cell medium was used. The weakened cytoplasmic membranes of dead cells allowed for the

Trypan Blue dye to penetrate the cell, turning the dead cells blue. Cells were placed in a

hemocytometer for counting, and percent viability was determined by dividing the number of

live cells by the total number of cells counted.

Results

Time course Viability

Time course experiments were conducted to determine the progression of apoptosis in HT2 cells exposed to drug treatments. Cells were exposed to camptothecin or staurosporine for time points between 0 and 24 hours. After exposure to treatment, cell viability was determined using trypan blue exclusion. Cellular viability with no drug treatment was found to be nearly 100%, but after 24 hours of exposure to chemical inducers of apoptosis, cell viability dramatically decreased (Fig. 1). The drop in cell viability indicates the effectiveness of the chosen drugs as inducers of apoptosis in HT2 cells. By examining the progression of cell death when exposed to the inducers, we were able to select treatment times that would yield the best results for apoptosis reversal.

Rescue Viability

Knowing that the chosen inducers of apoptosis were effective, HT2 cells were then tested for cursory viability during rescue from these inducers with Trypan Blue exclusion. Cells were exposed to camptothecin for 9 hours and then allowed to recover from treatment for 12, 24, 48, and 72 hours. Cell viability decreased to ~28% after 12 hours of rescue compared to ~89% viability directly after the 9-hour treatment (Fig. 2A). Viability after 12 hours increased continuously through to 72 hours at which point viability reached nearly 70%. In previous work, IL2 deprivation was observed to increase viability over all rescue periods, and this was corroborated by our current results. In one representative experiment, cells reached 98% viability in just 48 hours and 100% viability after 72 hours of recovery (Fig. 2B). Staurosporine-induced cells showed 96% viability after a one hour treatment. However, with as little as one hour of

treatment and as long as 72 hours of recovery in +IL 2 medium, cell viability dropped to zero (Fig. 2C). These findings were consistent with the zero percent viability of cells induced with 3- and 6-hour treatment times (Fig. 2C).

SYTOX Red Viability Analysis

Cell viability was additionally quantified using SYTOX Red fluorescent staining. Dead cells with permeable membranes take up the fluorescent red dye and exhibit a higher fluorescence intensity by flow cytometry analysis. In contrast with control HT2 cells maintained in IL2 medium, cells treated with camptothecin for 9 hours displayed two intensity peaks: a high-intensity peak of around 700, and a low fluorescence intensity peak at 10 (Fig. 3A, B). The low fluorescence peak was left-shifted compared to the control peak. The rescue trials corroborated the Trypan Blue exclusion data showing that, over the course of rescue, cell viability initially decreased, began to increase and by 72 hours post-rescue, a fluorescence peak indicative of healthy cells reemerged. The 12-hour rescue contained two peaks, a low fluorescence peak at around 20 and a high fluorescence peak at 200 (Fig. 3C). At 24 hours post-recovery, the fluorescence intensity of the low peak right-shifted to 30, but the high fluorescence peak intensity remained the same. However, the cell count of the high-intensity peak was increased compared to 12 hours (Fig. 3D).

SYTOX Red staining of cells exposed to staurosporine for one hour showed a high-intensity fluorescence peak at about 2000 and two low-intensity fluorescence peaks, one which aligned with the +IL 2 control peak and a second one that was left-shifted (Fig. 4B). All of the rescue trials displayed two fluorescence peaks: one that was left-shifted relative to the control peak around 20 and a high-intensity peak around 400 (Fig. 4C-E).

These data were congruent with the viability findings obtained with Trypan Blue exclusion. Though the time point at which viability was the lowest was different between SYTOX Red staining and Trypan Blue exclusion, they both indicated an eventual increase in viability as rescue time progressed. Both analyses also demonstrated the complete loss of viability of HT2 cells exposed to staurosporine after one hour of treatment.

Cell viability reached a minimum following camptothecin induction at 24 hours post rescue, with the greatest cell count in the high intensity (apoptotic) peak.

Cell cycle analysis

Healthy cells undergo a standard progression of growth and development known as the cell cycle. A cell's progression through the cell cycle is tracked by known changes in DNA content throughout its life cycle. Fluorescent staining of DNA allows for the analysis of DNA content within cells based on the fluorescence intensity, with higher intensity indicating higher DNA content. The flow cytometry analysis of a cell population for cell cycle analysis produces a frequency histogram indicating how many cells in a given population are at each stage of the cell cycle. For healthy cells, the distribution starts with an initial peak indicating the G1 phase at which point each cell has one complete copy of its genome. This is followed by a lower plateau of cells in S phase (DNA synthesis) at different stages of DNA replication. Then, there is a second peak at twice the DNA content of the first peak indicative of the G2/M phase at which point cells have two full copies of their genome. Apoptotic cells do not display this normal cell cycle distribution and acquire a Sub G1 peak. Cells in this peak display lower fluorescence intensity than cells with one full copy of the genome indicating the presence of fragmented DNA, a hallmark feature of apoptosis (Darzynkiewicz et. al. 2001). As apoptotic cells deviate

from the cell cycle distribution displayed by healthy cells, cell cycle analysis was used as a measure of apoptotic activity within HT2 cells exposed to the array of death inducers.

Previous studies have shown that IL2 deprivation of HT2 cells induces an accumulation of a Sub G1 peak at 24hrs of deprivation where 34.44% of cells fell in the Sub G1 range. After 48hrs of deprivation, 98.95% of cells were observed within the Sub G1 range. In contrast, if cells were rescued by reintroducing IL 2 growth factor after 24hrs of deprivation, the cell cycle redistribution will return to normal over a 72 hr recovery period (Lauzon, unpublished observations).

Camptothecin-exposed cells also showed an accumulation of Sub G1 cells compared to the +IL 2 control following a 9hr treatment (Fig. 5A, B). At 12 hours of recovery, nearly 82% of cells were observed in Sub G1 and apoptotic (Fig. 5C). From 12 to 72 hours of recovery, there was a progressive re-establishment of a normal cell cycle distribution pattern as well as a decrease in the percentage of the population in Sub G1 (Fig. 5D-F).

After one hour of staurosporine treatment, there was an increase in the percentage of the population in the Sub G1 peak from 3.34% as seen in healthy cells to 42%, but with the maintenance of some cells with a normal cell cycle distribution pattern (Fig. 6A, B). When a one hour treatment was combined with 72 hours of rescue, 99% of the cell population was observed in the Sub G1 range (Fig. 6C). This remained true for cells exposed to staurosporine treatment times of 3 and 6 hours (Fig. 6D, E). A return to a normal cell cycle distribution was never observed in staurosporine-treated cells even when using the longest recovery time of 72 hours (Fig. 6C-E).

Caspase activity

Caspase activity is a defining marker of apoptosis. The activation of caspases is responsible for many of the features of apoptosis. NucView 488 is a caspase substrate that emits green fluorescence when cleaved by active executioner caspases 3 and 7. Higher intensity fluorescence is indicative of greater caspase activity within a cell.

+IL 2 control cells displayed a single fluorescence peak at 25, which is indicative of a baseline fluorescence for healthy cells (Fig. 7A). Following 9 hrs. of camptothecin treatment, the healthy cell peak was right-shifted from 25 to approximately 50 and a second, high fluorescence peak emerged (Fig. 7B). The lower fluorescence peak fluctuated as to cell count throughout the rescue period, but remained around 50 at all of the recovery time points from 12 to 72 hours. The higher fluorescence peak reached its maximum fluorescence intensity at approximately 7000, immediately after the 9-hour camptothecin treatment (Fig. 7B). After 12 hours of rescue, peak fluorescence intensity dropped to around 600 (Fig. 7C). Between 12 and 72 hours, the high fluorescence peak fluctuated but did not exceed 1,000 (Fig. 7C-F). At 72 hours of the rescue period, the intensity and cell number within the high fluorescence peak were lower than at the initial treatment, but caspase activity remained elevated as compared to control +IL 2 cells (Fig. 7).

Cells exposed to staurosporine displayed an initial high fluorescence peak intensity of around 5,000 (Fig. 8A, B). After 72 hrs. of rescue for all treatment times tested (1, 3 and 6 hours), the high fluorescence peak intensity was consistently observed around 80. Caspase activity levels remained unchanged following rescue from staurosporine (Fig. C-E).

For both camptothecin and staurosporine treated cells, the high-intensity fluorescence peak reached its maximum intensity directly after treatment (Figs. 7, 8). This high caspase

activity was due to staurosporine inducing the apoptotic pathways that activate caspases. The reduction in peak fluorescence intensity displayed lower levels of caspase activation compared to when the inducer is present but was still elevated compared to healthy cells.

Discussion

This study aimed to determine whether the cellular process known as anastasis, or reversibility of apoptosis, was a feature of normal cell physiology, uncoupled from malignancy. Previous studies have shown applicability of anastasis to cancer cells, yet there is a paucity of studies investigating the physiological significance of anastasis in non-cancerous cells (Mohammed et. al. 2022, Sun et. al. 2017, Sun et. al. 2023, Tang and Tang 2018). The current study focused on the effects of different cell death inducers on anastasis in a non-transformed, IL 2–dependent cell line (HT2). Using camptothecin and staurosporine treatment as well as IL 2 deprivation, cells were induced to undergo apoptosis and then tested to determine if they could be rescued from death. We used flow cytometry to quantify fluorescence-based assays that assessed cell viability (SYTOX Red), executioner caspase activation (NucView 488), and cell cycle distribution via DNA content (Propidium Iodide). Analysis of these assays indicated that anastasis could be initiated within non-transformed HT2 cells exposed to IL2 deprivation and camptothecin but not staurosporine, as apoptosis was found to be irreversible following treatment with this inducer. Consequently, our findings strongly suggest that anastasis represents a normal homeostatic process, but is inducer-dependent. Taken together, this information has provided a better understanding of the conditions required to study the reversibility of apoptosis in HT2 cells.

Cell death is a process that involves the organized dismantling of the cell, rather than a singular event. Specifically, apoptosis involves the key features of membrane blebbing, nuclear and cytoplasmic condensation, and apoptotic body formation and has historically been thought to involve a point-of-no-return after which cell death becomes irreversible (Green and Kroemer 1998, Ketelut-Carneiro and Fitzgerald 2022). However in recent years, this viewpoint has come

under scrutiny by research showing the reversibility of apoptosis even following caspase activation and mitochondrial membrane permeabilization, two events once thought to be the 'point of no return' for apoptotic cell death (Tang et. al. 2008). Even though reversal has been shown to be an active physiological process, the removal of the inducer is not sufficient to immediately halt or reverse the progression of cellular apoptotic pathways. This led to the drop in viability that was observed in camptothecin treated cells by both Trypan Blue and SYTOX analysis between the initial treatment and the first recovery time point at 12 hrs. Though the precise moment at which a cell becomes irreversibly committed to die is still debated, our data with HT2 cells suggests that some cells may be at a point in the cell death pathway where reversal is not possible. The increase in viability, indicated by Trypan Blue viability analysis, as rescue progressed through 72 hours demonstrates that HT2 cells were able to be rescued from both IL 2 deprivation and camptothecin treatment. This was corroborated for staurosporine-treated cells by both SYTOX Red analysis and Trypan Blue viability assays. The reduction to zero percent viability after 72 hours of recovery time for all exposure times (1, 3, and 6 hours) indicates the inability of HT2 cells to be rescued from staurosporine treatment.

The gradual restoration of a normal cell cycle distribution in both IL 2-deprived and camptothecin-treated cells during the 72 hour rescue period is indicative of cells resuming normal activity and starting to grow and divide. The initial accumulation of cells in the Sub G1 (apoptotic) peak aligned with the SYTOX viability analysis indicating that a majority of the cells in the population were apoptotic or dead at 24 hrs of recovery. The decline in the percentage of the cell population within the Sub G1 peak between 24 and 72 hours occurred simultaneously as the number of metabolically active cells in the population increased and resumed mitosis. This is in agreement with SYTOX analysis which indicated that viability continuously increased from

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24 hours to 72 hours post rescue for cells exposed to camptothecin. This further suggests that increased viability is not solely due to the recovery of apoptotic cells but also the generation of new viable cells by mitosis. The accumulation of cells in the Sub G1 peak following staurosporine treatment and the lack of restoration of a normal cell cycle distribution pattern over the 72 hour rescue period is another indication that staurosporine-treated HT2 cells are unable to initiate anastasis.

The consistently elevated levels of caspase activity following 1-6 hours of staurosporine treatment and rescue are indicative of a pattern of caspase activity in dying cells. The high-intensity peaks correspond to elevated caspase activity as is needed for the progression of apoptosis. The consistency between the fluorescence intensity of the high-intensity peaks independent of the time exposed to the inducer shows the consistency in the level of caspase activity needed to induce apoptosis. The left-shifted lower-level fluorescence peak is indicative of fragmented dead cells, and these observations were corroborated by HT2 apoptotic bodies displaying low forward scatter (Data not shown). The small cell fragments have a smaller volume and thus appear to have low fluorescence intensity due to the limited amount of caspase in a fragment compared to a whole cell.

Caspase levels were also observed to remain elevated throughout the recovery of camptothecin-rescued cells. The lower intensity of the high fluorescence peak seen during rescue compared to the 9 hour control indicates comparatively lower executioner caspase activation. Yet, in comparison to the untreated control there was sustained caspase activation in the cell population rescued from camptothecin treatment. The functional significance of this finding is unclear as well as contradictory to the historical notion that caspase activation is indicative of the cell's commitment to die (Green and Kroemer 1998). Caspases have traditionally been thought to

be a 'point of no return' but the continued activation of caspases while cell viability and

replication increases implies that there is a more complex story. The constitutive activation of

caspases during anastasis indicates that caspases may even play a functional role in restoring

cellular homeostasis.

Future Work

The function of elevated caspase activity seen throughout the rescue of camptothecin-exposed cells remains unclear. Since caspases are functionally involved in the organized dismantling of the cell during apoptosis, this observation was initially surprising. There are many different types of caspases with activities and functions ranging from apoptosis to cell differentiation and NucView 488 staining can not differentiate between them (Shalini et. al. 2015). It is unknown which of the executioner caspases are active during this rescue period and thus we were unable to determine what function they may be performing in HT2 cells during this remodeling process. Further investigations using antibodies directed against the active forms of several executioner caspases (3, 6, 7) in western blots could be useful in distinguishing the various caspases that are active during the recovery process. By determining the type of caspases that are active at different time points during anastasis, we may be able to better understand why this activity remains high.

The use of caspase inhibitors may also provide a clue as to the functional involvement of elevated caspase activity during anastasis. The wreck and check method of investigation posits that you can determine the function of an element of a system by removing that element and examining the effects on the system. By blocking caspases and analyzing differences in outcomes between rescued cells with and without (or decreased) caspase activity, we may be able to further pinpoint the functional role that caspases play in cellular recovery from apoptosis.

Broad spectrum caspase inhibitors such as Z-VAD-fmk and p53 block all caspase activity (Belmokhtar et. al. 2001). Though caspase plays an important role in the propagation of many of the features of apoptosis, studies have shown cells can undergo apoptosis even when caspases are blocked and exhibit similar if not identical morphological changes (Lavoie et. al. 1998).

Staurosporine has been found to act in the cell both through a caspase-dependent "fast" pathway and a caspase-independent "slow" pathway (Belmokhtar et. al. 2001). This could also provide an explanation for the discrepancy between our results and previous studies that have indicated reversibility from staurosporine treatments for as long as 3 hours, which included the remarkable reincorporation of apoptotic bodies into a cell (Tang and Tang 2018). Caspase inhibitors would allow us to test whether the caspase-dependent or independent pathways are deployed by HT2 cells in response to the inducers of apoptosis used in this study. The rapid loss of viability in staurosporine-treated HT2 cells suggests that the fast-acting, caspase-dependent pathway may be preferentially triggered in these cells. HT2 cells exposed to staurosporine die rapidly when caspase is activated Therefore, if the caspase-dependent pathway is used by these cells, then staurosporine-induced HT2 cell apoptosis should be reversible in the presence of caspase inhibitors.

Lastly, it may be valuable to continue testing the response of HT2 cells to rescue from staurosporine-induced apoptosis with other variables. If the fast-acting, caspase-dependent pathway is being used by these cells, induction times shorter than one hour may produce desirable results. Dose-response trials testing different staurosporine concentrations may also provide useful insights, as previous studies using staurosporine as an inducer have ranged widely in their concentration (Sun et. al. 2017, Tang and Tang 2018).

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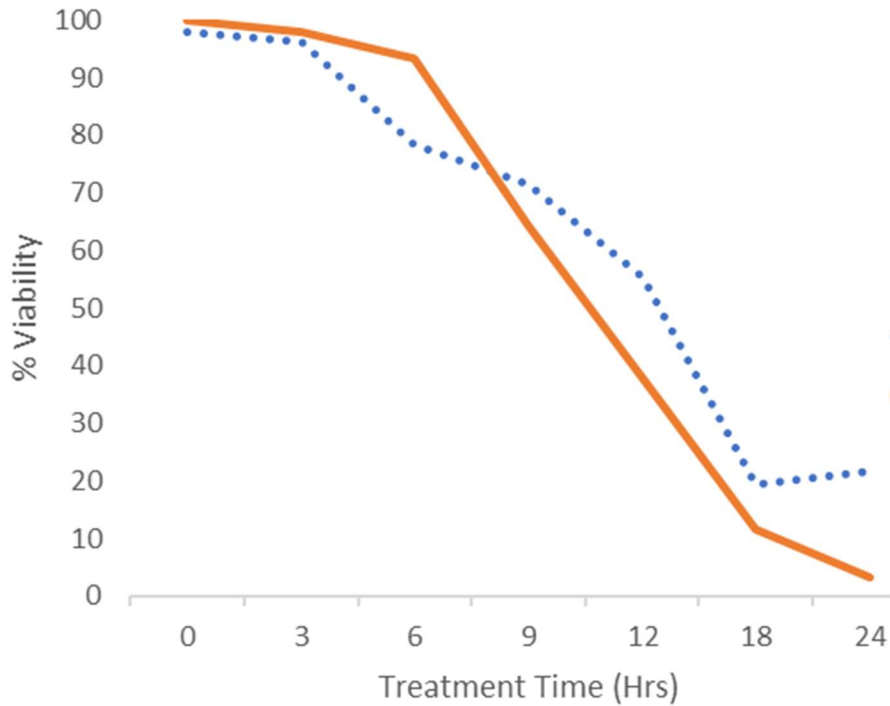
Figure 1. Time Course of HT2 cell viability when exposed to chemical inducers of apoptosis.
Cells were exposed to either camptothecin (solid orange line) or staurosporine (dotted blue line)
for up to 24 hours. Cells for each time point were stained using Trypan Blue dye and observed
under brightfield microscopy using a hemocytometer. Counts of living and dead cells were
recorded for each time point to determine cellular viability throughout the course of the
treatments. Both camptothecin and staurosporine produce at least an 80% decrease in viability by
24 hours of treatment.
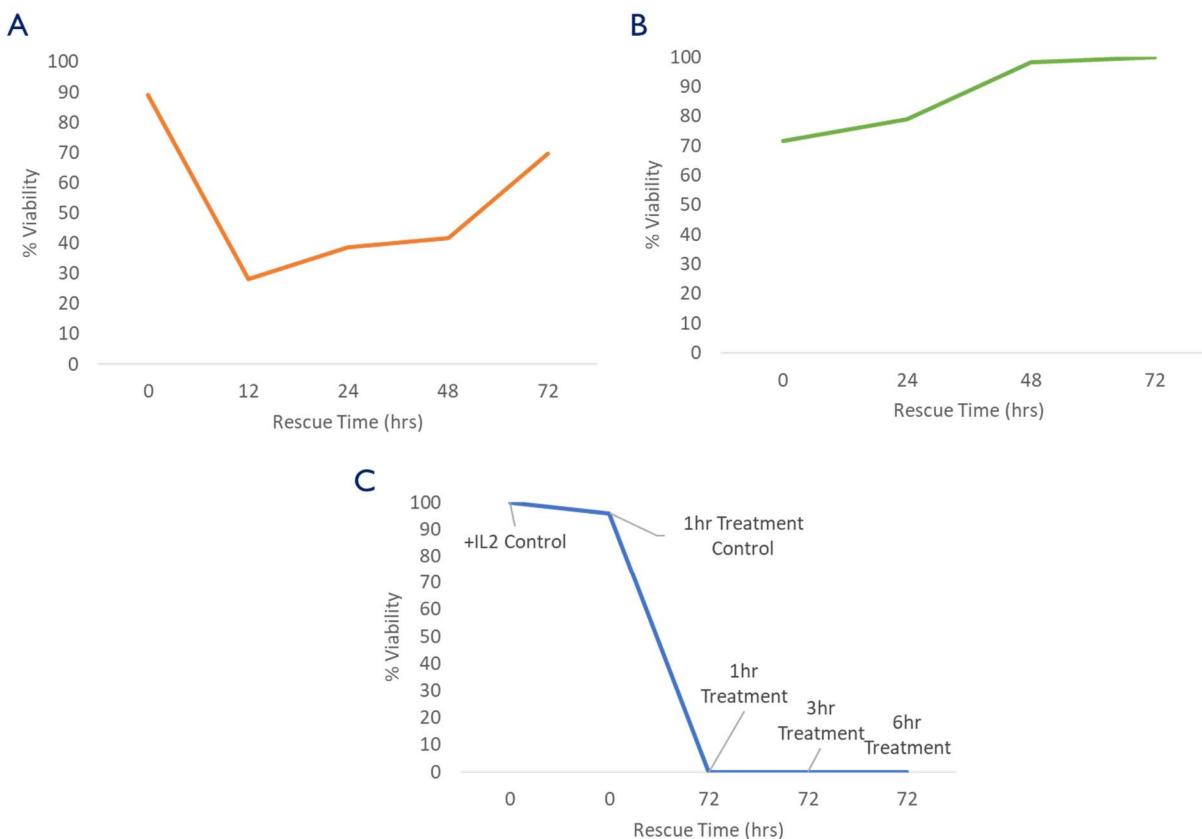
Figure 2. Viability of HT2 cells following treatment and rescue from inducers of apoptosis. (A) Cells treated with camptothecin for 9 hours decreased in viability at 12 hours of recovery but then steadily increased to 69.2% viability by 72 hours post rescue. (B) HT2 cells were deprived of the essential growth factor IL 2 for 24 hours at which point viability was 71.6%. During rescue, following the addition of IL 2, cells returned to 100% viability by 72 hours post rescue. (C) Cells were treated with staurosporine for 1, 3, and 6 hours and all were allowed to recover for 72 hours. Following a one-hour treatment with no rescue, cell viability was 96%, but after a 72 hour rescue, viability dropped to zero for all treatment times.
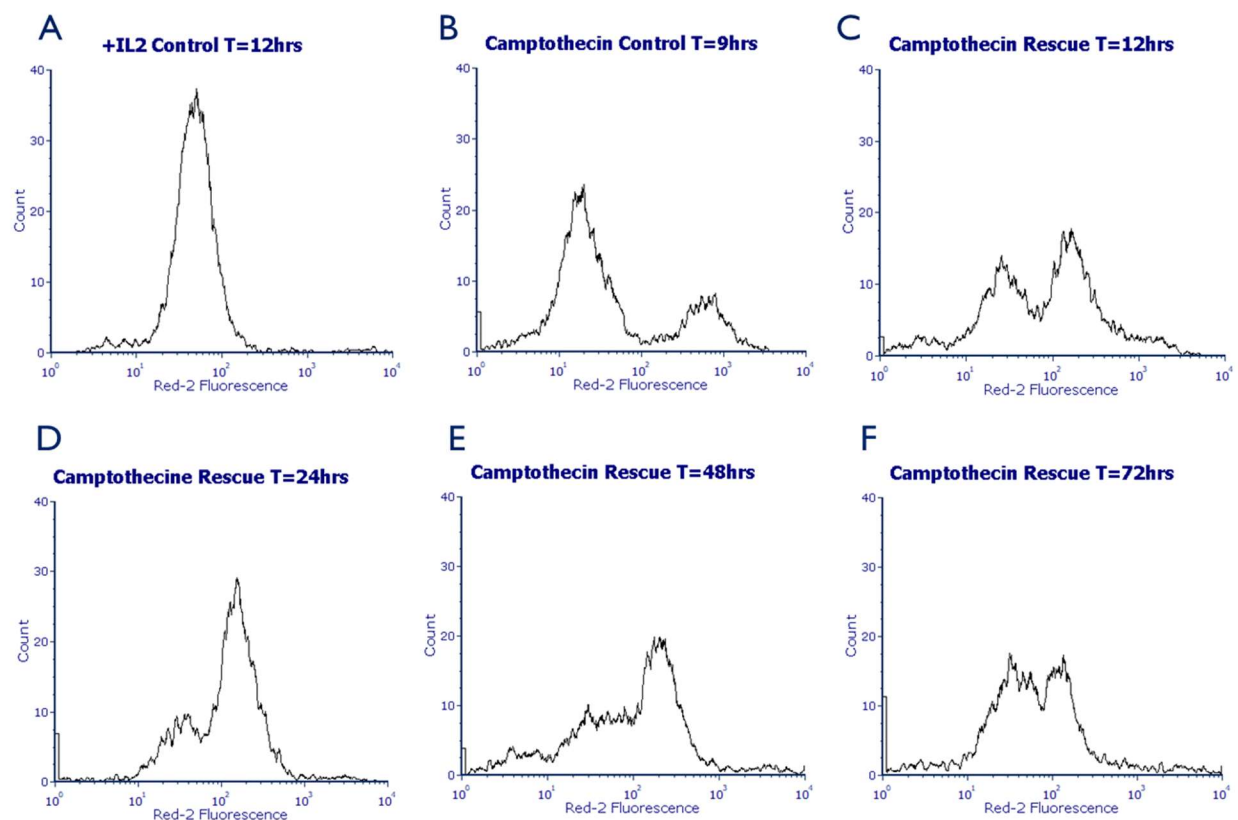
Figure 3. Flow cytometry determination of HT2 cell viability by SYTOX Red staining following camptothecin treatment and rescue. (A) The +IL 2 control displays a peak of baseline fluorescence indicative of a viable, non-apoptotic population. (B) HT2 cells were exposed to camptothecin for 9 hours and harvested for analysis directly following treatment. Cells were treated with camptothecin for 9 hours and then allowed to recover in +IL 2 medium for 12 (C), 24 (D), 48 (E), and 72 hours (F). SYTOX Red penetrates cells with compromised membranes leading to increased fluorescence in the Red2 channel. The emergence of higher fluorescence peaks in C-F is indicative of apoptotic cells. Longer recovery times correlated with a decrease in cells displaying the high fluorescence (apoptotic cells) peak and increase in the low fluorescence peak (viable cells).
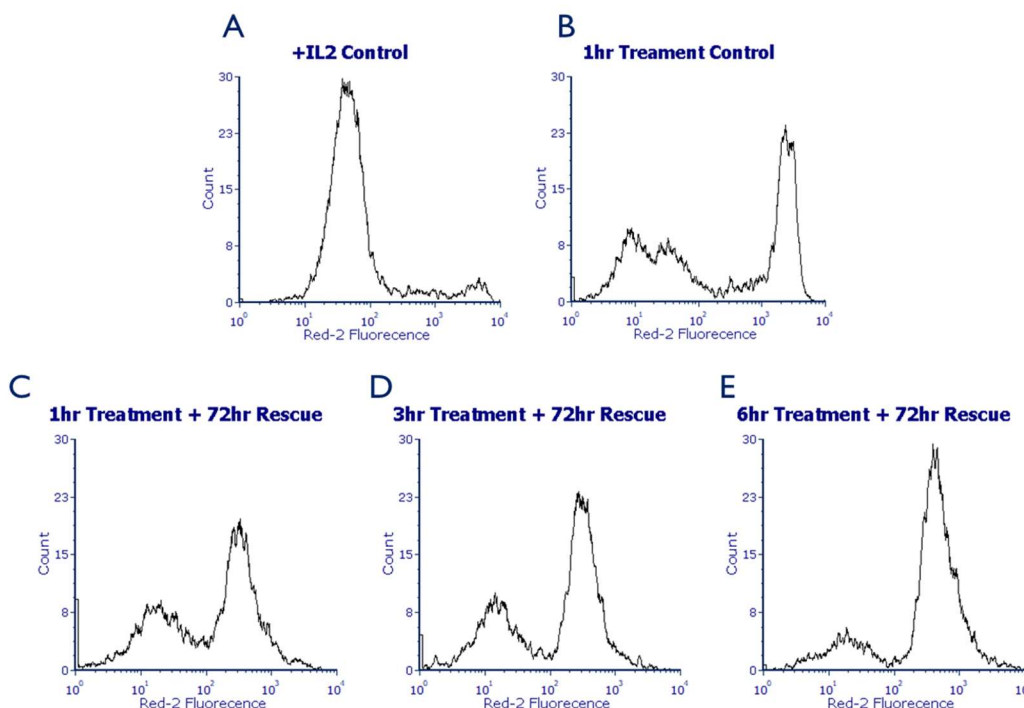
Figure 4. Flow cytometry determination of HT2 cell viability by SYTOX Red staining following staurosporine treatment and rescue. (A) The +IL 2 control displays a peak baseline fluorescence indicative of a viable, non-apoptotic population. (B) Cells were treated with staurosporine for one hour and analyzed directly following treatment and no rescue period. Cells were treated with staurosporine for 1 (C), 3 (D), and 6 hours (E) after which they were allowed to recover in +IL 2 medium for 72 hours. The high-intensity fluorescence peak indicates late-stage apoptotic cells with compromised cytoplasmic membrane integrity that allows for the fluorescent dye to penetrate. The lower fluorescence peak is left-shifted compared to the control peak indicative of a cell population in the early stages of apoptosis.
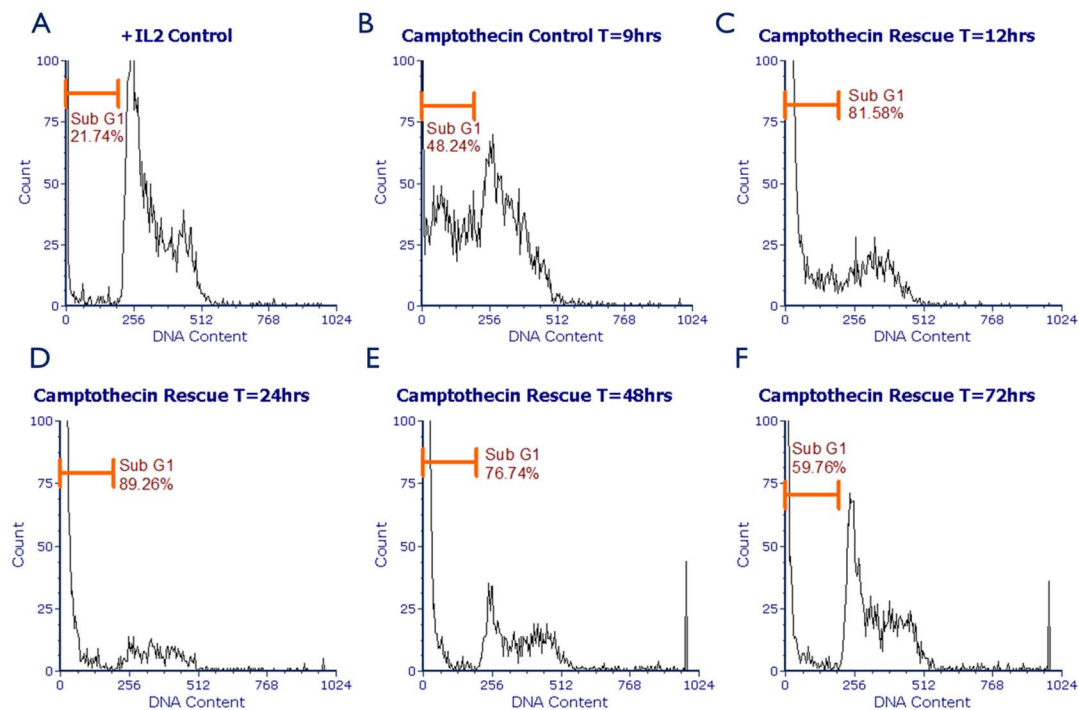
Figure 5. Flow Cytometry analysis of HT2 cell cycle distribution following camptothecin treatment and rescue. 5x105 HT2 cells were treated with propidium iodide for 30 minutes in the dark and subsequently analyzed for DNA content, indicative of cell cycle distribution. (A) Normal cell cycle distribution of HT2 cells depicting G1, S, and G2/M cell populations. (B) Cells exposed to camptothecin for 9 hours and analyzed directly following treatment show the emergence of a Sub G1 peak and disappearance of the normal cell cycle distribution pattern. In apoptosis, DNA is fragmented leading to less than the normal DNA content. Cells exposed to camptothecin for 9 hours and then allowed to recover for 12 (C), 24 (D), 48 (E), and 72 hours (F) develop a sub-G1 peak indicating apoptosis. As rescue proceeded the percentage of the cell population in the sub-G1 peak decreased and there was a restoration of a normal cell cycle distribution pattern.
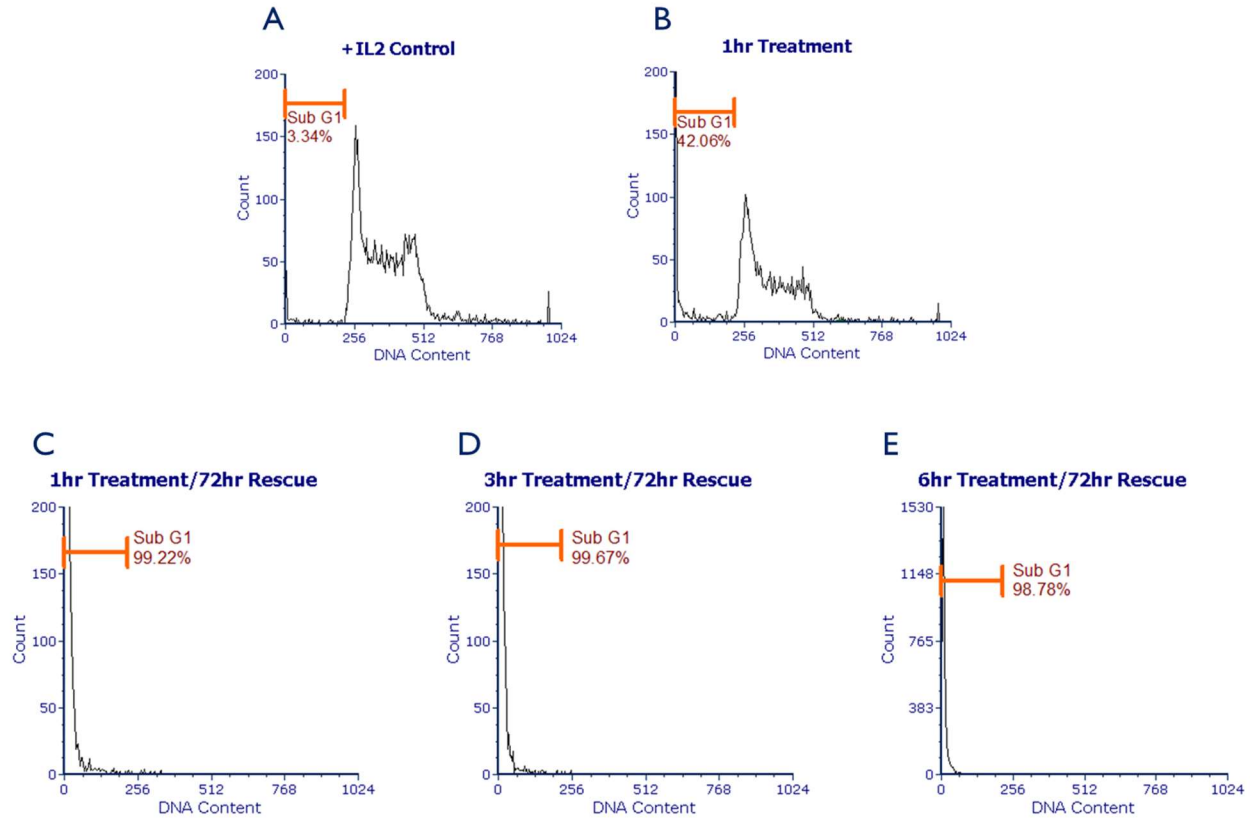
Figure 6. Flow Cytometry analysis of HT2 cell cycle distribution following staurosporine treatment and rescue. 5x105 HT2 cells were treated with propidium iodide for 30 minutes in the dark and subsequently analyzed for DNA content, indicative of cell cycle distribution. (A) Normal cell cycle distribution of HT2 cells, depicting G1, S and G2/M cell populations. (B) Cells exposed to staurosporine for one hour and analyzed directly following treatment depict the emergence of a Sub G1 peak. Cells exposed to staurosporine for 1 (C), 3 (D), and 6 hours (E) were allowed to recover in +IL 2 medium for 72 hours. Even after 72 hours of recovery time, all treatment periods resulted in nearly all cells accumulating in the Sub-G1 peak and lack of a normal cell cycle distribution pattern.
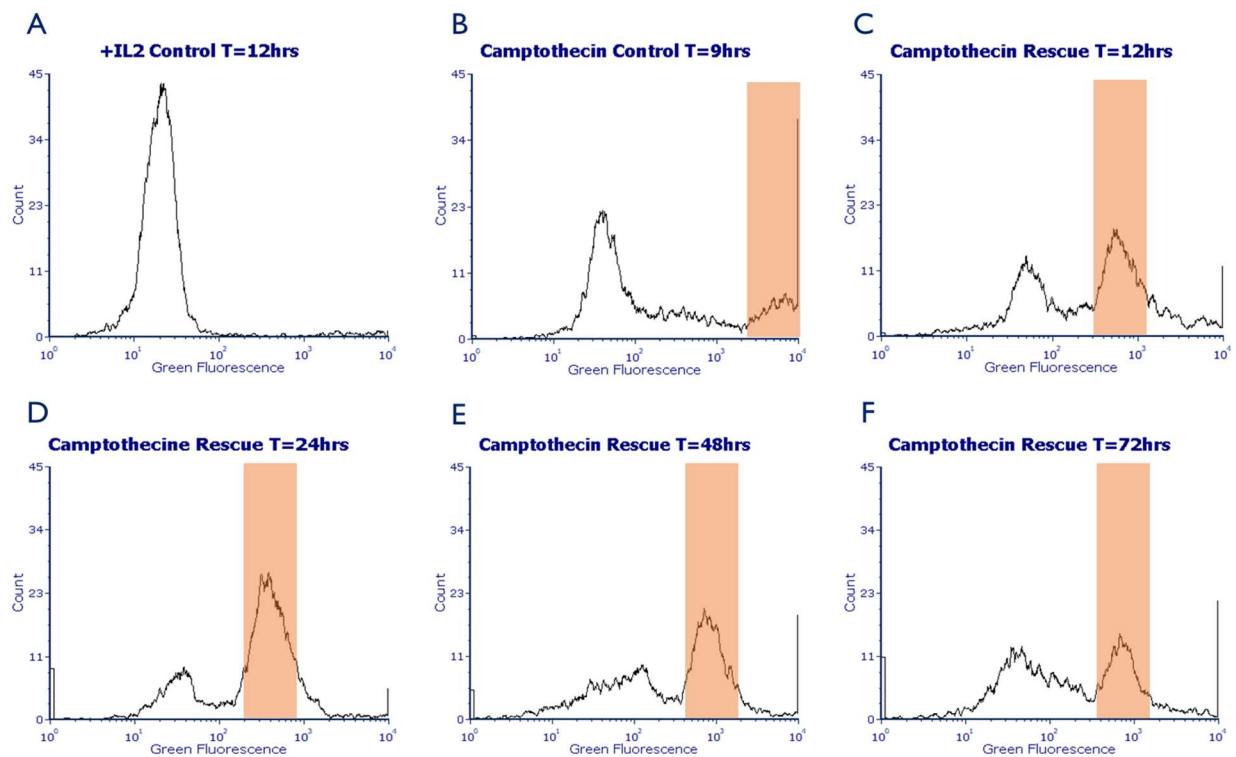
Figure 7. Flow cytometry analysis of caspase activity in HT2 cells following camptothecin treatment and rescue. 5x105 HT2 cells were treated with NucView 488 substrate for 20 minutes at room temperature and caspase 3/7 activity was quantified using flow cytometry. NucView 488 is a substrate of caspase that emits green fluorescence when cleaved with higher intensity fluorescence indicating higher caspase activity. (A) Viable HT2 cells depicting a baseline fluorescence caspase peak. (B) Cells treated with camptothecin for 9 hours display a right-shifted, lower intensity fluorescence peak indicative of early stage apoptosis. A high-intensity fluorescence peak is also observed, indicating high levels of caspase 3/7 activity and mid-late stage apoptosis. Cells exposed to camptothecin for 9 hours and then allowed to recover for 12 (C), 24 (D), 48 (E), and 72 hours (F) also display a right-shifted low-intensity peak as well as left-shifted high-intensity peak as compared to the 9 hour treated sample.
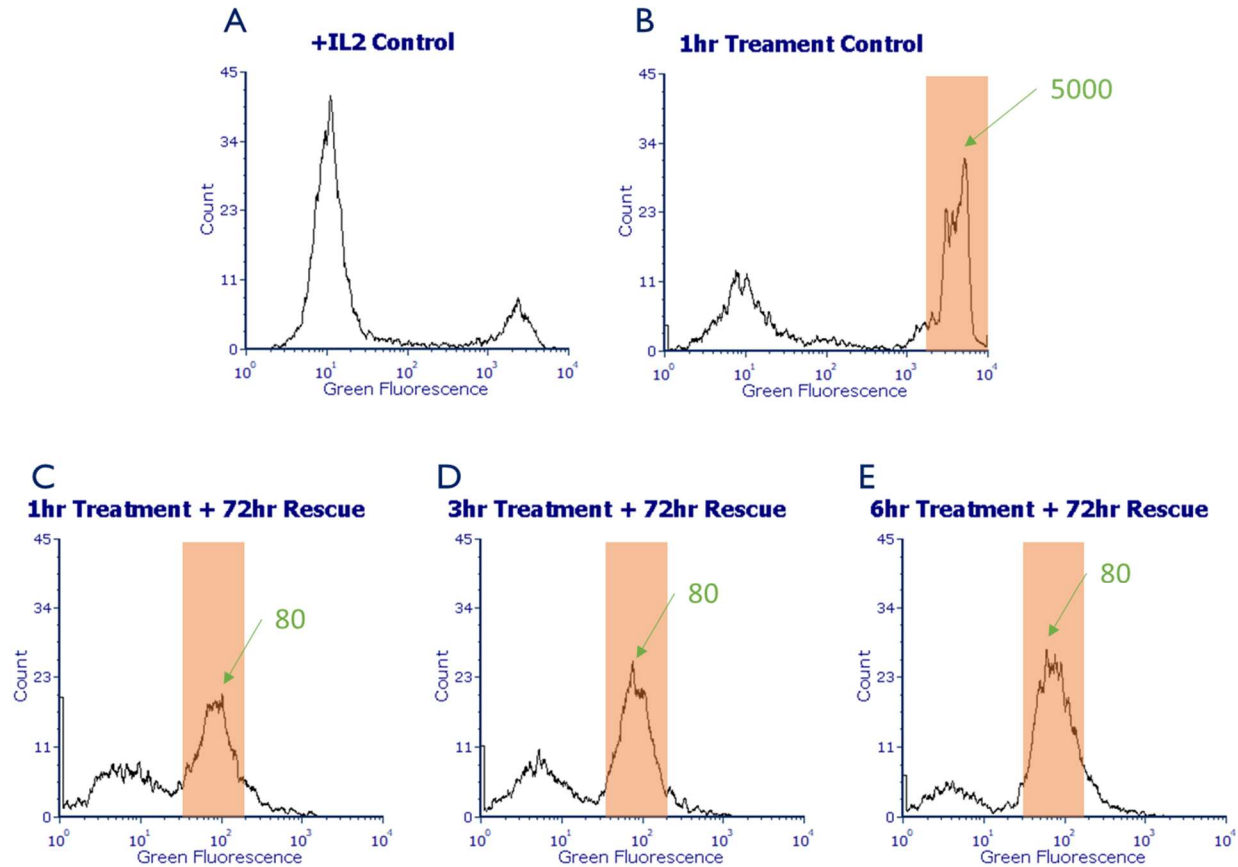
Figure 8. Flow Cytometry analysis of caspase activity in HT2 cells following staurosporine treatment and rescue. 5x105 HT2 cells were treated with NucView 488 substrate for 20 minutes at room temperature and caspase 3/7 activity was quantified using flow cytometry. NucView 488 is a substrate of caspase that fluoresces green when cleaved with higher intensity fluorescence indicating higher caspase activity. (A) Baseline fluorescence of viable HT2 cells stained with NucView 488. The high fluorescence peak observed in this experiment is likely due to cellular stress that occurred during processing prior to flow cytometry analysis. (B) Cells exposed to Staurosporine for 1 hour and analyzed immediately following treatment depict a low fluorescence caspase peak (viable cells) peak but also a prominent high-intensity peak indicative of high caspase activity and mid-late stage apoptotic cells. Cells exposed to Staurosporine for 1 (C), 3 (D), and 6 hours (E) were allowed to recover in +IL2 medium for 72 hours and revealed a

consistently elevated fluorescence peak indicative of caspase activity, as well as a left-shifted, lower intensity peak that did not align with the viable control peak. This latter peak corresponds to apoptotic bodies that result from cellular fragmentation which is highly prominent following staurosporine treatment.