# Dual-Payload Combination of Top1 and CHK Inhibitors: An Innovative Strategy to Synergistically Overcome Colorectal Cancer Resistance

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Poster No. 5632

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## Introduction

Dual-payload ADCs deliver two types of payloads to enhance efficacy through synergistic effects and mitigate resistance, thereby addressing the challenge of poorly responsive cancers and rapid patient relapses. The success of dual-payload ADCs hinges on the appropriate dual-payload combination.

DDR inhibitors are increasingly being evaluated as payloads for ADCs. To identify a rationally designed dual-payload combination that overcomes tumor heterogeneity and conventional resistance of colorectal cancer (CRC), synergistic pairs of Top 1 inhibitors and a group of DDR inhibitors with different mechanisms were screened and evaluated. A combination of a Top 1 inhibitor Exatecan and a CHK inhibitor Prexasertib showed significant synergy in CRC lines and ADC-related drug-resistant cells with both 2D and 3D cytotoxicity models. Mechanistic studies of this combination were further explored with cell cycle analysis in both LS411N and NCI-N87/DS-8201 R cells, revealing that the synergy stemmed from coordinated cell cycle arrest. Critically, the dual-payload combination did not show increased toxicity in a group of healthy human cell lines and limited or additive hematotoxicity in the $CD34^+$ HSPC proliferation and differentiation assays, indicating a promising candidate payload pair for dual-payload ADC development.

### DDR as potential combination targets for dual-payload ADC development

#### Dual-payload mechanism of action

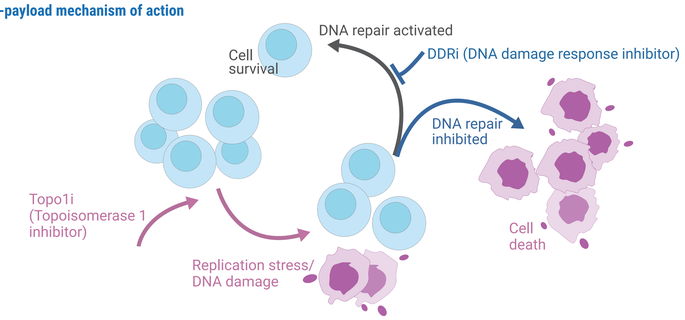



**Figure 1. Schematic principle of synthetic lethality.** Top1 inhibition traps DNA breaks at replication forks. Concurrent DDR blockade severs the repair escape route, leading to replication catastrophe and selective cell death, particularly in HR-deficient tumors.

## Results

### Cytotoxicity screening of different individual payloads in the CRC cell panel

| Payload | Target | Mechanism of action |
|---|---|---|
| DXd | Top1 | Stabilizes Top1-cc → SSB → replication-run-off DSBs |
| SN38 | Top1 | Identical to DXd; generates replication-associated DSBs |
| Exatecan | Top1 | Generates replication-associated DSBs |
| Azenosertib | WEE1 | Blocks WEE1 → abolishes G2/M checkpoint → forces cells with DNA damage into premature mitosis → mitotic catastrophe & apoptosis |
| Prexasertib | CHK1/2 | Prevents CHK1/2 activation → bypasses S/G2 arrest → cells enter mitosis with broken DNA |
| Lartesertib | ATM | Blocks ATM → impairs DNA DSB repair → abolishes G1/S & G2/M checkpoints → genomic instability & apoptosis |
| Ceralasertib | ATR | Blocks ATR kinase → abolishes replication-stress checkpoint → fork collapse & mitotic catastrophe |

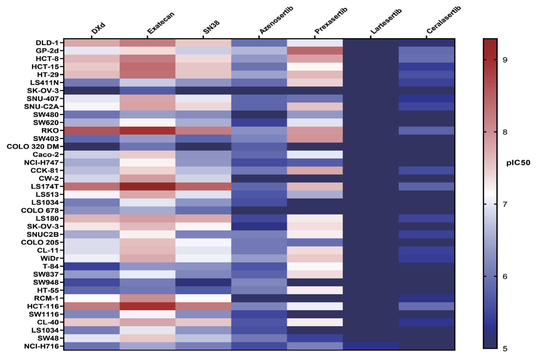
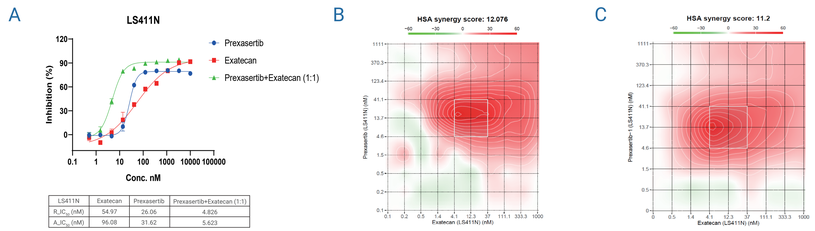



**Figure 2. Cytotoxic activities of different individual payloads across a CRC cell panel.** 39 CRC lines were treated with individual Top1 inhibitor or DDR related target (WEE1, CHEK, ATM, ATR) inhibitors for 3 days and cell viability was quantified by CTG; $pIC_{50}$ ($-\log_{10}(IC_{50})$) values were shown in the heat-map (dark red = high potency, dark blue = low potency).

### Combination of Prexasertib (a CHK inhibitor) and Exatecan (a Top1 inhibitor) showed *in vitro* synergistic effects




| LS411N | Exatecan | Prexasertib | Prexasertib+Exatecan (1:1) |
|---|---|---|---|
| R_$IC_{50}$ (nM) | 54.97 | 26.06 | 4.826 |
| A_$IC_{50}$ (nM) | 96.08 | 31.62 | 5.623 |

**Figure 3. Combination of Prexasertib and Exatecan showed synergistic effects in LS411N with either fixed-ratio combination or matrix combination cytotoxicity assays.** A. 1:1 ratio combination of dual-payload demonstrated ~5-fold A_$IC_{50}$ left-shift compared to Prexasertib group. B-C. Matrix combinations of dual-payload were tested in 2D (B) and 3D (C) LS411N cell models with 10×10 dose matrices. Grid data revealed pronounced synergy for Prexasertib and Exatecan (HSA synergy scores >10 in both 2D and 3D models), demonstrating synergistic activities.

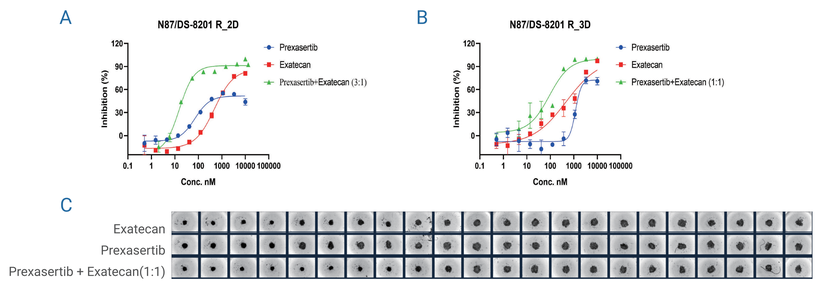



**Figure 4. Fixed-ratio combination of Prexasertib and Exatecan displayed synergistic effects in NCI-N87/DS-8201 resistant (R) cell models.** A. The combination group showed 5-fold A_$IC_{50}$ left-shift and ~40% maximum inhibition improvement compared to Prexasertib group in 2D model. B. The combination group displayed slight A_$IC_{50}$ difference and ~30% maximum inhibition improvement compared to Prexasertib group in 3D model. C. Representative images of 3D tumor spheroids treated with different drugs. These data indicated the potential of dual-payload combination to overcome resistance of DS-8201.

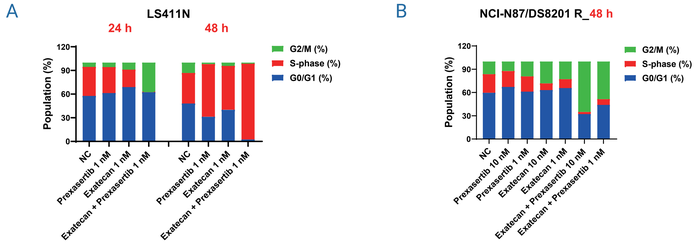



**Figure 5. Cell cycle analysis of LS411N and NCI-N87/DS-8201 R cells treated with dual-payload combination showed coordinated cell cycle arrest.** A. LS411N cells were treated with 1 nM of individual payload or the combination for 24 h and 48 h. The combination group induced coordinated G2/M phase arrest or significant S-phase accumulation after 24 h or 48 h incubation. B. NCI-N87/DS-8201 R cells were treated with different concentrations of test articles for 48 h. The combination group triggered robust G2/M phase arrests in a dose-dependent manner.

### Dual-payload combination exhibited minimal additive toxicity *in vitro*

| Progenitor Cell Type | Detected Pathway | Predictive Toxicity |
|---|---|---|
| **Primitive erythropoietic progenitor cell:** $CD34^+$ HSPCs induced into the erythropoietic lineage | Erythropoietic lineage | Anemia |
| **Primitive granulocyte-macrophage (GM) progenitor cell:** $CD34^+$ HSPCs induced into the myeloid-GM lineage | Myeloid-GM lineage | Neutropenia, Leukopenia |
| **Primitive megakaryocyte progenitor cell:** $CD34^+$ HSPCs induced into the megakaryocyte lineage | Megakaryocyte lineage | Thrombocytopenia |

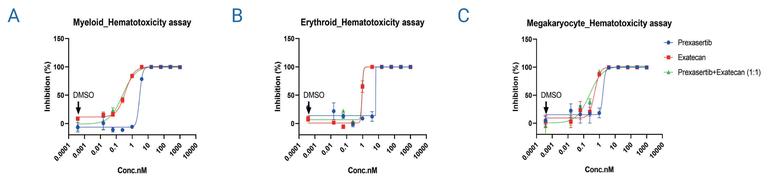



**Figure 6. Fixed-ratio combination of dual-payload exhibited minimal additive effects in hematotoxicity prediction assays.** $CD34^+$ hematopoietic stem and progenitor cells were differentiated into 3 different hematopoietic lineages to predict lineage-specific hematotoxicity *in vitro* . Drugs were added while inducing lineages (A. Erythropoietic, B. Myeloid, and C. Megakaryocyte lineages). The combination group did not show significant $IC_{50}$ shift or maximum inhibition improvement compared to Exatecan group in all these 3 assays.

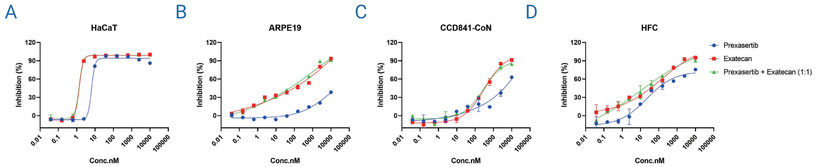



**Figure 7. Fixed-ratio combination of dual-payload did not show increased toxicity in representative cell lines of human healthy tissues.** Cytotoxicity of dual-payload tested in A. HaCaT (skin; keratinocyte), B. ARPE19 (eye; retinal pigmented epithelium), and C. FHC and CCD841-CoN (colon) showed similar or comparable toxicity with the Exatecan groups.

## Summary

This study established a rational strategy for dual-payload screening and demonstrated that simultaneously targeting Top1 and CHK can effectively overcome conventional and acquired resistance in CRC with synergistic effects, and minimal additive toxicity *in vitro* . The strategies and findings from this study can accelerate innovative research on dual-payload ADCs, especially in the proof-of-concept stage for dual-payload combination as a promising strategy to overcome cancer heterogeneity and resistance.