Cell cycle specific effects and associated DNA damage of selective inhibitors of nuclear export (SINE)

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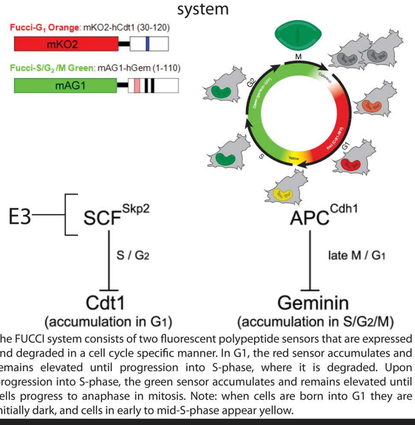
Background and Rationale

Anti-cancer responses to small molecule drugs or natural products are determined on the molecular and cellular scale. Understanding cell responses and fates following treatment using population average assays (e.g. immunoblotting), masks cell-cell variability and differences in timing, and discounts transient and rare responses. To more completely understand the complexity of drug response we must track molecular responses and cell fate choices simultaneously in individual cells in real time. The use of long-term longitudinal approaches to follow a given single cell or a cell population is a less common but very powerful approach that allows for the direct study of molecular response pathways, different phenotypes (e.g. cell death or cell division), observation of cell-to-cell variability within a population, and how these factors contribute to population response dynamics. XPO-1 inhibitors have been developed and are being evaluated as anti-cancer agents in the clinical setting. Termed selective inhibitor of nuclear export (SINE), these agents covalently bind XPO-1 and block XPO-1-dependent nuclear export of cargo proteins, including p53, p21 and p27. SINE effects on the cell cycle have been noted, but the effect(s) on the progression of individual cells, variability between cells and cell lines, and how these impact the overall response are unclear.

Abstract

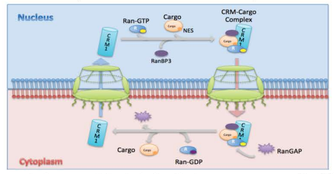
Nuclear export of proteins is fundamental for cell growth and function. Selinexor is a SINE compound that is in clinical development for the treatment of different cancers. Selinexor forms a slowly reversible covalent bond to Exportin-1 (XPO1), preventing its association with protein cargos, thereby resulting in their nuclear retention. XPO1 cargos include the majority of tumor suppressor proteins (TSP) and cell cycle regulators such as p53, p21 and p27 that have key roles in cancer progression and drug response. It is unclear how selinexor affects cell cycle progression in individual cells and the subsequent stress and fate of those cells. To elucidate Selinexor action, we developed cell lines that stably express fluorescent ubiquitin cell cycle reporters (FUCCI), and followed individual cells longitudinally using continuous time-lapse microscopy for 72 hours. We report that in fibrosarcoma-derived HT-1080 cells that express wildtype p53 and p21, 18% of the initial cell population became arrested with >90% in G1- or S-phase and 40% died with 64% from G1- or early S-phase after a cell cycle delay or arrest. We also found that 42% of cells divided, but the progeny died or arrested in G1- or S-phase of the next cell cycle – often after cell cycle arrest or slowed cell cycle progression. Using FUCCI, we tracked the response of cells treated acutely in specific cell cycle stages. Cells treated in G1-phase most often arrested or died in G1- or S-phase, whereas cells treated in G2-phase usually progressed to cell division. As FUCCI revealed S-phase progression defects and associated cell death, we further characterized this phenotype. Using nucleotide incorporation with fluorescent detection, we find that as soon as 2 hours after Selinexor fewer cells are undergoing DNA replication and those that are, are doing so inefficiently as both the rate and maximal levels of nucleotide incorporation are significantly reduced. S-phase arrest and progression defects may manifest as DNA double-strand breaks. We find a strong association between S-phase status and DNA damage. In some cells, the damage occurs within hours of Selinexor treatment and appears as a striking cluster of foci. At 8 hours, nearly 35-45% of cells contain DNA damage clusters. Importantly, the damage clusters sometimes repair as determined by fixed cell time-course analysis and live-cell microscopy. We are exploring the nature of these DNA damage structures and the mechanisms of their formation and repair. In summary, Selinexor is fast acting, shows cell cycle selectivity, results in DNA damage and is highly effective at arresting cell growth and inducing apoptosis in tumor cells. These data suggest that Selinexor may exert anti-cancer effects even on slow growing tumors where the bulk of the cell mass presents a G1-like state and that it likely combines well with other cell cycle targeted therapeutics.

Monitoring cell cycle progression using the FUCCI system



The FUCCI system consists of two fluorescent polypeptide sensors that are expressed and degraded in a cell cycle specific manner. In G1, the red sensor accumulates and remains elevated until progression into S-phase, where it is degraded. Upon progression into S-phase, the green sensor accumulates and remains elevated until cells progress to anaphase in mitosis. Note: when cells are born into G1 they are initially dark, and cells in early to mid-S-phase appear yellow.

Exportin-1 and Nuclear Export



Governs export of many proteins, including the tumor suppressors p53, $p21^{CIP1}$, p27.

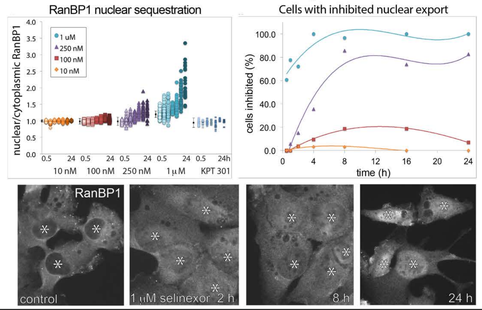
XPO-1 and Cancer

- Different cancers show elevated XPO-1, resulting in enhanced nuclear export and decreased function of many tumor suppressor proteins.
- These cancer cells maintain the ability to import XPO-1 cargos, they simply favor cytoplasmic localization.

Selective Inhibitors of Nuclear Export - SINE

SINE, including Selinexor, covalently bind Cys528 of XPO-1 and prevent cargo interaction, resulting in pharmacologically enforced nuclear sequestration and accumulation.

Nuclear Sequestration of RanBP1 following treatement with SINE



Summary of HT1080 FUCCI response to Selinexor

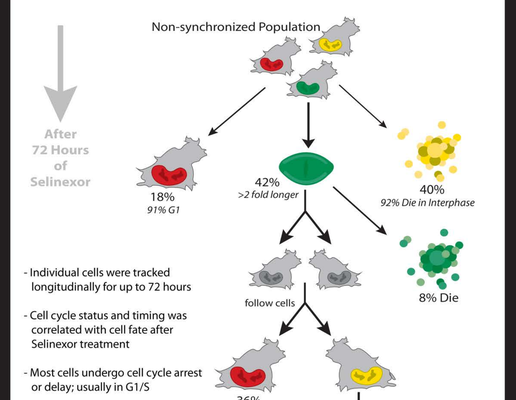
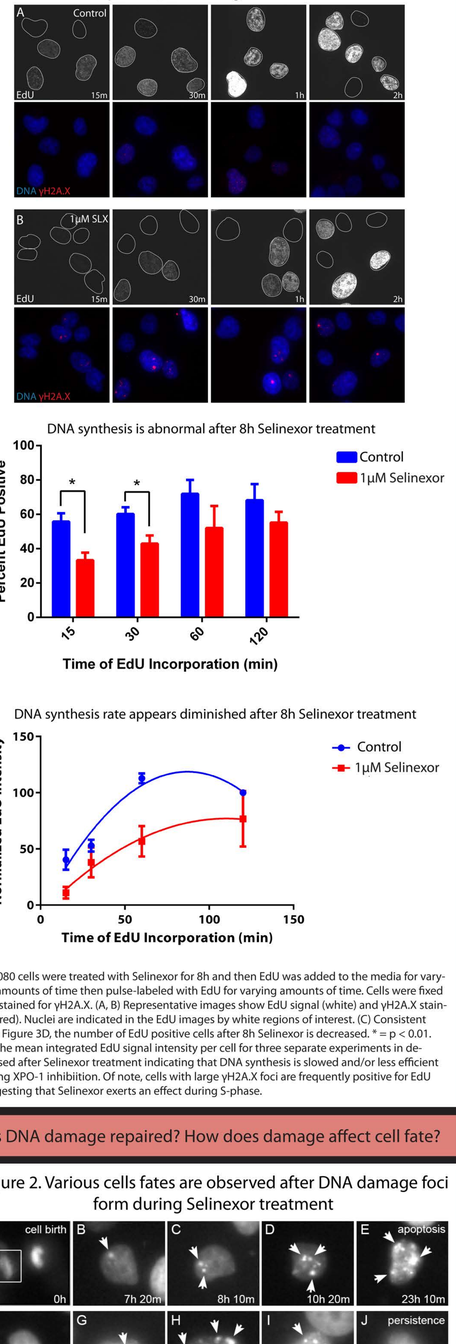


Table 1.	Treatment	
	Untreated	selinexor
Cell fate determination (all cells)	**% of total population**	
Death	0%	40%
Mitosis	100%	42%
Arrest	0%	18%
FUCCI status - cells that die (all cells)	**% of death population**	
G1-phase (hCdt1 only)	-	58%
early S-phase (hCdt1 and hGem)	-	6%
mid-late S/G2-phase (hGem only)	-	28%
mitosis (hGem only)	-	8%
FUCCI status - cells that arrest (all cells)	**% of arrested population**	
G1-phase (hCdt1 only)	-	91%
early S-phase (hCdt1 and hGem)	-	0%
mid-late S/G2-phase (hGem only)	-	9%
Cell fate determination (daughter cells)	**% of G1-phase cells**	
Death	0%	64%
Mitosis	100%	0%
Arrest	0%	36%
FUCCI status - cells that die (daughter cells)	**% of death population**	
G1-phase (hCdt1 only)	-	81%
early S-phase (hCdt1 and hGem)	-	2%
mid-late S/G2-phase (hGem only)	-	16%
mitosis (hGem only)	-	0%

	Treatment	
	Untreated	selinexor
FUCCI status - cells that arrest (daughter cells)	**% of arrested population**	
G1-phase (hCdt1 only)	-	96%
early S-phase (hCdt1 and hGem)	-	0%
mid-late S/G2-phase (hGem only)	-	4%
Fate determination - cells treated in G1-phase	**% of daughter cells**	
Death	0%	53%
Mitosis	100%	25%
Arrest	0%	22%
Fate determination - cells treated in early S-phase	**% of early S-phase cells**	
Death	0%	32%
Mitosis	100%	64%
Arrest	0%	4%
Fate determination - cells treated in S/G2/M-phase	**% of S/G2/M-phase cells**	
Death	0%	2%
Mitosis	100%	98%
Arrest	0%	0%
Average time observed per cell cycle phase	**time (hours)**	
G1-phase (hCdt1 only)	5	23
early S-phase (hCdt1 and hGem)	2	5
mid-late S/G2-phase (hGem only)	7	18
Mitosis	1	2

HT1080 FUCCI cells were treated with 1µM Selinexor and tracked longitudinally with time-lapse microscopy. The diagram is a graphical summary of cell fates observed after Selinexor treatment. The Table represents quantification of all observed responses of FUCCI cells in control conditions and after Selinexor treatment. Cell cycle stage upon death and when treated was correlated with response. The time spent in each FUCCI phase is also noted.

Figure 1. Selinexor treated cells show slowed DNA synthesis and accumulation of γH2A.X, indicating DNA double-strand breaks



HT1080 cells were treated with Selinexor for 8h and then EdU was added to the media for varying amounts of time then pulse-labeled with EdU for varying amounts of time. Cells were fixed and stained for γH2A.X. (A, B) Representative images show EdU signal (white) and γH2A.X staining (red). Nuclei are indicated in the EdU images by white regions of interest. (C) Consistent with Figure 3D, the number of EdU positive cells after 8h Selinexor is decreased. * = $p < 0.01$. (D) The mean integrated EdU signal intensity per cell for three separate experiments in decreased after Selinexor treatment indicating that DNA synthesis is slowed and/or less efficient during XPO-1 inhibition. Of note, cells with large γH2A.X foci are frequently positive for EdU suggesting that Selinexor exerts an effect during S-phase.

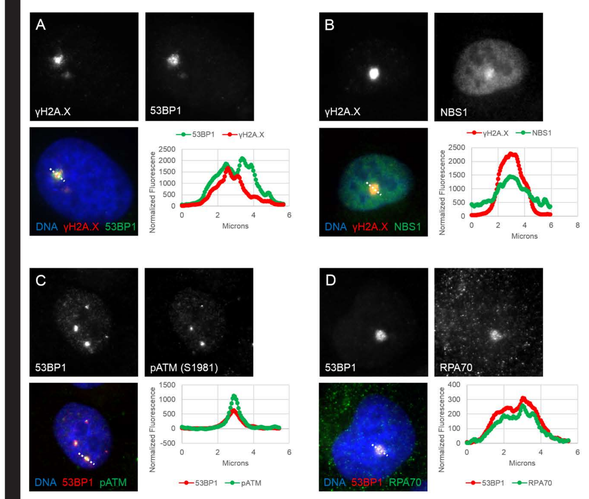
Is DNA damage repaired? How does damage affect cell fate?

Figure 2. Various cells fates are observed after DNA damage foci form during Selinexor treatment

(A) HT1080 expressing the mCherry-BP1-2 double-stranded DNA damage biosensor. This cell (box) was just born into the presence of 1µM Selinexor and showed no fluorescent foci. (B-E) The cell was directly tracked every ten minutes and DNA damage (foci, arrowheads) began forming after 7h and became more numerous over time until this cell died at 23h 10min. h = hours, m = minutes. (F-J) This cell also forms numerous foci, but appears to repair them and remains until the end of the time-lapse; we are now asking what the fate of these cells is.

Are DNA damage repair proteins recruited to break sites?

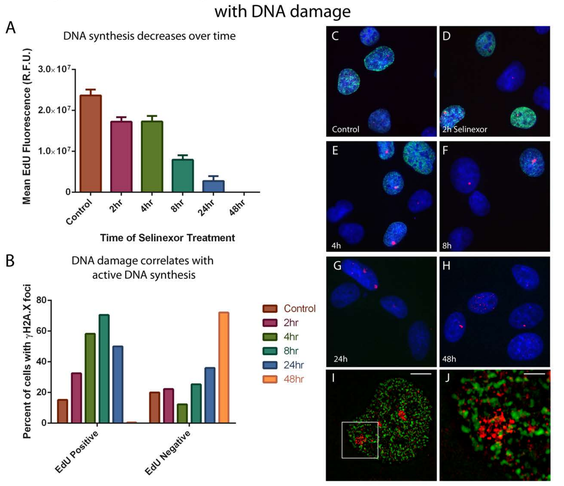
Figure 3. DNA damage repair pathway proteins are recruited to break sites following selinexor treatment



HT1080 cells were treated with 1µM selinexor for 8h then fixed and stained for multiple DNA damage repair proteins. (A) The histone variant H2A.X is phosphorylated in response to double strand breaks (γH2A.X). 53BP1 is recruited to and binds γH2A.X. A line scan demonstrates colocalization of both proteins in response to DNA damage. (B) NBS1 is a component of the MRN complex that is recruited to double strand breaks and is involved in end processing prior to homologous recombination. A line scan demonstrates colocalization of NBS1 with γ H2A.X. (C) Ataxia telangiectasia mutated kinase (ATM) is autophosphorylated at Serine 1981 in response to DNA damage. ATM has many targets including cell cycle checkpoint proteins and γH2A.X. A line scan demonstrates colocalization of ATM with 53BP1 after selinexor treatment. (D) Replication protein A subunit (RPA70) is involved with single strand DNA binding during DNA damage response. Colocalization of RPA70 with 53BP1 is shown in a line scan after selinexor treatment.

Does DNA damage correlate with active DNA synthesis?

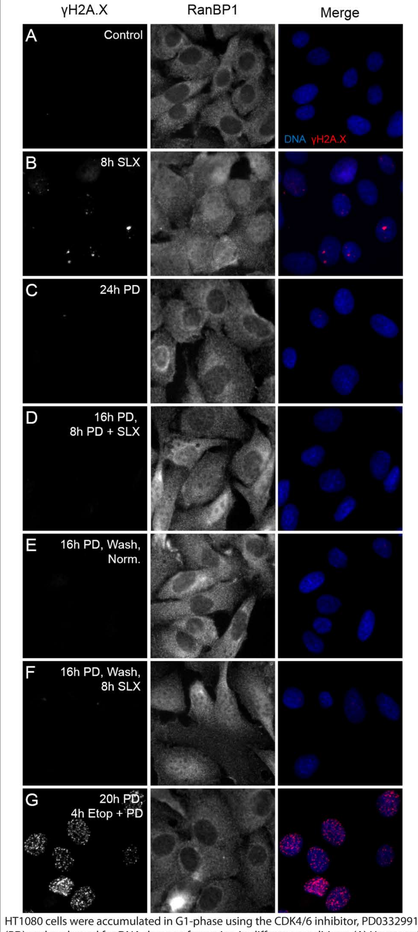
Figure 4. Active DNA synthesis decreases over time and correlates with DNA damage



HT1080 FUCCI cells were treated treated with Selinexor for increasing times. EdU was pulsed for 15min at the end of treatment. Cells were then fixed and stained for γH2A.X. (A) Mean fluorescence for EdU was quantified for each time point. Error bars represent SEM. (B) Cells were scored at each time point as positive or negative for EdU. γ H2A.X foci were identified using Find Maxima in ImageJ. The percent cells over time with 1 or more foci are indicated. (C-H) Representative images of cells treated with control, 2h, 4h, 8h, 24h and 48h Selinexor, respectively. Cells were stained for γH2A.X (red), EdU (green) and DNA (blue). (I-J) A single optical slice from Structured illumination microscopy of a cell treated with 1µM Selinexor for 8hr and stained for γH2A.X (red) and EdU (green). Scale bars represent 6µm (I) and 2µm (J).

DNA damage appears to be S-phase associated Can Selinexor cause damage in other cell states?

Figure 5. DNA damage does not occur in cells held in G1-phase following selinexor treatment



HT1080 cells were accumulated in G1-phase using the CDK4/6 inhibitor, PD0332991 (PD) and evaluated for DNA damage formation in different conditions. (A) Untreated cells have little damage and RanBP1, an XPO-1 cargo, is located in the cytosol. (B) Cells treated with 8h selinexor show large DNA damage foci. RanBP1 is sequestered in the nucleus demonstrating selinexor action. (C) Cells synchronized in G1-phase with 24h PD have little damage and normal cytosolic distribution of RanBP1. (D) Cells synchronized for 24h in PD with selinexor for the final 8h have little DNA damage. However, sequestered RanBP1 demonstrates selinexor action. (E) Cells were treated with 16h PD and then released into normal media. (F) Cells were treated with 16h PD and then released into media containing selinexor for 8h. Cells have little damage accumulation. It is not clear whether cells are actively cycling after release from PD. RanBP1 is sequestered in the nucleus, demonstrating selinexor action. (G) Cells were treated with 24h PD with Etoposide (Etop), a topoisomerase II inhibitor, for the final 4h. Etoposide is known to cause DNA double strand breaks regardless of cell cycle status.

Summary

Selinexor treatment results in cell cycle arrest and S-phase progression defects associated with striking γH2A.X foci. DNA damage leads to multiple cell fates, including repair and/or death. Damage appears to only occur in actively cycling cells, providing further evidence for S-phase association. In continuation of these studies, we are quantifying DNA damage and repair in live cells expressing FUCCI to establish its contribution to different cell fates and the cell cycle dependence of damage. Given this action of Selinexor, it is possible it may remain effective on cancer cells regardless of TP53 and/or p21CIP1 status. This is extremely important, as these are dysfunctional in many cancers.