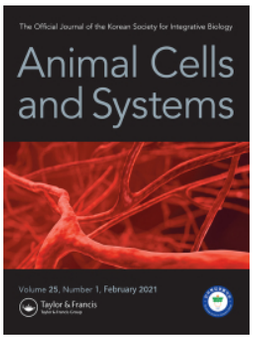

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Targeting MMR-deficient colorectal cancer with a potent small molecule UNI110

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ABSTRACT

Mismatch repair (MMR) deficiency is a hallmark of microsatellite instability (MSI) in hereditary non-polyposis colorectal cancer, Lynch syndrome, contributing to resistance against conventional chemotherapy and posing a significant therapeutic challenge. In this study, we introduce UNI110, a novel small molecule derived from Baicalein, engineered for enhanced selectivity against MMR-deficient cancer cells. UNI110 exhibits a remarkable sevenfold increase in potency over Baicalein, demonstrating significantly lower IC50 values and heightened cytotoxic effects in MMR-deficient cell lines. Mechanistically, UNI110 selectively induces DNA damage in MMR-deficient cancer cells, ultimately resulting in cell death. Furthermore, UNI110 disrupts homologous recombination (HR) repair by inhibiting the MSH2-MSH3 complex, specifically blocking the interaction between MSH2 and EXO1, thereby impairing long-range end resection during double-strand break (DSB) repair. These findings establish UNI110 as a promising lead compound for the targeted treatment of MMR-deficient colorectal cancers, offering a potential breakthrough in overcoming chemotherapy resistance and improving patient outcomes.

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Baicalein; homologous recombination; mismatch repair; UNI110; end resection

Introduction

The DNA mismatch repair (MMR) pathway is essential for maintaining genome integrity by recognizing and correcting mismatched bases and insertion-deletion loops (IDLs) that arise during DNA replication, recombination, or as a consequence of DNA damage (Kolodner and Alani 1994; Li 2008). In eukaryotic cells, this process is mediated by the MutSα complex (a heterodimer of MSH2 and MSH6), which primarily detects base – base mismatches and small IDLs, and the MutSβ complex (a heterodimer of MSH2 and MSH3), which recognizes larger IDLs. Upon binding to a mismatch or IDL, MutSα/β complexes recruit MutLα (MLH1-PMS2) or MutLβ (MLH1-PMS1) to form a ternary complex, which subsequently facilitates the recruitment of exonuclease 1 (EXO1) to degrade the erroneous DNA strand. This degradation enables polymerase δ to synthesize a new, corrected DNA strand (Jiricny 2006; Li 2008; Pećina-Šlaus et al. 2020). The critical role of MMR in genome maintenance is underscored by the fact that germline mutations in MMR-related genes confer a heightened risk of cancer, particularly Lynch syndrome (hereditary non-polyposis colorectal cancer, HNPCC) (Fishel 1999; Heinen et al. 2002; Jiricny 2006; Palomaki et al. 2009). Despite its high prevalence, colorectal cancer often exhibits resistance to standard chemotherapeutic regimens, posing significant challenges for effective treatment (Hewish et al. 2010).

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DNA double-strand breaks (DSBs) represent the most cytotoxic form of DNA damage due to their potential to compromise genomic integrity, leading to mutations and cell death if left unrepaired (Featherstone and Jackson 1999). DSBs are repaired through three primary pathways: non-homologous end joining (NHEJ), homologous recombination (HR), and polymerase theta (POLθ)-mediated alternative end-joining (TMEJ) (Scully et al. 2019). Among these, HR is particularly significant due to its high-fidelity repair mechanism, which utilizes a homologous template to accurately restore the original DNA sequence (Li and Heyer 2008). A critical step in HR initiation is DNA end resection, which generates long 3′-hydroxyl overhangs necessary for strand invasion and homology search. This process is initiated by the MRE11-RAD50-NBS1 (MRN) complex in conjunction with CtIP, which facilitates short-range resection (Williams et al. 2008; Huertas and Jackson 2009; Yun and Hiom 2009). Long-range end resection is subsequently mediated by EXO1 or DNA2, with helicases such as BLM and WRN playing a supporting role (Nimonkar et al. 2011). This end resection step is crucial in committing DSB repair toward HR instead of alternative pathways.

Recent studies have revealed a critical role of mismatch repair (MMR) proteins MSH2 and MSH3 in HR by promoting end resection (Oh et al. 2023). These proteins are recruited to DSB sites via their interaction with SMARCAD1**,** which facilitates EXO1 recruitment and enhances long-range end resection. Consequently, MMR proteins serve as key determinants in the DSB repair pathway choice, biasing repair toward HR.

Baicalein is a natural flavonoid derived from Scutellaria baicalensis and Scutellaria lateriflora. Traditionally used as an herbal supplement to support liver function, baicalein has recently gained attention for its antitumor properties (Chen et al. 2000; Taniguchi et al. 2008; Li-Weber 2009; Kim et al. 2013; Fu et al. 2014; Wang et al. 2015). It has been reported that baicalein inhibits the proliferation of various types of cancer cells, including oral, gastric and hepatocellular carcinoma cells, primarily through induction of cell cycle arrest (Chao et al. 2007; Cheng et al. 2012; Zheng et al. 2014; Mu et al. 2016; Alessandrini et al. 2018). In addition, baicalein exhibits anti-inflammatory activity by inhibiting Src tyrosine kinase and suppressing IL-6 production (Jelic et al. 2016). Recently, our group evaluated the efficacy of baicalein against mismatch repair (MMR)-deficient cancer cells and found that it exhibits preferential cytotoxicity by selectively increasing DNA damage in MMR-deficient cells (Zhang et al. 2016). However, its relatively high IC50 highlights the need for structural optimization to enhance its therapeutic potential.

In this study, we developed a novel Baicalein derivative, UNI110, which exhibits enhanced potency against MMR-deficient cancer cells compared to Baicalein. UNI110 treatment also resulted in a dose-dependent reduction in HR efficiency, similar to Baicalein. Mechanistically, UNI110 impairs end resection by inhibiting the interaction between MSH2 and EXO1, thereby preventing EXO1 recruitment to DSB sites. These findings suggest that UNI110 is a promising lead compound for selectively targeting MMR-deficient cancers by disrupting HR-mediated DNA repair.

Materials and methods

Chemicals

Baicalein (92081), sodium acetate (79714), acetic anhydride (242845), potassium carbonate (1.04928), 4-(trifluoromethyl)benzyl bromide (290564), ethyl acetate (58958) and Camptothecin (PHL89593) were purchased from Sigma-Aldrich.

Materials

Flash column chromatography was performed using Merck silica gel 60 (mesh 230−400). Analytic thin layer chromatography (TLC) was performed on Merck Silica Gel F254 plates (0.25 mm).

Cell culture and compound treatment

HEC59, HEC59-2 (received from Dr. Peggy Hsieh (NIH)'s laboratory) and HEK293T (received from Dr. Pamela Schwartzberg (NIH)'s laboratory) cells were cultured in DMEM/High Glucose (Hyclone) supplemented with 10% FBS (Millipore) and 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen) at 37°C and 5% CO_2. Additionally, HEC59-2 cells were maintained in 400 μg/ml G418 (Invitrogen). All cell lines were tested for

mycoplasma contamination and were authenticated by testing the expression of DNA repair proteins, MSH2 and MSH6 as described previously (Zhang et al. Cancer research). Baicalein (TCI) and UNI110 were dissolved in DMSO. Each compound was mixed with cell culture media and treated to cells for 24 hours or 48 hours.

Cell viability assay

Cells were plated in triplicate in white, flat bottom 96 well plates at a density of 5000 cells/well one day before each compound treatment. Each compound was treated for 24 hours, and cell viability was measured by Cell Titer-Glo Luminescent cell viability assay (Promega) according to the manufacturer's instruction.

Antibodies

Anti-MSH2 (ab52266), anti-EXO1 (ab95012) and anti RPA32 (ab2175) antibodies were purchased from Abcam. Anti-γ-H2AX (Ser139, 05-636) antibody was obtained from Merck Millipore. Anti-α-Tubulin was purchased from Santacruz.

RNA interference

siRNAs were transfected using RNAiMAX (Invitrogen) according to manufacturer's protocol. The following siRNAs were used: The siControl (5'-CGU ACG CGG AAU ACU UCG A-3'), siMSH2 (5'-AAU CUG CAG AGU GUU GUG CUU-3').

Immunoprecipitation assay

HEK293 T cells plated in 10 cm dishes were treated with 4 μM UNI110 for 24 hours, respectively. After 24 hours incubation, cells were harvested and lysed in IP buffer (100 mM Tris-HCl pH8.0, 250 mM NaCl, 1 mM EDTA, 1% NP-40, 5 mM $MgCl_2$, protease inhibitor cocktail (Roche), PMSF and Benzonase) at 4°C for 30 minutes. Cell lysates were incubated with 1 μg of MSH2 antibody for overnight at 4°C. To analyze the antibody captured binding complex, Dynabeads Protein G (Invitrogen) were used according to manufacturer's instruction. Beads were washed three times with IP buffer, then eluted by boiling in 2X sample buffer (Biorad), followed by SDS-PAGE and immunoblotting analysis. 2% of total cell lysates were used as input loading control.

Cell cycle analysis

Cells were harvested and fixed with 70% ethanol in phosphate-buffered saline (PBS) for a minimum of one hour. Following fixation, the cells were treated with 0.2 mg/mL RNase A in PBS at 37°C for one hour in the dark. DNA was subsequently stained with 10 μg/mL propidium iodide in PBS. Flow cytometry analysis was performed using a FACSVerse™ flow cytometer with BD FACSuite™ software (BD Biosciences). Data analysis was conducted using FlowJo software.

Immunoblotting

Cell extracts were obtained by incubating cells with RIPA buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 5 mM EDTA; 1% Triton X-100; 0.1% sodium dodecyl sulfate; 0.5% sodium deoxycholate) containing Halt™ Protease & Phosphatase Single-Use Inhibitor Cocktail and Benzonase® nuclease (250 units/μL, Enzynomics) on ice for one hour, sonicating and centrifugation. Proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% skim milk at room temperature for one hour and incubated with primary antibody at 4°C overnight. The blots were washed three times each for 10 minutes with Tris-Buffered Saline (TBS) with 0.05% Tween (TBS-T) and incubated with a horseradish peroxidase-conjugated secondary antibody (Enzo Life Sciences) at room temperature for one hour. Signal was detected by ChemiDoc (Bio-rad).

RPA retention assay using fluorescence-activated cell sorting (FACS)

HEK293T cells were pre-treated with 8 μM UNI110 for 24 hours, followed by a 1-hour treatment with 1 μM CPT and a 2-hour treatment with 4 μM Aphidicolin. Cells were subsequently permeabilized with 0.2% Triton X-100 in PBS and fixed with a solution of 3% paraformaldehyde and 2% sucrose in PBS. The samples were then incubated with anti-RPA32 antibody, followed by a secondary antibody conjugated with Alexa Fluor 488, each for a duration of 45 minutes. Subsequently, the samples were incubated with PBS containing 10 μg/ml propidium iodide (PI) and 100 μg/ml RNase A and were subjected to analysis by FACS.

Immunostaining

U2OS cells were treated with 8 μM UNI110 for 24 hours, followed by treatment with 1 μM CPT for 1 hour. Cells were permeabilized using CSK buffer (10 mM PIPES (pH6.8), 100 mM NaCl, 300 mM Sucrose, 3 mM $MgCl_2$, 1 mM EGTA (pH7.5)), fixed with 4% paraformaldehyde, and blocked with 10% FBS at room temperature for 30 minutes. Subsequently, the samples were stained with RPA2 antibody, followed by a secondary antibody conjugated with Alexa Fluor 488. Finally, mounting in permanent mounting medium with DAPI was used to stain the nucleus of cells.

Statistical analysis

The statistical analyses for individual experiments are delineated within the corresponding figure legends. All statistical analyses were conducted using GraphPad Prism (Version 9.0.0). Data are presented as mean ± standard deviation (SD) from triplicate samples. Significance levels are denoted by p values, where $p > 0.5$ is indicated as not significant (ns), $p < 0.05$ as (*), $p < 0.01$ as (**), $p < 0.001$ as (***), and $p < 0.0001$ as (****).

Results

Synthesis of UNI110

Baicalein has demonstrated high specificity toward MMR-deficient cancer cells; however, its clinical applicability is limited due to its relatively high IC50. To overcome this limitation, we synthesized a novel Baicalein derivative, 4-oxo-2-phenyl-7-((4-(trifluoromethyl)benzyl)oxy)-4H-chromene-5,6-diyl diacetate, designated as UNI110 (Figure 1). The synthesis of UNI110 was carried out as follows (Ding et al. 2011): Baicalein (770 mg,

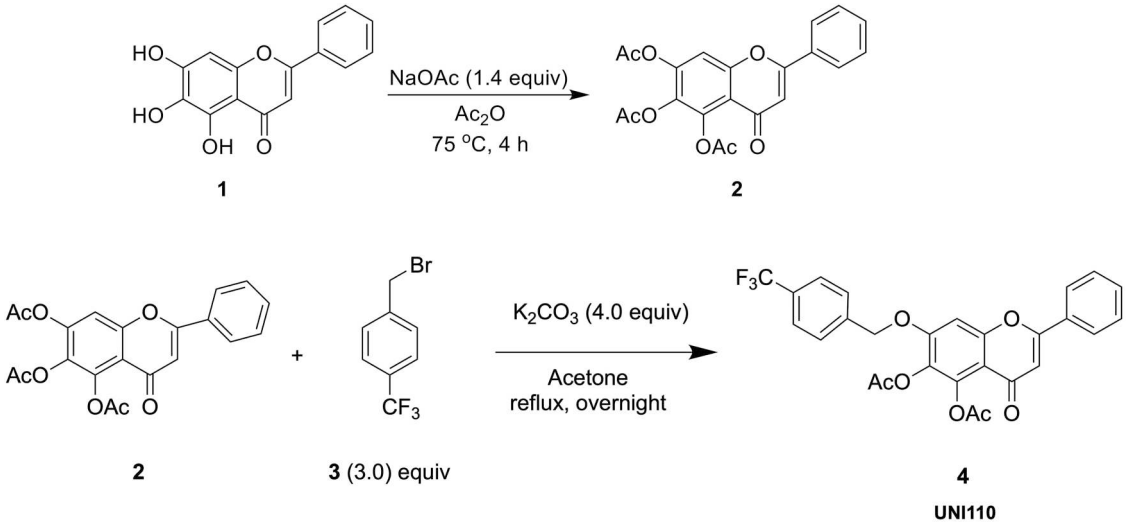

Figure 1. Synthesis of UNI110. Scheme of UNI110 synthesis. Baicalein (1), 4-oxo-2-phenyl-4H-chromene-5,6,7-triyl triacetate (2), benzyl bromide derivative (3), and UNI110 (4).

2.85 mmol (**1**)) and sodium acetate (NaOAc, 320 mg, 3.99 mmol) were placed in a dry sealed tube, followed by the addition of 5 mL of acetic anhydride. The reaction mixture was heated to 75°C, and progress was monitored via thin-layer chromatography (TLC). After 4 hours, complete consumption of the starting material was observed, upon which ice-cold water was added, resulting in the precipitation of a white solid. The precipitate was collected by filtration, washed with ethanol, and dried, yielding 1.03 g (2.6 mmol, 91%) of 4-oxo-2-phenyl-4H-chromene-5,6,7-triyl triacetate (**2**). The obtained compound was characterized, and its analytical data were consistent with previously reported values (Ding et al. 2011).

A mixture of compound **2** (60 mg, 1.0 equiv) and potassium carbonate (K_2CO_3, 4.0 equiv) was prepared in a sealed reaction tube, followed by the addition of acetone as the solvent. Subsequently, 4-(trifluoromethyl)-benzyl bromide (3.0 equiv (**3**)) was introduced, and the reaction mixture was refluxed overnight. The reaction progress was monitored by thin-layer chromatography (TLC), and upon completion, the solvent was removed under reduced pressure. The resulting crude product was purified by column chromatography using a gradient of ethyl acetate in hexane (15–25%) as the eluent, yielding UNI110 (**4**) (Figure 1). The synthesized compound was characterized by ^{19}F, ^{1}H, and ^{13}C NMR spectroscopy, along with high-resolution mass spectrometry (HRMS), as shown in Figures S1–S4 of the Supplementary Information.

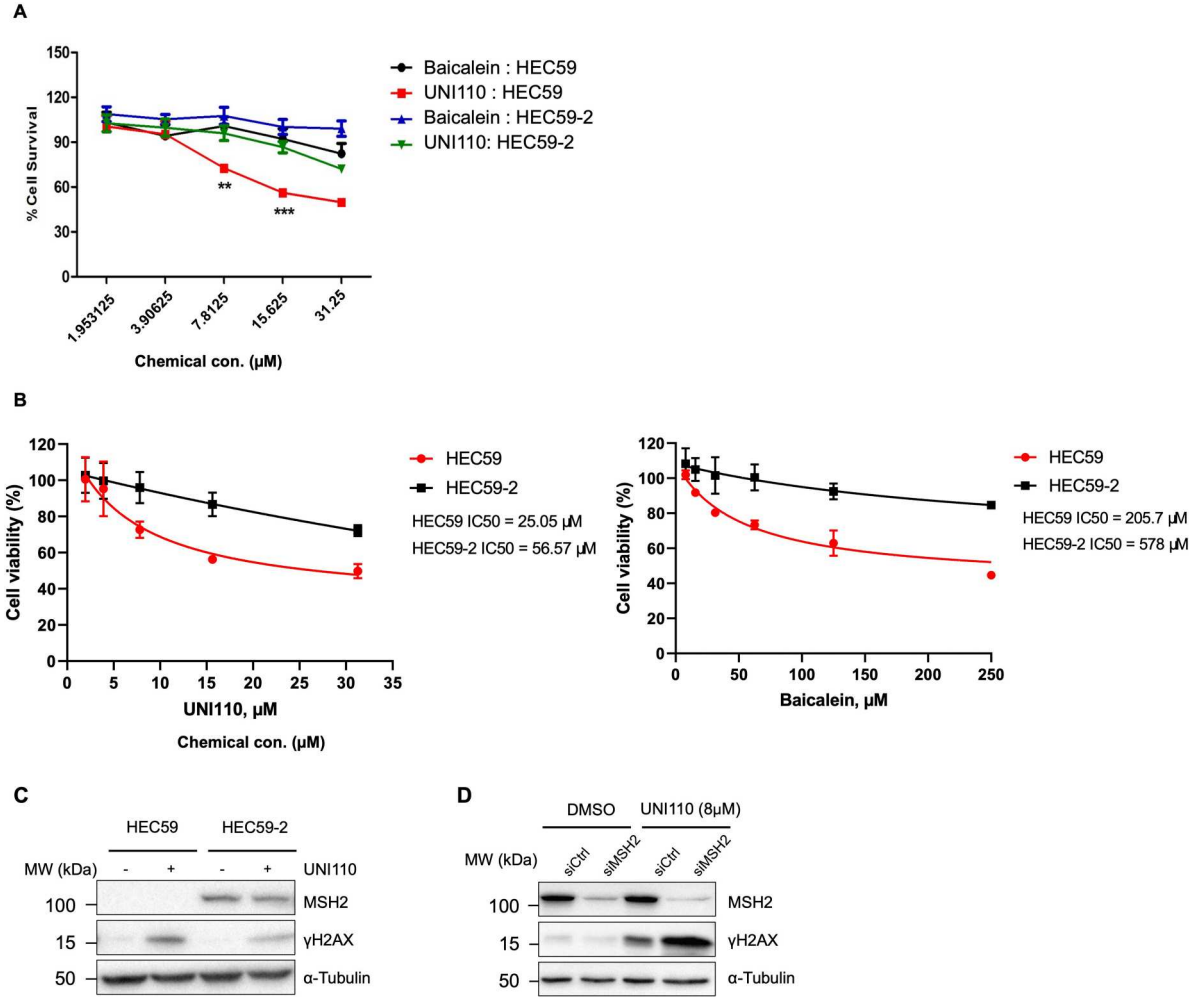

Figure 2. UNI110 selectively kills MutSa deficient cells. A, B. HEC59 and HEC59-2 cells were treated with the indicated doses of UNI110 for 24 hours. Cell viability was measured by Cell Titer-Glo reagent. Data are represented as mean ± SD ($n = 3$). The IC50 was determined using non-linear regression analysis. C. HEC59 and HEC59-2 cells were treated with 8 μM UNI110 for 24 hours. Isolated proteins were subjected to immunoblotting using the indicated antibodies. D. HEK293T cells were transfected with control and MSH2 siRNAs. The following day, cells were treated with 8 μM UNI110 for 24 hours. Isolated proteins were then analyzed using immunoblotting with the indicated antibodies.

UNI110 selectively kills MutSα deficient cells

To evaluate the effect of UNI110 on the survival of MutSα-deficient cancer cells, we utilized HEC59 cells (MutSα-deficient endometrial cancer cell line) and HEC59-2 cells (HEC59 cells complemented with human chromosome 2 to restore MutSα function). Similar to baicalein, UNI110 exhibited selective cytotoxicity against MutSα-deficient HEC59 cells (Figure 2A). Notably, UNI110 demonstrated cytotoxic effects at a significantly lower concentration than baicalein. The half-maximal inhibitory concentration (IC50) values for baicalein were 205.7 μM in HEC59 cells and 578 μM in HEC59-2 cells. In contrast, the IC50 values for UNI110 were 25.05 μM in HEC59 cells and 56.57 μM in HEC59-2 cells (Figure 2B), indicating that UNI110 selectively induces cytotoxicity in MutSα-deficient cancer cells with approximately sevenfold greater potency than baicalein.

To further validate this observation, we assessed DNA damage accumulation by measuring γ-H2AX levels following UNI110 treatment. HEC59 cells exhibited a higher accumulation of DNA damage compared to HEC59-2 cells upon treatment with UNI110 (Figure 2C). Consistently, MSH2-depleted cells also displayed increased γ-H2AX accumulation following UNI110 treatment (Figure 2D). These findings suggest that UNI110 could serve as a potential therapeutic agent for MMR-deficient cancers, offering improved efficacy and specificity compared to baicalein.

UNI110 decreases the efficiency of HR

Our group recently discovered that the MMR proteins MSH2 and MSH3 play a critical role in HR by facilitating DNA end resection. Specifically, these proteins recruit EXO1 to sites of DNA DSBs, thereby promoting HR. Furthermore, previous studies have shown that baicalein treatment reduces HR efficiency (Oh et al. 2023). Based on these findings, we investigated the effect of UNI110 on HR. To assess HR efficiency, we employed the well-established DR-GFP reporter assay (Pierce et al. 1999). RAD51 inhibitor B02 reduced the efficiency of HR. Treatment with UNI110 resulted in a dose-dependent reduction in HR efficiency (Figure 3A). One possible explanation for this decrease is that UNI110 induces G1-phase cell cycle arrest, as HR predominantly occurs during the S and G2 phases. To test this hypothesis, we analyzed cell cycle distribution following UNI110 treatment. However, rather than inducing G1 arrest, UNI110 treatment led to a modest increase in the S-phase population, indicating that the observed reduction in HR efficiency is not due to alterations in cell cycle progression (Figure 3B). These findings suggest that UNI110 impairs HR through a mechanism independent of cell cycle regulation.

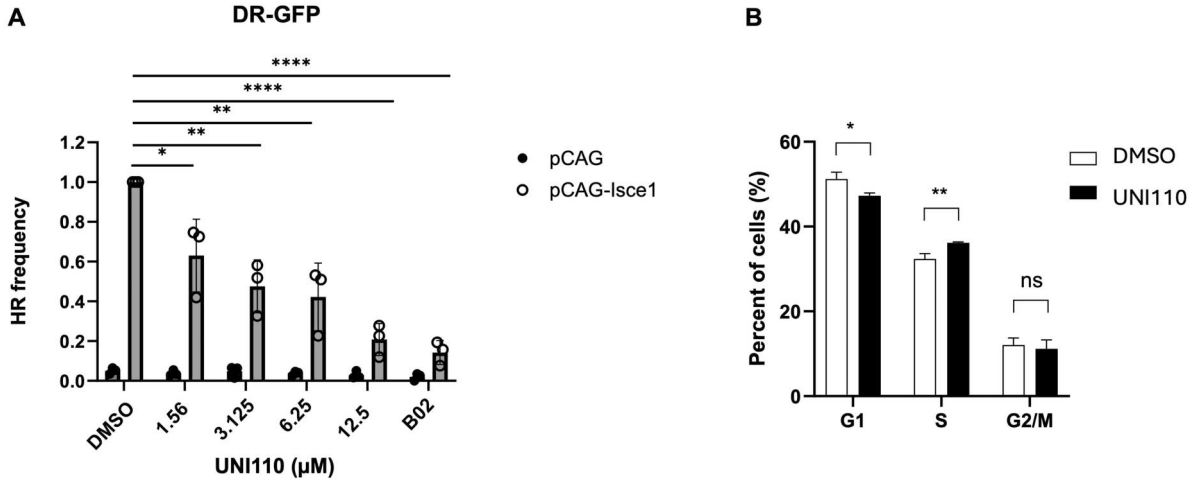

Figure 3. UNI110 decreases the efficiency of HR. A. HR reporter cell lines were treated with the indicated concentrations of UNI110 for 48 hours. GFP positive cells were analyzed by FACS. RAD51 inhibitor B02 was used as positive control. The data are represented as mean ± SD (n =3), and statistical significance was assessed using an unpaired t-test. B. HEK293T cells were treated with 8 μM UNI110 for 24 hours. The cell cycle profiles were analyzed by FACS. The data are represented as mean ± SD (n =3), and statistical significance was assessed using an unpaired t-test.

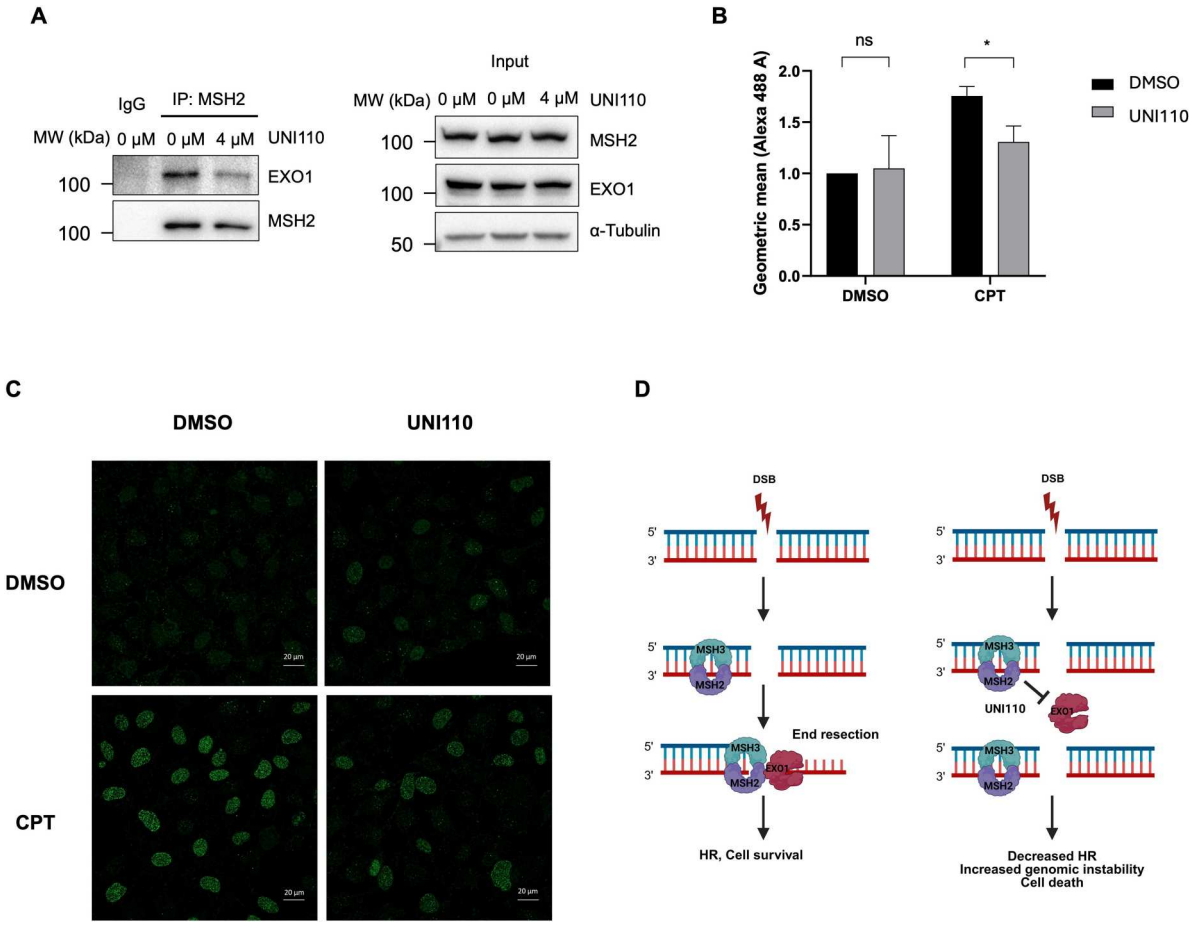

Figure 4. UNI110 decreases the efficiency of HR. A. HEK293T cells were treated with 4 μM UNI110 for 24 hours. Extracts were immunoprecipitated using an MSH2 antibody, and the interacting proteins were subsequently analyzed by immunoblotting with the specified antibodies. B. HEK293T cells were treated with 8 μM UNI110 for 24 hours, followed by treatment with 1 μM CPT and 4 μM Aphidicolin. Subsequently, the cells were permeabilized and incubated with an anti-RPA32 antibody. After staining with an Alexa Fluor 488-conjugated secondary antibody, the cells were analyzed by FACS. The data are presented as mean ± SD (n =3), and statistical significance was assessed using two-way ANOVA. C. U2OS cells were treated with 8 μM UNI110 for 24 hours, followed by a 1-hour treatment with 1 μM CPT. The cells were permeabilized and fixed, then incubated with a primary antibody followed by an Alexa Fluor 488-conjugated secondary antibody. Nuclei were stained with DAPI for visualization. Scale bar: 20 μm. D. Schematic representation of the mechanism.

UNI110 reduces end resection efficiency

The initial steps of HR involve DNA end resection, a process in which DSB ends are processed to generate long single-stranded DNA (ssDNA). Previous studies have shown that MSH2 and MSH3 promote end resection by facilitating the recruitment of EXO1 to DSB sites through direct binding (Oh et al. 2023). To investigate the effect of UNI110 on this interaction, we performed immunoprecipitation assays to assess MSH2-EXO1 binding following UNI110 treatment. Our results indicate that UNI110 treatment disrupts the interaction between MSH2 and EXO1 (Figure 4A). To further evaluate the impact of UNI110 on end resection, we measured RPA32 accumulation following treatment with the topoisomerase I inhibitor camptothecin (CPT), as RPA32 binds and stabilizes the ssDNA generated during end resection. UNI110 treatment resulted in a significant reduction in end resection efficiency in both biochemical assays, as demonstrated by decreased RPA retention and immunostaining (Figure 4B, and C). These findings suggest that UNI110 impairs HR efficiency by inhibiting end resection, likely through disruption of the MSH2-EXO1 interaction (Figure 4D).

Discussion

The critical role of the MMR pathway in maintaining genomic stability is well established, with mutations in MMR-related genes predisposing individuals to various cancers, most notably Lynch syndrome, which

significantly increases the risk of colorectal and other malignancies (Kolodner and Alani 1994; Li 2008). Despite this well-documented association, MMR-deficient cancers often exhibit resistance to conventional chemotherapy, posing a major challenge in their treatment (Hewish et al. 2010).

Baicalein, a natural flavonoid with known antitumor properties, has demonstrated selective cytotoxicity against MMR-deficient cancer cells (Zhang et al. 2016). However, its relatively high IC50 limits its clinical utility as a therapeutic agent. To address this limitation, we designed and synthesized UNI110, a novel baicalein derivative, which exhibits a sevenfold increase in potency against MMR-deficient cancer cells.

Therapeutic agents that induce DNA damage often trigger cell death in cancer cells by activating DNA damage response pathways (Cheung-Ong et al. 2013). The phosphorylation of H2AX at serine 139 (γH2AX) is a well-established marker of DNA damage and is mediated by ATM kinase upon the accumulation of DNA DSBs (Burma et al. 2001). Our findings indicate that UNI110 treatment significantly increases γH2AX levels specifically in MutSα-deficient cancer cells and in MSH2-knockdown cells. This suggests that UNI110 induces the accumulation of DNA damage in MSH2-deficient cells, potentially leading to increased cytotoxicity and cell death. Given its lower IC50 compared to baicalein, UNI110 holds significant promise as a targeted therapeutic for MMR-deficient cancers. However, further characterization – including toxicity assessments, pharmacokinetic profiling, and *in vivo* efficacy studies – is necessary to fully evaluate its therapeutic potential.

MMR proteins, particularly MSH2 and MSH3, have been implicated in HR by promoting DNA end resection (Oh et al. 2023). Additionally, baicalein has been reported to suppress HR efficiency, providing a rationale for investigating the effects of UNI110 on this critical DNA repair pathway. Our study demonstrates that UNI110 treatment significantly impairs HR by inhibiting DNA end resection, an effect mediated by the disruption of the MSH2-EXO1 interaction.

MSH2-MSH3 plays a critical role in HR by facilitating the recruitment of EXO1 to DSB sites and enhancing its processivity (Oh et al. 2023). Beyond recruitment, MSH2-MSH3 has been shown to actively modulate DNA structure, inducing conformational changes that enhance EXO1 accessibility and long-range resection. Furthermore, MSH2-MSH3 suppresses TMEJ, thereby biasing DSB repair toward high-fidelity HR. Our results show that UNI110 disrupts the MSH2-EXO1 interaction, leading to decreased end resection activity and reduced RPA32 accumulation on ssDNA, ultimately impairing HR.

HR repair is tightly regulated in a cell cycle-dependent manner, with maximal activity occurring in the S and G2 phases when a sister chromatid is available as a repair template (Jasin and Rothstein 2013; Zhao et al. 2017). During the G1 phase, HR is largely inactive, and cells rely on alternative repair mechanisms, such as NHEJ (Mao et al. 2008). While G1-phase arrest is often associated with HR suppression, our cell cycle analysis revealed that UNI110 treatment resulted in a modest increase in the S-phase population rather than G1 arrest. This suggests that the observed reduction in HR efficiency is not attributable to cell cycle effects but rather to direct inhibition of end resection.

In summary, UNI110 is a novel and potent baicalein derivative that selectively targets MMR-deficient cancer cells with enhanced efficiency. Its ability to inhibit HR through disruption of the MSH2-EXO1 interaction further establishes its potential as a therapeutic agent for MMR-deficient tumors. Future studies in preclinical models will be essential to assess its *in vivo* efficacy, safety profile, and therapeutic applicability.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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