CDC7 Kinase Inhibition by SRA141 Induces a Potentially Novel Caspase-Dependent Tumor Cell Apoptosis Associated with Altered DNA Replication and Cell Cycle Dynamics

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Background

- **During S-phase, Cell division cycle 7 (CDC7) kinase, together with its partner protein Dbf4 or Drf1, phosphorylates and activates the MCM2-7 helicase thereby initiating DNA replication origin firing.**
- **Owing to its important role in DNA replication, and its overexpression in various neoplasms (e.g. colorectal and breast cancer), CDC7 is an attractive therapeutic target with emerging clinical validation in oncology.[1]**
- **While the precise mechanism of CDC7 inhibitor-mediated anti-tumor activity remains to be determined, preclinical studies investigating the inhibition of CDC7 using siRNA and small molecules demonstrate differential sensitivity of tumor cells as a consequence of a p53-dependent DNA replication checkpoint that is operational only in non-transformed cells.[2]**
- **We previously reported that SRA141, a clinic-ready, potent, selective, orally bioavailable CDC7 inhibitor, is cytotoxic to multiple tumor cell lines *in vitro*. In addition, SRA141 treatment demonstrates robust anti-tumor efficacy in colorectal and leukemia xenograft models.**
- **To further understand the mechanism of SRA141-induced cell death, we explored the effects of the compound on DNA replication and cell cycle dynamics in several tumor cell lines.**
- **Our findings reveal a potentially novel mechanism of cytotoxicity for CDC7 inhibitors that is distinct from agents that cause replication fork collapse or cyclin-dependent kinase inhibition, and thus may define a new class of cancer therapeutic agents with a differentiated anti-tumor profile.**

Translational Significance

- **SRA141 does not induce G1 cell cycle arrest or replication stress, thereby distinguishing it from cyclin-dependent kinase inhibitors and DNA damage response targeting agents. Rather, SRA141 alters DNA replication dynamics and delays cell cycle progression, ultimately resulting in caspase-dependent cell death associated with mitotic accumulation.**
- **Our findings reveal a potentially novel mechanism of cytotoxicity for CDC7 inhibitors, and thus may define a new class of cancer therapeutic agents with a differentiated anti-tumor profile. This differentiated mechanism of action supports a potentially unique spectrum of clinical deployment opportunities as both monotherapy as well as in combination with pro-apoptotic and mitotic disrupting agents.**

References

1. **Cdc7-Dbf4 kinase overexpression in multiple cancers and tumor cell lines is correlated with p53 inactivation. Bonte et al. *Neoplasia* 2008**
2. **Cdc7 Inhibition Reveals a p53-Dependent Replication Checkpoint That Is Defective in Cancer Cells. Montagnoli et al. *Cancer Research* 2004**
3. **The level of origin firing inversely affects the rate of replication fork progression. Zhong et al. *Journal of Cell Biology* 2013**

Results

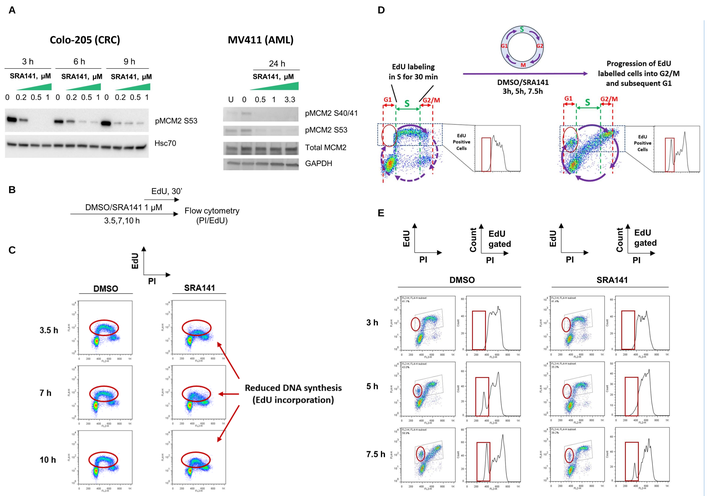

Figure 1: SRA141 inhibits phosphorylation of CDC7 cellular substrate MCM2, slows DNA replication and delays cell cycle progression. (A) Phosphorylation of MCM2 (S40 and S53) was reduced by SRA141 in a dose-dependent manner, demonstrating robust CDC7 inhibitory activity. Total MCM2 and housekeeping protein Hsc70 were unchanged. (B) Colo-205 cells were treated with DMSO or SRA141 and the incorporation of EdU, added during the last 30 min, was assessed by flow cytometry of PI stained cells. (C) SRA141 treatment significantly reduced the incorporation of EdU indicating reduction in the overall DNA synthesis likely due to the inhibition of origin firing. (D) EdU pulse-chase treatment scheme in Colo-205 cells is shown. (E) SRA141 treatment resulted in a reduction of EdU positive cells that moved through the cell cycle and reached the subsequent G1 phase (red circles in EdU plot, red box in PI cell cycle plot), indicating a slowed progression through S-phase.

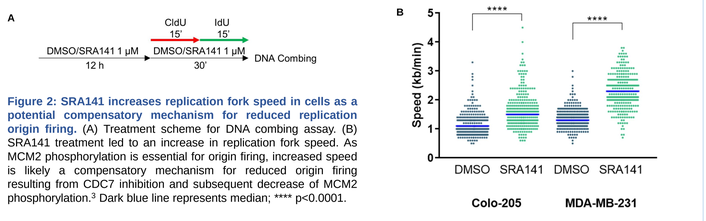

Figure 2: SRA141 increases replication fork speed in cells as a potential compensatory mechanism for reduced replication origin firing. (A) Treatment scheme for DNA combing assay. (B) SRA141 treatment led to an increase in replication fork speed. As MCM2 phosphorylation is essential for origin firing, increased speed is likely a compensatory mechanism for reduced origin firing resulting from CDC7 inhibition and subsequent decrease of MCM2 phosphorylation.[3] Dark blue line represents median; **** p<0.0001.

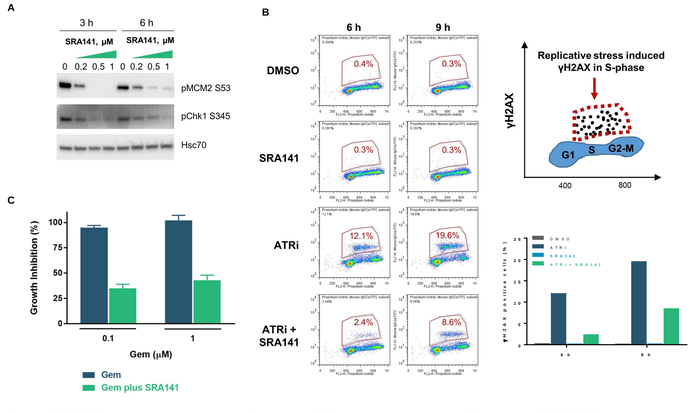

Figure 3: SRA141 abrogates intrinsic replicative stress (RS) and is antagonistic to RS inducers. (A) SRA141 treatment led to a reduction in levels of RS marker pChk1 S345 in Colo-205 cells. The level of pMCM2 S53 was also reduced consistent with CDC7 inhibition, while housekeeping protein Hsc70 remained unchanged. (B) The ATR inhibitor (VE822; 2 µM) was used to induce RS (γH2AX in S-phase) in MDA-MB-231 cells in the presence or absence of SRA141. SRA141 treatment did not induce γH2AX on its own and was antagonistic to the VE822-mediated induction of γH2AX, suggesting a novel MOA that is distinct from that of DDR targeting agents (such as ATRi, Chk1i and WEE1i). (C) HT-29 cells were treated for 96 h with the RS-inducing chemotherapy, gemcitabine, resulting in near 100% growth inhibition. Addition of SRA141 (1 µM) for the last 72 h substantially reduced the growth inhibitory effects of gemcitabine further supporting its antagonism of RS-inducing agents.

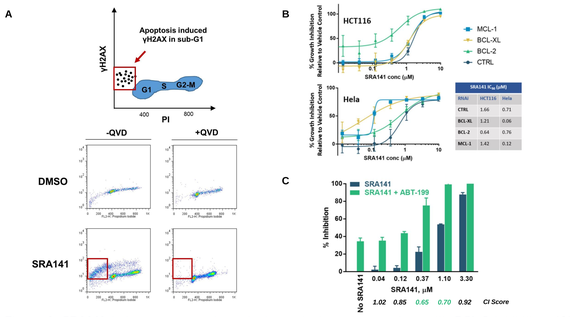

Figure 4: SRA141 induces caspase-dependent apoptosis and synergizes with BCL-2 inhibition. **(A)** Model depicts flow cytometry analysis of γH2AX as a function of cell cycle (PI) in order to distinguish source of γH2AX as either apoptosis (sub G1) or replication stress (S-phase). Colo-205 cells were incubated with SRA141 (500 nM; 24h) in the presence or absence of a pan-caspase inhibitor, QVD (20 µM), to block apoptosis. The disappearance of sub-G1 γH2AX with QVD treatment indicates that DNA damage induced by SRA141 is a consequence of apoptosis. **(B)** RNAi knockdown of BCL-2 family anti-apoptotic genes synergizes with SRA141. Cells were treated with control (CTRL), BCL-XL, BCL-2 and MCL-1 RNAi for 24h, followed by treatment with SRA141 for 72h and cell viability assessment. **(C)** Further support for caspase-dependent apoptosis was demonstrated by the synergy of the BCL-2 inhibitor, ABT-199, and SRA141 in MOLM-13 cells following 72h treatment. Combination indices (Bliss Independence analysis) were calculated; CI scores < 0.7 indicate synergy, while CIs between 0.7 and 1.3 indicate additivity.

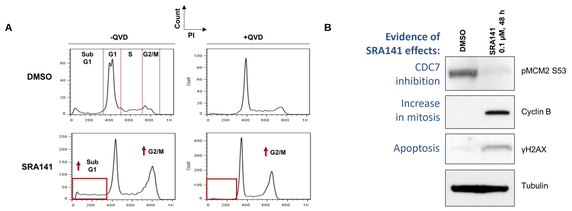

Figure 5. SRA141-mediated cell death coincides with mitotic markers of G2/M and accumulation of cells in mitosis. **(A)** Colo-205 cells were treated with SRA141 (500 nM; 24h) in the presence or absence of QVD (20µM) and analyzed by flow cytometry. SRA141 led to a substantial accumulation of cells in G2/M, which was accompanied by cell death as evidenced by the accumulation of cells in sub G1. QVD eliminated sub G1 accumulation indicating an apoptotic mechanism of cell death. In contrast, the mitotic accumulation was caspase-independent as it was observed in the presence and absence of QVD. **(B)** SRA141 treatment in Colo-205 cells led to an increase of the mitotic marker, Cyclin B, consistent with accumulation of cells in mitosis and a DNA damage marker, γH2AX, consistent with apoptosis. pMCM2 S53 levels were also reduced confirming CDC7 inhibition. **(C)** Cells were treated for 48h with CDC7 inhibitors or a CHK1 inhibitor as a negative control, and the percentage of mitotic cells was determined by immunocytochemistry. All three CDC7 inhibitors led to a concentration-dependent accumulation of cells in mitosis, while Chk1 inhibition had no effect.

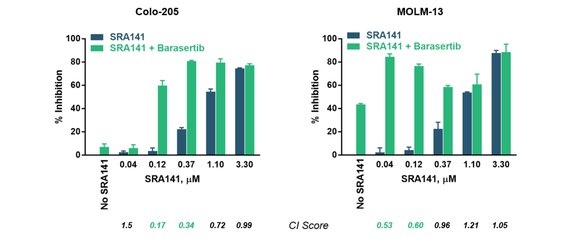

Figure 6. SRA141 demonstrates synergistic activity in combination with a small molecule Aurora B kinase inhibitor. Based on the observed SRA141-mediated accumulation of cells in mitosis, we tested whether SRA141 cytotoxicity could be enhanced by barasertib, an agent that further disrupts mitosis. Cells were treated with 10 nM barasertib in the presence of SRA141 for 72 h and cell viability was determined. Combination indices were calculated as described in Figure 4C. SRA141 was highly synergistic with barasertib, reinforcing its novel MOA and potential clinical utility in combination with certain mitotic disruptors.

Summary

- **SRA141 is a clinic-ready, potent, orally bioavailable selective inhibitor of CDC7 kinase that was previously shown to display potent antiproliferative activity against various tumor cell lines. SRA141 also displays favorable PK properties and robust anti-tumor activity evidenced by complete and partial regressions in colorectal and leukemia xenograft models.**
- **Consistent with its role in abrogating origin firing, SRA141 strongly inhibited MCM2 phosphorylation which was accompanied by a reduction in the rate of DNA synthesis. SRA141 caused a corresponding increase in DNA replication fork speed, suggesting engagement of a compensatory mechanism triggered by a reduction in replication origin firing.**
- **In contrast to inhibitors of DNA replication checkpoint kinases, SRA141 was shown to be antagonistic to replication stress inducers, as demonstrated by its ability to counteract ATR inhibitor-induced S-phase DNA damage and oppose gemcitabine-mediated growth inhibition.**
- **SRA141 treatment caused an accumulation of cells in mitosis as evidenced by elevated cyclin B levels and an increase in the percentage of cells in mitosis. Studies are ongoing to determine whether mitotic accumulation is associated with under-replicated DNA. Similar accumulation of cells in mitosis was demonstrated using other CDC7 inhibitors, suggesting a potentially novel mechanism of action for this class of agents that is distinct from other DNA damage response targeted drugs.**
- **Synergistic cytotoxicity between SRA141 and inhibitors of both Aurora kinase B and anti-apoptotic proteins provides further support for a mechanism of cell death involving mitotic dysregulation and Bcl-2 family mediated apoptosis.**

Proposed SRA141 Mechanism of Action

SRA141 alters replication dynamics through the inhibition of CDC7-mediated phosphorylation of MCM2-7 leading to a reduction of origin firing and a compensatory increase in replication fork speed. As the cells progress through S-phase without finishing replication, under-replicated DNA delays subsequent mitosis and ultimately leads to mitotic catastrophe and apoptotic cell death.