

Discovery of ART5537: A Potent and Selective Small-Molecule Probe for EXO1

Sam E. Mann,* Owen A. Davis, Jörg Bomke, Irina Cornaciu, Elias Elinati, Bruce Follows, Alessandro Galbiati, Lerin Geo, Diego Grande, Catherine Jorand-Lebrun, Claudio A. Lademann, Julien Lefranc, Birgitta Leuthner, Bethany Mason, Claire L. McWhirter, Djordje Musil, Ulrich Pehl, Carl Petersson, Andrea Pica, Maria Filipa Pinto, Eeson Rajendra, Christin Rakers, Ana Toste Rêgo, Helen M. R. Robinson, Daniel Schwarz, Graeme C. M. Smith, Fiona J. Sorrell, Frank T. Zenke, Robert A. Heald, and Lars T. Burgdorf



Cite This: *J. Med. Chem.* 2025, 68, 26432–26447



Read Online

ACCESS |



Metrics & More

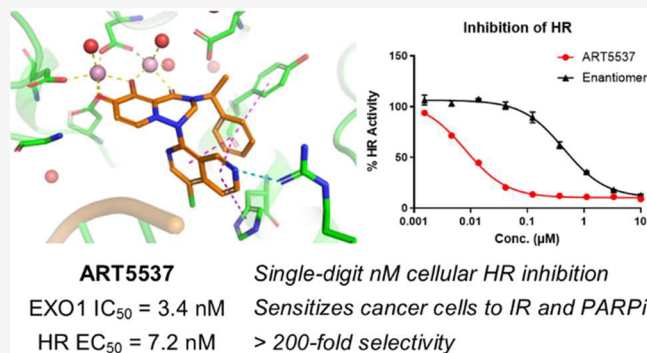


Article Recommendations



Supporting Information

ABSTRACT: Exonuclease 1 (EXO1) is emerging as a target of interest in oncology due to its involvement in multifaceted DNA metabolic processes, particularly in homologous recombination (HR). Evidence is building that *BRCA1*-deficient cancers are sensitive to loss of EXO1, suggesting therapeutic potential for treating certain subsets of patients. However, EXO1 remains under-explored, with very few reported inhibitors, and there is a paucity of good quality, potent, and selective pharmacological tools to explore its biology. Here, we describe a metal-chelating fragment screen, which resulted in highly selective, submicromolar EXO1 hits. Our subsequent structure-based design and optimization led to the discovery of ART5537, the first highly potent and selective EXO1 inhibitor. We demonstrate that inhibition of EXO1 leads to potent suppression of HR in cells and that the HR inhibition of ART5537 is driven exclusively by EXO1. Furthermore, we show that ART5537 sensitizes cancer cells to ionizing radiation (IR) and synergizes with PARP inhibitors (PARPi).



INTRODUCTION

Exonuclease 1 (EXO1) is a member of the human *RAD2* family of structure-specific nucleases, which plays a variety of essential roles in DNA metabolic processes such as replication and repair.^{1,2} Multiple diverse functions have been ascribed to EXO1, which contributes to the maintenance of genome stability via involvement in double strand break repair (DSBR)^{3–6} and mismatch repair (MMR),^{7,8} as well as translesion DNA synthesis (TLS), nucleotide excision repair (NER), and checkpoint activation, among others.^{9–12}

The primary role of EXO1 is to digest double stranded DNA (dsDNA) in the 5′ to 3′ direction to generate 3′ single stranded DNA (ssDNA) overhangs during homologous recombination (HR).^{3,5,13} This resection activity generates sections of ssDNA, which are then coated with replication protein A (RPA) and in turn activate the ATR signaling pathway to initiate repair by HR.¹⁴ Structural insights into the mechanism of the exonucleolytic cleavage reaction have shown how concerted global movements in the catalytic domain guide the terminal 5′ nucleotide into position for Mg²⁺-activated hydrolysis of its scissile phosphodiester. Iterative repeats of this process result in a screw-like rotation and translocation of the

DNA double helix toward the active site to enable the processive digestion of the 5′ strand.¹⁵ Co-ordination of the two catalytic Mg²⁺ ions is therefore a logical strategy for inhibiting EXO1.

Loss of *Exo1* in mice results in MMR defects, which in turn lead to elevated microsatellite instability and increased mutation rates, as well as increased cancer susceptibility and sterility.¹⁶ EXO1 is tightly regulated to minimize excessive nucleolytic activity, as elevated expression of EXO1 can lead to genome instability and has been associated with poor prognosis in breast and uterine cancers, among others.^{17,18} There is also emerging evidence that EXO1 plays a role in protecting *BRCA1*-deficient cancer cells against toxic DNA lesions that result from impaired single strand annealing (SSA)

Received: September 9, 2025

Revised: November 11, 2025

Accepted: November 24, 2025

Published: December 8, 2025



ACS Publications

© 2025 American Chemical Society

26432

<https://doi.org/10.1021/acs.jmedchem.5c02593>
J. Med. Chem. 2025, 68, 26432–26447

activity, which has accelerated interest in pursuing EXO1 as a therapeutic target as a result.^{19,20}

Notwithstanding recent interest as a target in oncology, there are very few reported EXO1 inhibitors in the literature. Early *N*-hydroxyurea-containing FEN1 inhibitors were reported to be equipotent on EXO1,²¹ although to the best of our knowledge this finding was not followed-up. More recently, double-digit micromolar inhibitors of EXO1 have been reported.²² Therefore, there is a clear need for more advanced chemical matter to fully explore the consequences of pharmacological inhibition of EXO1.

RESULTS AND DISCUSSION

Metal-Chelating Fragment Screen. Following the success of our recently reported metal-chelating fragment screen to identify novel FEN1 inhibitors,^{23,24} we set out to explore the applicability of this approach to other nucleases, commencing with EXO1. Reversing the order of the primary screening cascade, we first screened our bespoke collection of over 300 metal-chelating fragments with an EXO1 biochemical assay, then filtered hits by FEN1 activity to give a preliminary read-out on the specificity and selectivity of EXO1 inhibition. A shortlist of the top hits was ranked on EXO1 potency, ligand efficiency and selectivity over FEN1, then assessed for activity against two additional RAD2 family members GEN1 and XPG. Consistent with our experience on FEN1, several fragments displayed inherent selectivity toward EXO1 and could easily have served as starting points for hit-expansion (data not shown). However, compound **1** (Figure 1) had by far the most

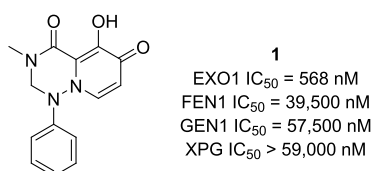
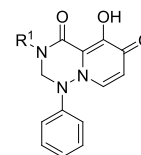


Figure 1. Structure of fragment hit **1** and biochemical selectivity data.

compelling profile, displaying submicromolar EXO1 activity, excellent LE and LLE, and ≥ 70 -fold selectivity over each of the closely related RAD2 family members FEN1, GEN1 and XPG. The identification of selective, submicromolar hits for two different nucleases demonstrates that our metal-chelating fragment screening approach can be successfully applied to multiple targets.

Fragment Hit-Expansion. We initiated our hit-expansion campaign by fragment growing of compound **1** from the “northern” *N*-substituent (Table 1). Alternative alkyl substituents were not well tolerated, with ethyl **2**, isopropyl **3** and methylenecyclohexyl **4** all losing potency and FEN1 selectivity relative to methyl **1**. However, addition of a benzyl *N*-substituent to give **5** resulted in a 5-fold increase in EXO1 potency and concurrent improvement in FEN1 selectivity, albeit with a modest reduction in LE and LLE relative to **1**. A further breakthrough was identified upon incorporation of an α -methyl substituent, which gave an additional 10-fold increase in potency, while retaining good LE, LLE and FEN1 selectivity. The stereochemistry at the α -methyl position was critical to this improvement, with the (*S*)-enantiomer **6** being preferred over (*R*)-**7**, suggesting a clear conformational preference of the pendant phenyl substituent.

Table 1. Fragment Growing



Cmpd	R ¹	EXO1 IC ₅₀ (nM) ^a	LE	LLE ^b	FEN1 selectivity ^c
1	CH ₃	568	0.44	6.11	70
2	CH ₂ CH ₃	646 ^d	0.41	5.63	9.5
3	CH(CH ₃) ₂	1,170 ^d	0.38	4.87	2.8
4	(CH ₂) ₅	5,750 ^d	0.28	2.96	0.50
5	CH ₂ CH ₂ Ph	102	0.38	5.12	162
6	CH(CH ₃)CH ₂ Ph	13	0.41	5.51	180
7	CH(CH ₃)CH ₂ Ph	82	0.37	4.72	78

^aData represent $n \geq 2$ unless otherwise indicated. Full tables of assay data including n and SD are available in the Supporting Information.

^bLLE calculated using XLogP, which correlated well with measured LogD throughout the investigation (see the Supporting Information).

^cFold-selectivity with respect to FEN1. ^dSinglicate value.

As an orthogonal read-out of on-target activity, we also verified that **6** and **7** bind to EXO1 by SPR (see Supporting Information). As with the biochemical and HR read-outs, there was a clear stereochemical dependence of EXO1-binding, with **6** displaying 5-fold higher affinity than **7** ($K_D = 73$ nM for **6** and 366 nM for **7** respectively).

With the EXO1 activity displaying a clear stereochemical dependence at the α -methyl position, we next sought to investigate the cellular consequences of EXO1 inhibition with **6**, using its weaker enantiomer **7** as a control.

EXO1 Inhibition Suppresses Homologous Recombination. We recently reported the development of a suite of cellular reporter assays that enable quantitative measurement of the activity of several of the major DSB repair pathways, including homologous recombination (HR).²⁵ Given its integral role in DNA 5'-end resection,⁵ which is required for normal DSB repair by HR, we hypothesized that inhibition of EXO1 nuclease activity would disrupt repair via the HR pathway. To confirm that our HR reporter assay was sensitive to loss of EXO1 activity, we compared the relative HR activity in HAP1 EXO1 KO cells to that in parental EXO1-expressing HAP1 cells (Figure 2A,B). Consistent with literature reports, we observed a ~50% reduction in HR activity in the HAP1 EXO1 KO cells.³ In-line with the genetic data, we also observed a dose-dependent reduction in HR activity in response to treatment with both **6** and **7** in our standard screening assay format in HEK293 cells, with the more active **6** displaying 10-fold higher potency than its weaker enantiomer **7** (Figure 2C,D). Intriguingly, we observed a greater reduction in HR activity following compound treatment than in the EXO1 KO, a phenomenon we explored in more detail with a later compound (vide infra).

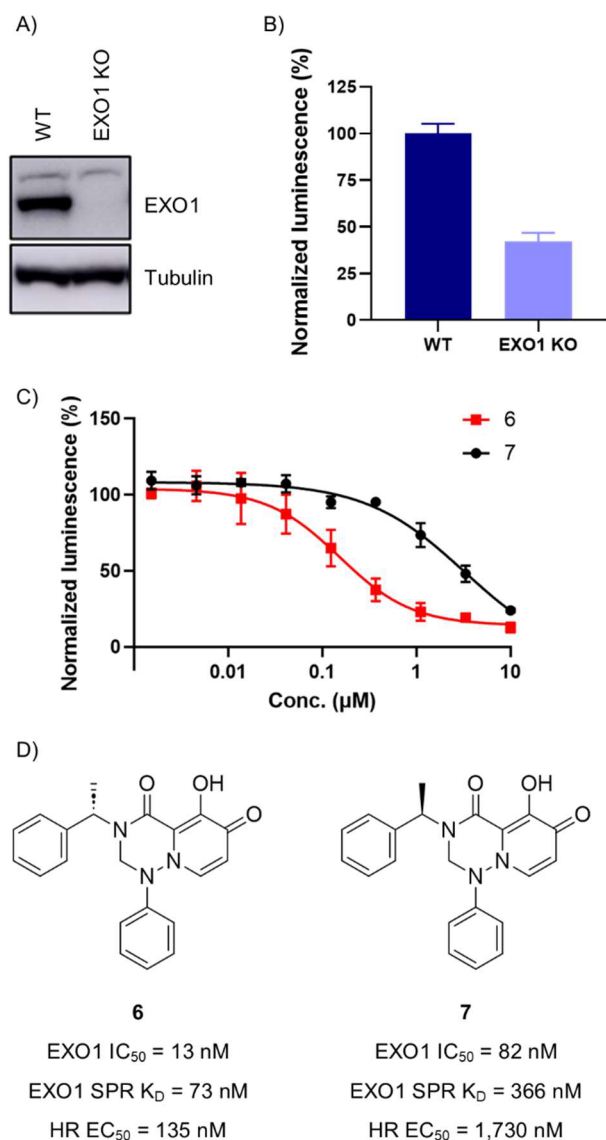


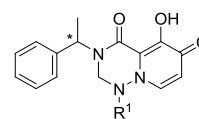
Figure 2. EXO1-dependency of HR inhibition. (A) Western blot of the HAP1 EXO1 KO line used for validation of EXO1-dependency of HR reporter activity. (B) Bar plot showing a 50% reduction in HR activity in the HAP1 EXO1 KO compared to WT. (C) Dose response curves showing inhibition of HR activity in HEK293 cells following treatment with compounds 6 and 7. (D) Structures of enantiomers 6 and 7 with associated biochemical, SPR, and HR inhibition data, showing a clear stereochemical preference for the (*S*)-enantiomer 6.

Taken together, these data strongly suggest that the observed reduction in HR activity is a direct consequence of EXO1 inhibition.

Hit-Optimization. We conducted a further SAR expansion exercise on the “northern” *N*-substituent, focusing on phenyl ring substitution and incorporation of alternative (hetero)-aromatic systems, however, none of these offered any improvement over the unsubstituted phenyl 6 (data not shown).

Next, our attention turned to expansion of the SAR at the “southern” *N*-substituent, again focusing on phenyl ring substitution and incorporation of heteroatoms (Table 2). To maximize synthetic efficiency and mitigate the number of chiral purifications required, we decided to conduct the initial SAR exploration on the racemic mixtures at the α -methyl benzyl

Table 2. Hit-Expansion



Cmpd	R ¹	*	EXO1 IC ₅₀ (nM) ^a	LLE ^b	FEN1 selectivity ^c	HR EC ₅₀ (nM)	mLogD
6	Ph	(<i>S</i>)-	13	5.51	180	135	2.1
8	2-F-Ph	(<i>Rac</i>)-	35	4.98	266	224	n.d.
9	2-Cl-Ph	(<i>Rac</i>)-	16	4.81	316	171	2.4
10	2-Me-Ph	(<i>Rac</i>)-	18	5.11	316	285	2.3
11	2-pyridyl	(<i>Rac</i>)-	n.d.	n.d.	n.d.	346	1.2
12	3-F-Ph	(<i>Rac</i>)-	35	4.98	158	n.d.	n.d.
13	3-Cl-Ph	(<i>Rac</i>)-	15 ^d	4.83	229	n.d.	2.5
14	3-Me-Ph	(<i>Rac</i>)-	40	4.75	58	n.d.	n.d.
15	3-pyridyl	(<i>Rac</i>)-	54	6.20	543	n.d.	n.d.
16	4-F-Ph	(<i>Rac</i>)-	29	5.06	646	613 ^d	n.d.
17	4-Cl-Ph	(<i>Rac</i>)-	19 ^d	4.73	108	n.d.	2.5
18	4-Me-Ph	(<i>Rac</i>)-	42	4.73	216	n.d.	n.d.
19	4-pyridyl	(<i>Rac</i>)-	240 ^d	5.55	417	n.d.	n.d.
20		(<i>S</i>)-	9.7	5.64	748	56	2.0
21		(<i>R</i>)-	431	4.00	93	329	1.9

^aData represent $n \geq 2$ unless otherwise indicated. Full tables of assay data including n and SD are available in the Supporting Information.

^bLLE calculated using XLogP, which correlated well with measured LogD throughout the investigation (see the Supporting Information).

^cFold-selectivity with respect to FEN1. ^dSinglicate value. n.d. = not determined.

position. Briefly, small lipophilic substituents such as methyl and halogens gave the best activity; methyl and chloro were broadly equivalent, with a mild preference for chloro at the 3- and 4-positions. Cognizant of the impact of increased lipophilicity,²⁶ we were keen to introduce heteroatoms to keep LogD within a reasonable range. 2- and 3-pyridyl analogues 11 and 15 were well tolerated, retaining good levels of HR and EXO1 activity respectively, while giving the desired reduction in LogD. The 4-pyridyl analogue 19 was much weaker.

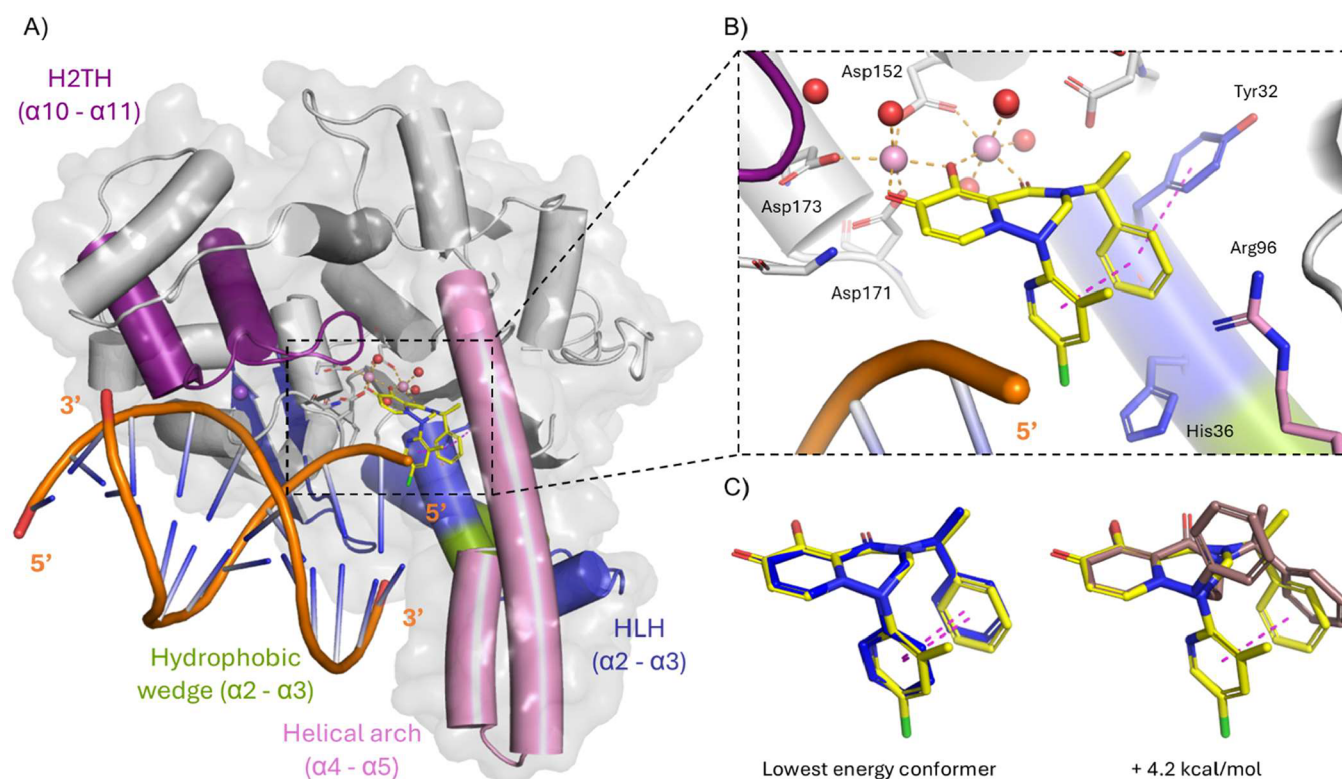


Figure 3. X-ray cocrystal structure of the ternary complex of compound **20** bound to EXO1-DNA (PDB 9SEB). (A) Overview of the structure, showing compound **20** (yellow) bound in the active site, nestled under the helical arch (pink cylinders) and chelating the catalytic Mg^{2+} ions (pink spheres), with the DNA substrate (orange helix) translocated by approximately one bp out of the active site relative to literature structure PDB 5V06 in the previously described “screw” motion (see the [Supporting Information](#) for more details). (B) Close-up of the active site, showing the detailed binding mode of **20** (yellow) with the intra- and intermolecular π -stacking interaction between the pyridyl and α -methyl benzyl substituents and Tyr32 (lilac). (C) Calculated lowest energy conformer of **6** (blue, left) and the next lowest energy conformer (maroon, right) overlaid with the X-ray binding pose of **20** (yellow), showing a clear preference for the bioactive conformation.

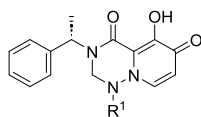
Combining the preferred substitutions into a single molecule and reinstating the (*S*)- α -methyl group gave compound **20**, which displayed a modest increase in both EXO1 and HR inhibition, while improving FEN1 selectivity and retaining desirable LogD and high LLE. Consistent with the earlier enantiomeric pair, the (*R*)-enantiomer **21** was also much weaker in both assays, further supporting the dependency of HR inhibition on EXO1.

X-ray Co-crystal Structure Informs Design of ART5537. Following unsuccessful efforts to cocrystallize **6** and **20** with apo EXO1, we decided to attempt to crystallize the ternary complex of a compound bound to EXO1-DNA under modified literature conditions.¹⁵ Literature DNA-bound structures of EXO1 such as PDB 5V06 show the phosphodiester backbone of the terminal 5' nucleotide bound to the catalytic metal ions (Mn^{2+} in this case, which is used to both slow the rate of nucleolytic cleavage relative to Mg^{2+} and provide an anomalous signal to facilitate crystallization and structure determination), which could potentially preclude compounds from accessing the active site. To rule this out, we first demonstrated by SPR that compounds such as **6** and **20** are equally capable of binding to EXO1 in the presence or absence of an added DNA substrate. In fact, some compounds even displayed a mild preference for binding in the presence of DNA (see [Supporting Information](#)). Satisfied that compound binding is compatible with, and possibly even facilitated by, the presence of DNA, we attempted to crystallize **20** with EXO1 in the presence of a

modified version of the PDB 5V06 substrate oligo. Cognizant of the subtle differences between Mn^{2+} and Mg^{2+} binding in metalloenzymes,^{27–29} we decided to use the more physiologically relevant Mg^{2+} in the crystallization buffer, but modified the substrate to contain a judiciously placed phosphorothioate at the terminal nucleotide to prevent enzymatic turnover. This strategy was rewarded almost immediately, with large single crystals forming under several sparse matrix screening conditions overnight. Crystals diffracted to 2.1 Å and we were able to solve the structure, which to the best of our knowledge is the first cocrystal structure of a small molecule bound to EXO1 and the first structure of a ternary DNA- and small molecule-bound complex of any DNA repair nuclease ([Figure 3](#)).

Our structure reveals that **20** binds in the active site via coordination of the two Mg^{2+} ions in an analogous fashion to previously reported small molecule cocrystal structures of FEN1,^{21,23} ([Figure 3A](#)). Subtle movements in the helical arch and substrate oligo relative to literature EXO1-DNA structure PDB 5V06 are required to accommodate the ligand, with the DNA being translocated outward from the active site by approximately one nucleotide in the previously described “screw” motion ([Figure 3A](#) and [Supporting Information](#)).¹⁵ Closer inspection of the binding mode of **20** reveals an intriguing folded conformation, whereby the α -methyl benzyl group participates in an extended intra- and intermolecular π -stacking interaction with the pyridyl substituent and Tyr32 ([Figure 3B](#)). We were keen to understand whether this

Table 3. Optimization of “Southern” Substituent



Cmpd	R ¹	EXO1 IC ₅₀ (nM) ^a	LLE ^b	FEN1 selectivity ^c	HR EC ₅₀ (nM)	mLogD
22		7.9	5.36	133	20	1.9
23		14	5.25	106	41	n.d.
24		8.1 ^d	5.50	271	27	1.7
25		31	5.93	1,390	156	n.d.
26		35	6.00	124	41	n.d.
27		12	6.48	532	13	1.1
28		9.5	6.44	248	19	1.3
29 (ART5537)		3.4	6.41	583	7.2	1.7

^aData represent $n \geq 2$ unless otherwise indicated. Full tables of assay data including n and SD are available in the [Supporting Information](#). ^bLLE calculated using XLogP, which correlated well with measured LogD throughout the investigation (see the [Supporting Information](#)). ^cFold-selectivity with respect to FEN1. ^dSinglicate value. n.d. = not determined.

interesting bioactive conformation was favorable and readily accessible, so we calculated the five lowest energy conformers of **6** as a simple surrogate for **20** using DFT (Maestro Jaguar module, (M06–2X)/SOLV), see [Supporting Information](#)). Of these, the two lowest energy conformers overlaid almost perfectly with the X-ray binding conformation of **20**, while the next lowest energy conformer that did not resemble the bioactive conformation was +4.2 kcal/mol higher in energy ([Figure 3C](#)). Taken together, these results confirm that the bioactive conformation is low in energy and readily accessible, likely explaining the high affinity and strong stereochemical dependency of EXO1 activity for **6** and **20**.

The X-ray structure also revealed opportunities for further optimization of **20**. Close proximity of the pyridyl substituent

to His36 suggested that extending the aromatic system with a fused bicyclic aryl group could potentially introduce an additional π -stacking interaction. The guanidine group of Arg96 also appeared accessible for targeting with a hydrogen bond acceptor, which we reasoned could be readily incorporated into a suitable fused bicyclic heteroaryl system, such as a naphthyridine.

Accordingly, we next investigated a concise set of fused bicyclic heteroaryl ring systems to replace the pyridyl N-substituent of **20** ([Table 3](#)). Extending the aromatic system with a fused phenyl ring to give isoquinoline **22** gave an encouraging increase in potency, albeit with a modest reduction in LLE. Azaindole regioisomers **23** and **24** also displayed good levels of potency, with the latter also retaining

high LLE. However, the designs we were most interested in were naphthyridines **25** to **28**, some of which had the potential to form a hydrogen bonding interaction with Arg96. Based on our modeling of these designs into the X-ray structure of **20**, we predicted that of the four possible regioisomers of the naphthyridine, the 2,7-analogue **27** would be the most likely to form the desired hydrogen bond. All four naphthyridine regioisomers were tolerated, with the 2,7-analogue **27** giving the best combination of potency, LLE and FEN1 selectivity, retaining very good biochemical and cell activity despite relatively high polarity ($m\text{LogD} = 1.1$). Finally, reincorporation of the 4-chloro substituent c.f. **20** gave **29** (**ART5537**), which displays single digit nanomolar biochemical EXO1 and cellular HR inhibition, while retaining excellent LLE and FEN1 selectivity, with modest lipophilicity ($m\text{LogD} = 1.7$).

X-ray Structure of ART5537. To validate our structure-based design hypothesis, we crystallized the ternary complex of **ART5537** and EXO1-DNA in an analogous fashion to compound **20** (vide supra). The resulting cocrystal structure confirms both the extended four-centered inter-intra-intermolecular π -stacking interaction between Tyr32- α -methyl benzyl-naphthyridine-His36, and the targeted hydrogen bond between the naphthyridine 7-position and Arg96 (Figure 4).

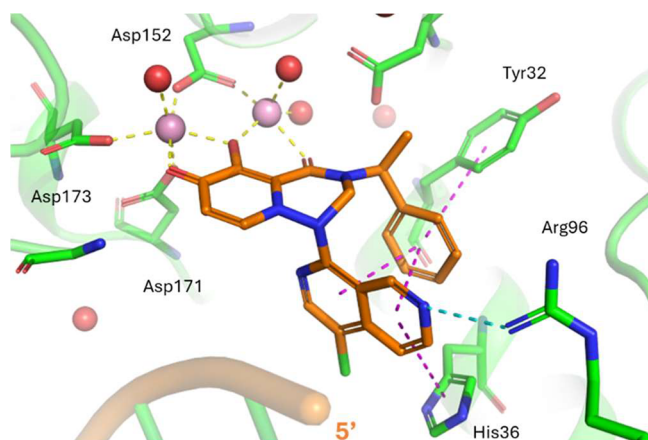


Figure 4. X-ray cocrystal structure of **ART5537** bound to EXO1-DNA (PDB 9SMO). The X-ray binding mode of **ART5537** (orange) bound in the active site of EXO1 (green) showing the planar tridentate chelation of the two catalytic Mg^{2+} ions (pink spheres), the extended four-centered inter-intra-intermolecular π -stacking interaction between Tyr32- α -methyl benzyl-naphthyridine-His36, and the targeted hydrogen bond between the naphthyridine and Arg96.

Cellular Phenotypes of ART5537. Consistent with previous analogues, the (*R*)-enantiomer of **ART5537** (**30**) is much weaker in the biochemical assay and has lower affinity for EXO1 by SPR (Figure 5A). As such, we reasoned that **30** would be a good control for exploring the EXO1-driven phenotypes of **ART5537**.

First, as an orthogonal readout of cellular target engagement with **ART5537**, we built a NanoBRET assay, using a tracer derived from compound **20** (see Supporting Information). In-line with its potent biochemical inhibition of EXO1, **ART5537** displays unambiguous dose-dependent cellular target engagement with an EC_{50} of 55 nM in this format (Figure 5B). **ART5537** is the most potent inhibitor of HR of all compounds tested in this study, with an EC_{50} of 7.2 nM in the HR reporter assay (Figure 5C). In-line with data for previous enantiomer pairs, both the cellular target engagement and HR inhibition of

ART5537 display a clear stereochemical dependence, with its enantiomer **30** being 20- to 50-fold weaker, respectively.

Importantly, to confirm that HR inhibition is driven specifically by EXO1, we demonstrate that **ART5537** is inactive in HAP1 EXO1 KO cells and that there is no further suppression of HR beyond the $\sim 50\%$ reduction observed previously with genetic KO of the target (Figure 5D,E).

Finally, we wanted to explore the apparent dominant negative effect of EXO1 inhibition on HR i.e. inhibition of EXO1 resulted in a greater reduction in HR activity than genetic ablation of the target (vide supra). To do this, we generated a U-2 OS Flp-In line constitutively expressing GFP2-tagged EXO1 to facilitate imaging of the colocalization of EXO1 and chromatin by confocal microscopy. We observed that **ART5537**, but not the weaker enantiomer **30**, induces a dose-dependent retention of GFP2-EXO1 on chromatin, as determined by an increase in GFP2 signal in treated cells following extraction with cytoskeletal (CSK) buffer (Figure 5F,G). Retention of inactive EXO1 at sites of DNA damage may not only prevent DNA repair by EXO1, but also prevent access of complementary nucleases to the damaged site, thus potentially accounting for the observed dominant negative effect of EXO1 inhibition compared to protein loss.

To explore the therapeutic potential of EXO1i, we conducted 7-day viability assays in a representative selection of *BRCA1* mutant cancer cell lines using the CellTiter-Glo (CTG) readout. To our surprise, only four of the eight *BRCA1* mutant lines displayed modest sensitivity to **ART5537** at the concentrations tested and there was no obvious dependence on *BRCA1* in two pairs of isogenic lines. **ART5537** kills MDA-MB-436 and HCC1937 (*BRCA1* null), SUM149PT (*BRCA1* $\Delta 11q$ hypomorph), and HCC38 (*BRCA1* promoter methylated) breast cancer cells in the low micromolar range, with the cells displaying a modest level of resistance to the weaker enantiomer **30** (Figure 6A). These data are potentially indicative of a mild EXO1-dependent sensitivity in these lines, although there appears to be a disconnect between the potent target engagement of **ART5537** and cell kill. Consistent with this finding, **ART5537** was found not to be broadly cytotoxic in a panel of 93 cancer cell lines, or in two additional noncancer lines (see Supporting Information).

Monotherapy activity notwithstanding, we were keen to explore the combination potential of **ART5537**. Given the robust suppression of HR by EXO1i, we wanted to probe the potential for synergy between **ART5537** and PARP inhibitors (PARPi), which are clinically approved for the treatment of HR deficient (HRD) cancers. We first screened the combination of **ART5537** and olaparib in 5-day cell viability assays across the same small panel of 93 cancer cell lines, observing a trend toward synergy in $\sim 50\%$ of the panel (Figure 6B). No synergy was observed in PBMC (peripheral blood mononuclear cells) or IMR-90 (normal lung fibroblasts) acting as two representative noncancer lines, suggesting that the combination is not ubiquitously cytotoxic. To confirm that the observed synergy is driven by inhibition of EXO1, we generated two independent EXO1 KO clones in HCT116 as a representative of lines showing sensitivity in the screen (Figure 6C). In-line with the screening data, we observed a robust synergy in expanded 7-day viability matrices in WT HCT116 cells treated with the combination of **ART5537** and olaparib, which was completely absent in both of the EXO1 KO clones (Figure 6D). Consistent with this trend, the EXO1 KO cells were highly sensitive to olaparib monotherapy

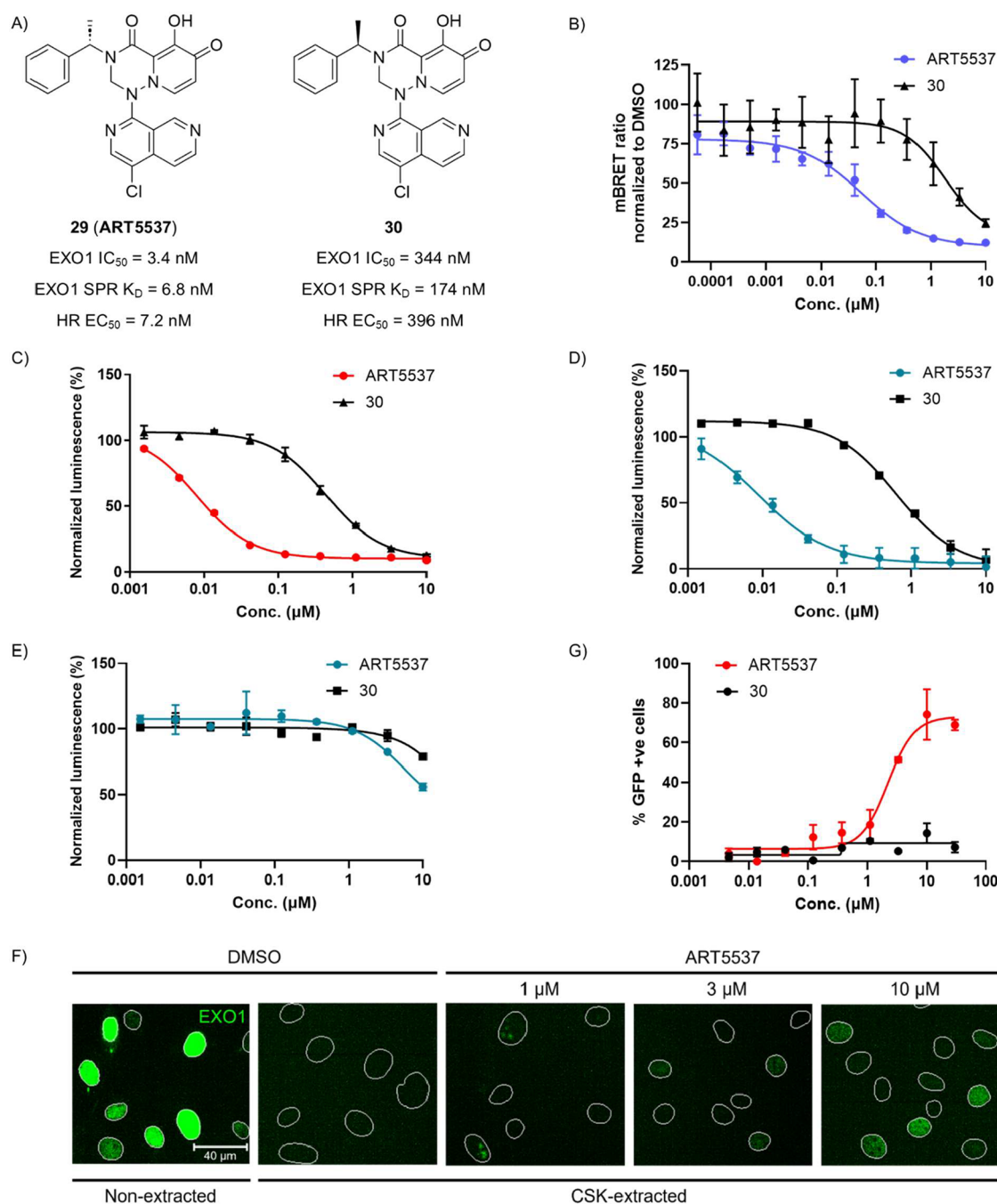


Figure 5. ART5537 inhibits HR via EXO1 inhibition. (A) Structures, biochemical, SPR, and cell data for ART5537 and its enantiomer 30. (B) NanoBRET curves (technical quadruplicate) demonstrating potent, stereochemistry-dependent target engagement of EXO1. (C) HR reporter assay curves for ART5537 and 30 in HEK293 cells, showing the stereochemical dependence of HR inhibition. (D, E) Inhibition of the HR DSBR reporter in HAP1 parental cells (D) and HAP1 EXO1 KO (E) showing no further effect of ART5537 over KO of EXO1. Data are representative of $n \geq 2$ unless otherwise indicated. (F) Representative confocal microscopy images showing the localization of GFP2-tagged EXO1 (green) and DNA (white masks, counter-stained with 4',6-diamidino-2-phenylindole (DAPI)) in untreated cells and CSK-extracted cells treated with DMSO and increasing concentrations of ART5537. The increase in green fluorescence indicates a greater quantity of GFP2-EXO1 bound to chromatin. (G) Quantification of the dose-response in the percentage of GFP-positive cells from (F) following treatment with ART5537 (red curve) and enantiomer 30 (black curve, images not shown) normalized to non-CSK extracted cells. Data represent mean $\pm$ SEM of $n = 2$ biological replicates.

treatment, whereas the WT HCT116 cells were resistant (Figure 6E). Taken together, these data strongly support a potential role for EXO1i as a combination therapy with PARPi.

Finally, to probe the effect of EXO1i in the potentiation of ionizing radiation (IR), we conducted colony formation assays with ART5537 and 30 in HCT116 cells following irradiation (Figure 6F–H). Consistent with literature reports demonstrat-

ing that knockdown of EXO1 renders cells more susceptible to IR,³ inhibition of EXO1 with ART5537 results in a > 40-fold sensitization relative to non-irradiated cells (Figure 6F,H). ART5537 potently sensitizes cells irradiated with 3×1 Gy (EC₅₀ = 144 nM), whereas non-irradiated cells are much more resistant, only becoming sensitive at high concentrations (EC₅₀ = 6.1 μM). In contrast, treatment with enantiomer 30 does not

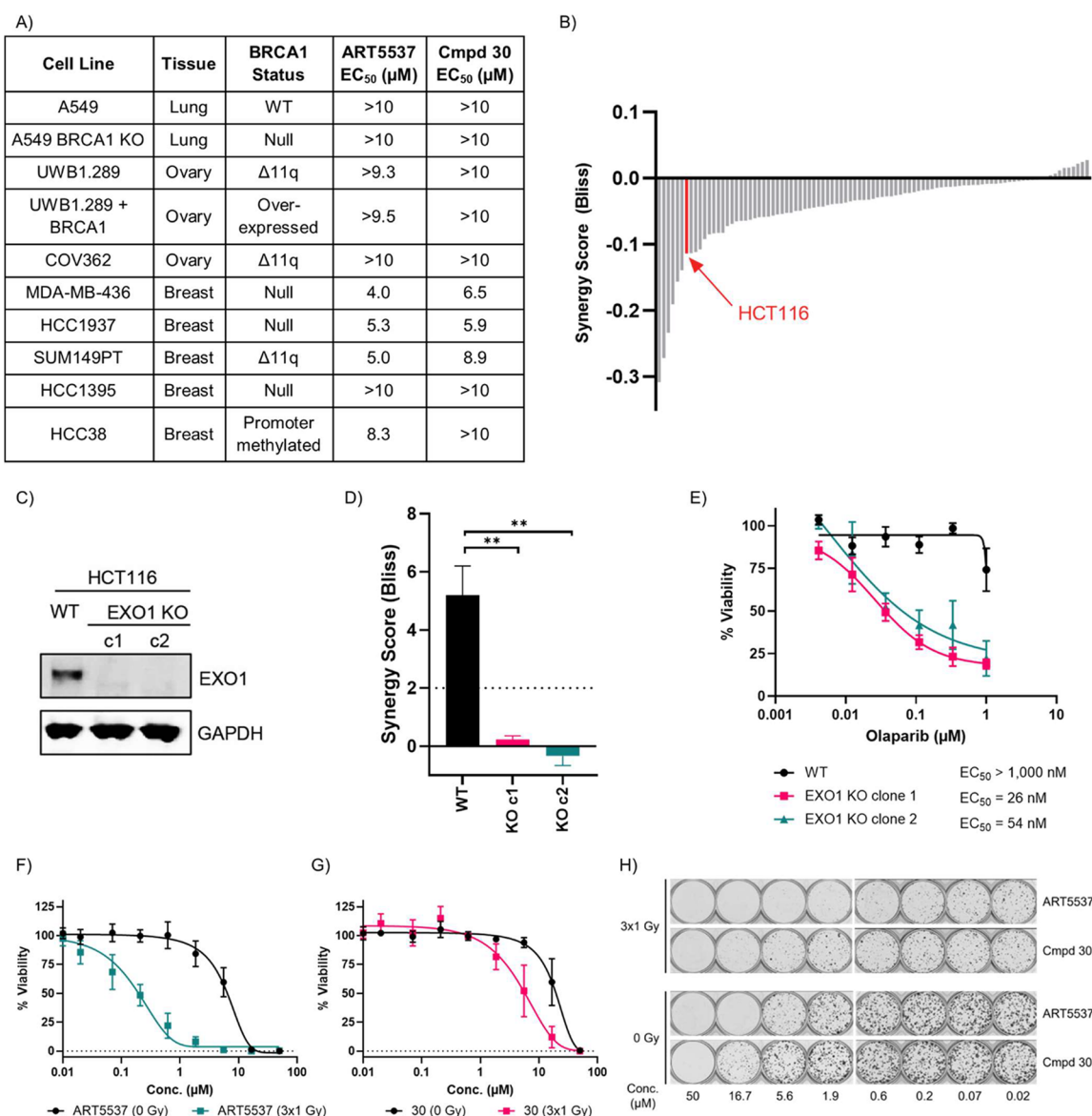


Figure 6. Cellular phenotypes of ART5537. (A) Table of monotherapy cell viability data (7-day CTG) of ART5537 and 30 in 8 representative BRCA1 mutant cancer lines, including two pairs of BRCA1-isogenic lines. Data represent the mean of $n \geq 3$ biological repeats. Fully annotated tables including n and SD, as well as representative curves for sensitive lines are available in the [Supporting Information](#). (B) Synergy scores (Bliss) for the combination of ART5537 and olaparib across a panel of 95 cell lines (comprising 93 cancer lines and two representative noncancer lines), as assessed by measuring sulforhodamine B (SRB)-based cell viability after 5 days of treatment. In this formulation, the Bliss synergy score ranges from +1 to -1, with negative values indicating synergistic potential. (C) Western-blot validation of EXO1 protein loss in HCT116 EXO1 KO cell lines generated by CRISPR Cas9-based editing. (D) Synergy scores (Bliss) from AlamarBlue-quantified cell viability matrices of ART5537 and olaparib in HCT116 wild-type and EXO1 KO cell lines. Bars represent the mean Bliss synergy score $\pm$ SEM of $n = 3$ biological replicates. In this formulation, the synergy score is continuous and positive values >2 indicate synergy. Significance was determined using a two-tailed t test. (E) Olaparib single agent AlamarBlue viability assay data from the experiment in (D) showing that loss of EXO1 sensitizes HCT116 cells to the PARPi. Data represent mean $\pm$ SEM of $n = 3$ biological replicates. (F, G) Colony formation assays (CFA) were performed in HCT116 cells following treatment with ART5537 (F) or 30 (G) with or without 3×1 Gy ionizing radiation. Data represent mean $\pm$ SEM of $n = 3$ biological replicates. (H) Representative images of individual wells in the CFA from (F) and (G).

result in a strong sensitization to IR, again supporting an EXO1-driven phenotype (Figure 6G,H).

Selectivity and ADME Profile of ART5537. The broader nuclease selectivity profile of ART5537 is also supportive of EXO1-driven phenotypes, with ≥ 200 -fold selectivity over a panel of the wider nuclease superfamily (Table 4). We also confirmed that ART5537 has suitable physicochemical properties for use as a cell probe with high thermodynamic solubility and good passive permeability, as measured by Caco-2. Unfortunately, ART5537 is highly unstable in human and

mouse liver microsomes and displayed high CL_p and low oral bioavailability ($F_{po} < 5\%$) in mouse in vivo, which precludes its use as an in vivo tool compound. Optimization of the DMPK profile of ART5537 will be described elsewhere in due course.

Compound Synthesis. A robust and generic synthesis was developed, which enabled access to all compounds described herein via the same simple sequence of steps (Scheme 1). Briefly, the R¹ substituent was incorporated via T₃P-mediated amide coupling with commercially available 3-(benzyloxy)-4-oxo-4H-pyran-2-carboxylic acid. Treatment of the resulting

Table 4. Full Profile of ART5537

assay	activity
EXO1 IC ₅₀ (nM)	3.4
DNA2	17,800-fold ^a
GEN1	5560-fold ^a
MRE11	5790-fold ^a
MUS81	26,100-fold ^a
SLX1/4	2640-fold ^a
SNM1A	>33,300-fold ^a
TREX1	6630-fold ^a
XPF	>19,700-fold ^a
XPG	209-fold ^a
thermodynamic solubility pH 7.4 (μM) ^b	851
Caco-2 passive (10 ⁻⁶ cm/s)/(efflux ratio)	7.8 (13)
microsome CL _{int} (μL/min/mg) h/m	38/170
Obs CL _p (mL/min/kg) ^c	111
t _{1/2} (h) ^c	0.7
V _{ss} (L/kg) ^c	5.7

^aFold-selectivity with respect to indicated nuclease. ^bThermodynamic solubility generated on lyophilized amorphous material. ^cIn vivo mouse PK parameters following 0.2 mg/kg iv dosed in 40% PEG 200 in water; h = human, m = mouse; Data represent $n \geq 2$ unless otherwise indicated.

pyranone amide with aqueous ammonium hydroxide gave the 4-pyridone, which readily underwent *N*-amination with *O*-(2,4-dinitrophenyl)hydroxylamine, followed by condensation with formaldehyde to form the triazinanone ring. The R² substituent was then installed via Buchwald-Hartwig amination and a final LiCl-mediated benzyl deprotection furnished the desired compounds.

CONCLUSIONS

Submicromolar hit **1** was identified from a biochemical fragment screen against EXO1 using a bespoke metal-chelating fragment library. Consistent with our experience on FEN1, this approach yielded a fragment hit with excellent LE and LLE, and inherent selectivity over three closely related RAD2 family members. Fragment growing of **1** rapidly led to improvements in potency, with the discovery of a key α -methyl benzyl substituent, which displayed a strong stereochemical preference for the (*S*)-enantiomer **6**. Further optimization of **6**

resulted in the identification of ART5537, a highly potent and selective small molecule probe for EXO1.

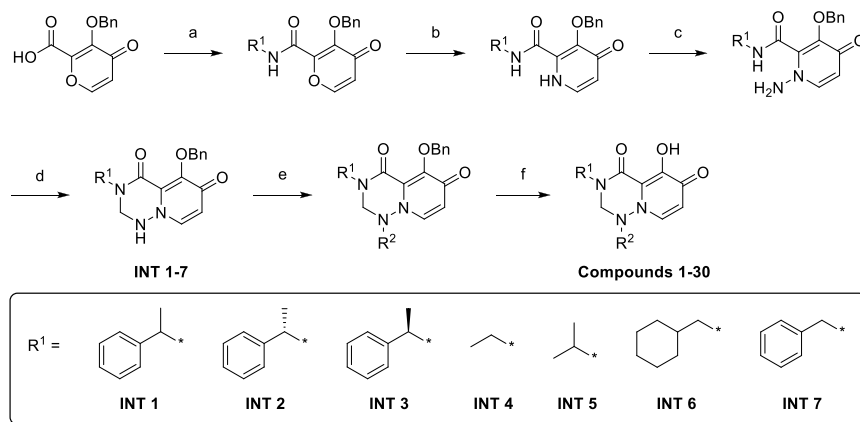
This effort was facilitated by the elucidation of the first small molecule cocrystal structures of EXO1, which revealed an interesting binding mode for compounds **20** and ART5537, wherein the bioactive conformation is biased by an intramolecular π -stacking interaction between the two core *N*-substituents.

ART5537 inhibits HR at single digit nanomolar concentrations in cells (HR EC₅₀ = 7.2 nM). We confirmed the EXO1-dependency of HR inhibition in an EXO1 KO, demonstrating that there is no further off-target-driven suppression of HR signal in the absence of the nuclease. Taken together, these results strongly support the EXO1 activity of ART5537 being responsible for inhibition of HR.

Interestingly, we observe a greater reduction in cellular HR activity in response to treatment with compound than that which is caused by genetic KO of the EXO1 gene (vide supra). In support of a possible dominant-negative effect, we show that ART5537 induces a dose-dependent retention of EXO1 on chromatin. This observation is reminiscent of the behavior of PARPi, where compounds such as olaparib are known to induce a similar retention of PARP1 on chromatin,^{30–32} broadly referred to in the literature as “trapping”. Such dominant-negative type interactions have been well described for PARPs, whereby sensitivity to the alkylating agent MMS is weaker in PARP1 KO cells than in those treated with PARPi,^{33–36} supporting the concept of PARP “trapping”. Taken together, our data are potentially indicative of a trapping mechanism for EXO1, although further work is required to probe the relationship between EXO1 inhibition, chromatin retention, and the observed phenotypic behavior of ART5537.

Finally, we demonstrate that ART5537 is potentially synergistic with olaparib in around 50% of a panel of 93 cancer cell lines, having validated the dependence of the observed synergy on EXO1 in HCT116 as a representative of lines displaying sensitivity to the combination. No synergy was observed in two representative noncancer lines, suggesting that the combination is not ubiquitously cytotoxic. We also show that ART5537 sensitizes cells to IR, which is consistent with literature reports on the role of EXO1 in DSB repair following irradiation. Despite potent cellular target engagement of EXO1, we found that ART5537 has only modest monotherapy

Scheme 1. Compound Synthesis: General Procedure 1



(a) R¹-NH₂, T₃P, DIPEA, DCM, rt, 2 h; (b) NH₄OH, EtOH, 60 °C, 2 h; (c) *O*-(2,4-dinitrophenyl)hydroxylamine, K₂CO₃, DMF, rt, 14 h; (d) 37% aqueous formaldehyde, toluene, 80 °C, 14 h; (e) R²-X (X = Br or Cl), XPhos Pd G3, K₃PO₄, dioxane, 80 °C, 2 h; (f) LiCl, DMF, 100 °C, 1 h.

activity in a selection of *BRCA1* mutant cancer cell lines with no clear and confirmed dependence on *BRCA1* loss in isogenic cell lines. Further work is required to probe the apparent disconnect between the potent EXO1 inhibition by ART5537 and monotherapy in the context of *BRCA1* mutations. It is well-reported that the interplay of different DDR processes can be highly context-dependent^{37–39} and many small molecule modulators of DDR targets benefit from synergistic activity as combinations.^{40,41} Further work may be required to determine the full translational potential of EXO1 as a target for cancer therapy.

In summary, we have identified ART5537 as a potent and selective small molecule probe, which we encourage the community to make use of to further explore EXO1 biology.

EXPERIMENTAL SECTION

Synthetic Chemistry. All final compounds were purified to $\geq 95\%$ purity and $\geq 95\%$ ee (where stereochemistry is defined) by HPLC, flash column chromatography, and SFC as necessary, and were characterized by proton NMR and LCMS. All chemicals were purchased from commercial suppliers and used as received unless otherwise indicated.

General Procedure 1 (Scheme 1). Step a: Amide Formation. A mixture of 3-(benzyloxy)-4-oxo-4H-pyran-2-carboxylic acid (CAS: 119736-16-2, 1.0 equiv), DIPEA (3.0 equiv), *R*¹-amine (1.5 equiv) and T₃P (2.0 equiv) in DCM was stirred at rt for 2 h. Upon completion, the reaction mixture was quenched by addition of water and the resulting mixture was extracted with DCM (3 $\times$). The combined organic layers were washed with brine (3 $\times$), dried over Na₂SO₄ and concentrated in vacuo to give the amide product, which was used without further purification.

Step b: Pyridone Formation. The amide product from step a (1.0 equiv) was dissolved in a 1:1 mixture of EtOH/28% aq. NH₄OH solution and the mixture was heated at 60 °C for 4 h. Upon completion, the reaction mixture was cooled to rt and diluted with EtOH. The resulting precipitate was removed by filtration and the filtrate was concentrated in vacuo to give the pyridone product, which was used without further purification.

Step c: Pyridone *N*-amination. A mixture of pyridone product from step b (1.0 equiv), O-(2,4-dinitrophenyl)hydroxylamine (CAS: 17508-17-7, 0.9 equiv) and K₂CO₃ (1.5 equiv) in DMF was stirred at rt for 14 h. Upon completion, the reaction mixture was filtered and the filtrate was directly purified by prep-HPLC (C18 column, MeCN/water +0.05% FA) to give the *N*-aminopyridone product.

Step d: Triazinanone Formation. A mixture of the *N*-aminopyridone product from step c (1.0 equiv) and 37% aq. formaldehyde solution (1.0 equiv) in toluene was stirred at 80 °C for 14 h. Upon completion, the reaction mixture was cooled to rt and the precipitate was collected by filtration to give the triazinanone product, which was used without further purification.

Step e: Triazinanone *N*-arylation. A mixture of triazinanone product from step d (1.0 equiv), R²-X (1.2 equiv), XPhos Pd G3 (CAS: 1445085-55-1, 0.1 equiv) and K₃PO₄ (3.0 equiv) in 1,4-dioxane was stirred at 80 °C for 2 h under a nitrogen atmosphere. Upon completion, the reaction mixture was cooled to rt and concentrated in vacuo. The residue was purified by flash chromatography on silica gel (DCM/MeOH) to give the *N*-aryl product.

Step f: O-Benzyl Deprotection. A mixture of the *N*-aryl product from step e (1.0 equiv) and LiCl (10 equiv) in DMF was stirred at 100 °C for 1 h. Upon completion, the reaction mixture was cooled to rt, diluted with water and purified by prep-HPLC (C18 column, MeCN/water +0.05% FA) to give the final compound.

5-(Benzyloxy)-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-*f*][1,2,4]triazine-4,6-dione (INT 1). The title compound was prepared via general procedure 1 steps a–d, using 1-phenylethan-1-amine (CAS: 618-36-0) in step a. *m/z* ES⁺ [M + H]⁺ 376.2.

(S)-5-(Benzyloxy)-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-*f*][1,2,4]triazine-4,6-dione (INT 2). The title compound was prepared via general procedure 1 steps a–d, using (S)-1-phenylethan-1-amine (CAS: 2627-86-3) in step a. *m/z* ES⁺ [M + H]⁺ 376.2; ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.69 (d, *J* = 7.5 Hz, 1H), 7.59–7.49 (m, 2H), 7.44–7.23 (m, 9H), 6.22 (d, *J* = 7.5 Hz, 1H), 5.72 (q, *J* = 7.2 Hz, 1H), 5.21–5.07 (m, 2H), 4.62–4.46 (m, 1H), 4.34–4.21 (m, 1H), 1.58 (d, *J* = 7.2 Hz, 3H).

(R)-5-(Benzyloxy)-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-*f*][1,2,4]triazine-4,6-dione (INT 3). The title compound was prepared via general procedure 1 steps a–d, using (R)-1-phenylethan-1-amine (CAS: 3886-69-9) in step a. *m/z* ES⁺ [M + H]⁺ 376.1.

5-(Benzyloxy)-3-ethyl-2,3-dihydro-1H-pyrido[2,1-*f*][1,2,4]triazine-4,6-dione (INT 4). The title compound was prepared via general procedure 1 steps a–d, using ethylamine in step a. *m/z* ES⁺ [M + H]⁺ 300.2; ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.70 (d, *J* = 7.6 Hz, 1H), 7.53 (d, *J* = 7.2 Hz, 2H), 7.37–7.27 (m, 4H), 6.20 (d, *J* = 7.6 Hz, 1H), 5.08 (s, 2H), 4.56 (d, *J* = 8.0 Hz, 2H), 3.24 (q, *J* = 7.0 Hz, 2H), 1.11 (t, *J* = 7.0 Hz, 3H).

5-(Benzyloxy)-3-isopropyl-2,3-dihydro-1H-pyrido[2,1-*f*][1,2,4]triazine-4,6-dione (INT 5). The title compound was prepared via general procedure 1 steps a–d, using isopropylamine in step a. *m/z* ES⁺ [M + H]⁺ 314.3; ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.71 (d, *J* = 7.6 Hz, 1H), 7.53 (d, *J* = 7.2 Hz, 2H), 7.37–7.25 (m, 4H), 6.20 (d, *J* = 7.6 Hz, 1H), 5.08 (s, 2H), 4.59–4.51 (m, 3H), 1.14 (d, *J* = 6.4 Hz, 6H).

5-(Benzyloxy)-3-(cyclohexylmethyl)-2,3-dihydro-1H-pyrido[2,1-*f*][1,2,4]triazine-4,6-dione (INT 6). The title compound was prepared via general procedure 1 steps a–d, using cyclohexanemethanamine in step a. *m/z* ES⁺ [M + H]⁺ 368.4; ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.70 (d, *J* = 7.6 Hz, 1H), 7.52 (d, *J* = 6.8 Hz, 2H), 7.40–7.29 (m, 4H), 6.19 (d, *J* = 7.6 Hz, 1H), 5.07 (s, 2H), 4.55 (d, *J* = 7.6 Hz, 2H), 3.28–3.27 (m, 2H), 1.67–1.59 (m, 6H), 1.19–1.15 (m, 3H), 0.92–0.89 (m, 2H).

3-Benzyl-5-(benzyloxy)-2,3-dihydro-1H-pyrido[2,1-*f*][1,2,4]triazine-4,6-dione (INT 7). The title compound was prepared via general procedure 1 steps a–d, using benzylamine in step a. *m/z* ES⁺ [M + H]⁺ 362.3; ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.71 (d, *J* = 7.6 Hz, 1H), 7.53–7.51 (m, 2H), 7.43 (t, *J* = 7.6 Hz, 1H), 7.37–7.27 (m, 8H), 6.21 (d, *J* = 7.6 Hz, 1H), 5.10 (s, 2H), 4.68 (s, 2H), 4.59 (d, *J* = 7.6 Hz, 2H).

5-Hydroxy-3-methyl-1-phenyl-2,3-dihydro-1H-pyrido[2,1-*f*][1,2,4]triazine-4,6-dione (1). The title compound was prepared via general procedure 1 steps e–f, using 5-(benzyloxy)-3-methyl-2,3-dihydro-1H-pyrido[2,1-*f*][1,2,4]triazine-4,6-dione (CAS: 2413415-09-3) in step e. *m/z* ES⁺ [M + H]⁺ 272.1; ¹H NMR (300 MHz, D₂O) δ 7.63 (d, *J* = 7.2 Hz, 1H), 7.39–7.27 (m, 2H), 7.20–7.09 (m, 1H), 6.82–6.74 (m, 2H), 6.42 (d, *J* = 7.2 Hz, 1H), 5.22 (s, 2H), 2.86 (s, 3H).

3-Ethyl-5-hydroxy-1-phenyl-2,3-dihydro-1H-pyrido[2,1-*f*][1,2,4]triazine-4,6-dione (2). The title compound was prepared via general procedure 1 steps e–f, using INT 4 and bromobenzene in step e. *m/z* ES⁺ [M + H]⁺ 286.2; ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.78–7.76 (m, 1H), 7.36 (d, *J* = 8.0 Hz, 2H), 7.11 (t, *J* = 7.6 Hz, 1H), 6.78 (d, *J* = 8.0 Hz, 2H), 6.18 (d, *J* = 7.6 Hz, 1H), 5.39 (br s, 2H), 3.32–3.27 (m, 2H), 0.79 (t, *J* = 7.2 Hz, 3H).

5-Hydroxy-3-isopropyl-1-phenyl-2,3-dihydro-1H-pyrido[2,1-*f*][1,2,4]triazine-4,6-dione (3). The title compound was prepared via general procedure 1 steps e–f, using INT 5 and bromobenzene in step e. *m/z* ES⁺ [M + H]⁺ 300.3; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.40 (br s, 1H), 7.81 (d, *J* = 7.6 Hz, 1H), 7.36 (t, *J* = 8.0 Hz, 2H), 7.11 (t, *J* = 7.6 Hz, 1H), 6.80 (d, *J* = 8.0 Hz, 2H), 6.20 (d, *J* = 7.6 Hz, 1H), 5.47 (br s, 1H), 5.22 (br s, 1H), 4.48–4.44 (m, 1H), 1.08 (br s, 3H), 0.72 (br s, 3H).

3-(Cyclohexylmethyl)-5-hydroxy-1-phenyl-2,3-dihydro-1H-pyrido[2,1-*f*][1,2,4]triazine-4,6-dione (4). The title compound was prepared via general procedure 1 steps e–f, using INT 6 and bromobenzene in step e. *m/z* ES⁺ [M + H]⁺ 354.3; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.36 (br s, 1H), 7.81 (d, *J* = 6.0 Hz, 1H), 7.39–7.35 (m, 2H), 7.13–7.10 (m, 1H), 6.81–6.79 (m, 2H), 6.22–6.18

(m, 1H), 5.43–5.38 (m, 2H), 2.91–2.89 (m, 2H), 1.47–1.32 (m, 4H), 1.08–0.77 (m, 7H).

3-Benzyl-5-hydroxy-1-phenyl-2,3-dihydro-1H-pyrido[2,1-f]-[1,2,4]triazine-4,6-dione (5). The title compound was prepared via general procedure 1 steps e–f, using INT 7 and bromobenzene in step e. m/z ES⁺ [M + H]⁺ 348.2; ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.18 (br s, 1H), 7.81 (d, J = 7.6 Hz, 1H), 7.26–7.06 (m, 6H), 6.99–6.97 (m, 2H), 6.65 (d, J = 7.6 Hz, 2H), 6.21 (d, J = 7.6 Hz, 1H), 5.42 (s, 2H), 4.54 (br s, 2H).

(S)-5-Hydroxy-1-phenyl-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-f]-[1,2,4]triazine-4,6-dione (6). The title compound was prepared via general procedure 1 steps e–f, using INT 2 and bromobenzene in step e. m/z ES⁺ [M + H]⁺ 362.2; ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.76 (d, J = 7.5 Hz, 1H), 7.53–6.29 (br m, 10H), 6.20 (d, J = 7.5 Hz, 1H), 5.59 (q, J = 7.2 Hz, 1H), 5.36 (d, J = 13.5 Hz, 1H), 5.10 (d, J = 13.5 Hz, 1H), 1.68–1.02 (m, 3H); Chiral SFC (Cellulose SZ, MeOH/DCM + 0.2% MSA) gave >99% ee.

(R)-5-Hydroxy-1-phenyl-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-f]-[1,2,4]triazine-4,6-dione (7). The title compound was prepared via general procedure 1 steps e–f, using INT 3 and bromobenzene in step e. m/z ES⁺ [M + H]⁺ 362.2; ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.76 (d, J = 7.5 Hz, 1H), 7.66–6.33 (br m, 10H), 6.20 (d, J = 7.5 Hz, 1H), 5.59 (q, J = 7.2 Hz, 1H), 5.36 (d, J = 13.5 Hz, 1H), 5.10 (d, J = 13.5 Hz, 1H), 1.66–0.99 (m, 3H); Chiral SFC (Cellulose SZ, MeOH/DCM + 0.2% MSA) gave >99% ee.

1-(2-Fluorophenyl)-5-hydroxy-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-f]-[1,2,4]triazine-4,6-dione (8). The title compound was prepared via general procedure 1 steps e–f, using INT 1 and 1-bromo-2-fluorobenzene in step e. m/z ES⁺ [M + H]⁺ 380.2; ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.79 (d, J = 7.5 Hz, 1H), 7.27–6.89 (br m, 8H), 6.41–6.30 (m, 1H), 6.24 (d, J = 7.5 Hz, 1H), 5.65 (q, J = 7.2 Hz, 1H), 5.33–5.07 (m, 1H), 4.89–4.64 (m, 1H), 1.55–1.14 (m, 3H).

1-(2-Chlorophenyl)-5-hydroxy-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-f]-[1,2,4]triazine-4,6-dione (9). The title compound was prepared via general procedure 1 steps e–f, using INT 1 and 1-bromo-2-chlorobenzene in step e. m/z ES⁺ [M + H]⁺ 396/398; ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.70 (d, J = 7.5 Hz, 1H), 7.39–7.26 (m, 2H), 7.26–7.16 (m, 2H), 7.13–7.01 (m, 2H), 6.99–6.84 (m, 2H), 6.51 (d, J = 7.8 Hz, 1H), 6.20 (d, J = 7.5 Hz, 1H), 5.63 (q, J = 7.2 Hz, 1H), 5.29–5.10 (m, 1H), 4.92–4.72 (m, 1H), 1.50–1.10 (m, 3H).

5-Hydroxy-3-(1-phenylethyl)-1-(*o*-tolyl)-2,3-dihydro-1H-pyrido[2,1-f]-[1,2,4]triazine-4,6-dione (10). The title compound was prepared via general procedure 1 steps e–f, using INT 1 and 1-bromo-2-methylbenzene in step e. m/z ES⁺ [M + H]⁺ 376.2; ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.79 (d, J = 7.5 Hz, 1H), 7.23–7.03 (m, 6H), 7.02–6.90 (m, 2H), 6.41–6.34 (m, 1H), 6.31 (d, J = 7.5 Hz, 1H), 5.58 (q, J = 7.2 Hz, 1H), 5.12–5.02 (m, 1H), 4.68–4.59 (m, 1H), 2.09 (s, 3H), 1.37–1.21 (m, 3H).

5-Hydroxy-3-(1-phenylethyl)-1-(pyridin-2-yl)-2,3-dihydro-1H-pyrido[2,1-f]-[1,2,4]triazine-4,6-dione (11). The title compound was prepared via general procedure 1 steps e–f, using INT 1 and 2-bromopyridine in step e. m/z ES⁺ [M + H]⁺ 363.2; ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.24–7.99 (m, 1H), 7.74–7.60 (m, 2H), 7.30–7.03 (m, 6H), 6.61–6.47 (m, 1H), 6.20 (d, J = 7.5 Hz, 1H), 5.61 (q, J = 7.2 Hz, 1H), 5.52–5.41 (m, 1H), 5.35–5.18 (m, 1H), 1.46 (d, J = 7.2 Hz, 3H).

1-(3-Fluorophenyl)-5-hydroxy-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-f]-[1,2,4]triazine-4,6-dione (12). The title compound was prepared via general procedure 1 steps e–f, using INT 1 and 1-bromo-3-fluorobenzene in step e. m/z ES⁺ [M + H]⁺ 380.1; ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.77 (d, J = 7.5 Hz, 1H), 7.51–6.73 (br m, 7H), 6.59–6.31 (m, 1H), 6.21 (d, J = 7.5 Hz, 1H), 6.15–5.92 (m, 1H), 5.64 (q, J = 7.2 Hz, 1H), 5.46–5.27 (m, 1H), 5.21–5.01 (m, 1H), 1.66–1.06 (m, 3H).

1-(3-Chlorophenyl)-5-hydroxy-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-f]-[1,2,4]triazine-4,6-dione (13). The title compound was prepared via general procedure 1 steps e–f, using INT 1 and 1-bromo-3-chlorobenzene in step e. m/z ES⁺ [M + H]⁺ 396/398; ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.81 (d, J = 7.5 Hz, 1H), 7.41–6.89

(br m, 7H), 6.84–6.46 (m, 1H), 6.42–6.10 (m, 2H), 5.63 (q, J = 7.2 Hz, 1H), 5.37 (d, J = 13.5 Hz, 1H), 5.13 (d, J = 13.5 Hz, 1H), 1.59–1.18 (m, 3H).

5-Hydroxy-3-(1-phenylethyl)-1-(*m*-tolyl)-2,3-dihydro-1H-pyrido[2,1-f]-[1,2,4]triazine-4,6-dione (14). The title compound was prepared via general procedure 1 steps e–f, using INT 1 and 1-bromo-3-methylbenzene in step e. m/z ES⁺ [M + H]⁺ 376.2; ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.79 (d, J = 7.5 Hz, 1H), 7.29–6.83 (br m, 7H), 6.76–5.86 (m, 3H), 5.60 (q, J = 7.2 Hz, 1H), 5.34 (d, J = 13.5 Hz, 1H), 5.06 (d, J = 13.5 Hz, 1H), 2.15 (s, 3H), 1.56–1.12 (m, 3H).

5-Hydroxy-3-(1-phenylethyl)-1-(pyridin-3-yl)-2,3-dihydro-1H-pyrido[2,1-f]-[1,2,4]triazine-4,6-dione (15). The title compound was prepared via general procedure 1 steps e–f, using INT 1 and 3-bromopyridine in step e. m/z ES⁺ [M + H]⁺ 363.2; ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.39–8.18 (m, 1H), 7.80 (d, J = 7.5 Hz, 1H), 7.57–6.62 (br m, 8H), 6.22 (d, J = 7.5 Hz, 1H), 5.63 (q, J = 7.2 Hz, 1H), 5.41 (d, J = 13.8 Hz, 1H), 5.14 (d, J = 13.8 Hz, 1H), 1.68–0.98 (m, 3H).

1-(4-Fluorophenyl)-5-hydroxy-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-f]-[1,2,4]triazine-4,6-dione (16). The title compound was prepared via general procedure 1 steps e–f, using INT 1 and 1-bromo-4-fluorobenzene in step e. m/z ES⁺ [M + H]⁺ 380.1; ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.74 (d, J = 7.5 Hz, 1H), 7.46–6.71 (br m, 8H), 6.59–6.31 (m, 1H), 6.20 (d, J = 7.5 Hz, 1H), 5.62 (q, J = 7.2 Hz, 1H), 5.31 (d, J = 13.8 Hz, 1H), 5.06 (d, J = 13.8 Hz, 1H), 1.62–0.98 (m, 3H).

1-(4-Chlorophenyl)-5-hydroxy-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-f]-[1,2,4]triazine-4,6-dione (17). The title compound was prepared via general procedure 1 steps e–f, using INT 1 and 1-bromo-4-chlorobenzene in step e. m/z ES⁺ [M + H]⁺ 396/398; ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.75 (d, J = 7.5 Hz, 1H), 7.62–6.66 (br m, 8H), 6.52–6.27 (m, 1H), 6.20 (d, J = 7.5 Hz, 1H), 5.63 (q, J = 7.2 Hz, 1H), 5.34 (d, J = 13.8 Hz, 1H), 5.09 (d, J = 13.8 Hz, 1H), 1.64–0.99 (m, 3H).

5-Hydroxy-3-(1-phenylethyl)-1-(*p*-tolyl)-2,3-dihydro-1H-pyrido[2,1-f]-[1,2,4]triazine-4,6-dione (18). The title compound was prepared via general procedure 1 steps e–f, using INT 1 and 1-bromo-4-methylbenzene in step e. m/z ES⁺ [M + H]⁺ 376.2; ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.81 (d, J = 7.5 Hz, 1H), 7.32–6.90 (br m, 7H), 6.68–6.36 (m, 2H), 6.32 (d, J = 7.5 Hz, 1H), 5.58 (q, J = 7.2 Hz, 1H), 5.32 (d, J = 13.5 Hz, 1H), 5.06 (d, J = 13.5 Hz, 1H), 2.23 (s, 3H), 1.42–1.21 (m, 3H).

5-Hydroxy-3-(1-phenylethyl)-1-(pyridin-4-yl)-2,3-dihydro-1H-pyrido[2,1-f]-[1,2,4]triazine-4,6-dione (19). The title compound was prepared via general procedure 1 steps e–f, using INT 1 and 4-bromopyridine in step e. m/z ES⁺ [M + H]⁺ 363.2; ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.40 (br s, 2H), 7.77 (d, J = 7.5 Hz, 1H), 7.33–7.09 (m, 5H), 6.81 (br s, 2H), 6.27 (d, J = 7.5 Hz, 1H), 5.68 (q, J = 7.2 Hz, 1H), 5.50 (d, J = 13.8 Hz, 1H), 5.30 (d, J = 13.8 Hz, 1H), 1.50 (d, J = 7.2 Hz, 3H).

(S)-1-(5-Chloro-3-methylpyridin-2-yl)-5-hydroxy-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-f]-[1,2,4]triazine-4,6-dione (20). The title compound was prepared via general procedure 1 steps e–f, using INT 2 and 2-bromo-5-chloro-3-methylpyridine (CAS: 65550-77-8) in step e. m/z ES⁺ [M + H]⁺ 411/413; ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.09–7.92 (m, 1H), 7.61–7.40 (m, 2H), 7.22–6.93 (m, 5H), 6.24–6.03 (m, 1H), 5.94–5.55 (m, 1H), 5.32–4.95 (m, 1H), 4.77–4.60 (m, 1H), 2.11 (s, 3H), 1.40 (d, J = 7.2 Hz, 3H).

(R)-1-(5-Chloro-3-methylpyridin-2-yl)-5-hydroxy-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-f]-[1,2,4]triazine-4,6-dione (21). The title compound was prepared via general procedure 1 steps e–f, using INT 3 and 2-bromo-5-chloro-3-methylpyridine (CAS: 65550-77-8) in step e. m/z ES⁺ [M + H]⁺ 411/413; ¹H NMR (300 MHz, CD₃OD) δ 8.00 (s, 1H), 7.59 (d, J = 7.2 Hz, 1H), 7.42–7.28 (m, 1H), 7.25–7.17 (m, 1H), 7.16–7.06 (m, 2H), 7.02–6.88 (m, 2H), 6.38 (d, J = 7.2 Hz, 1H), 5.89–5.80 (m, 1H), 5.39–5.13 (m, 1H), 4.71–4.50 (m, 1H), 2.01 (s, 3H), 1.67–1.48 (m, 3H).

(S)-5-Hydroxy-1-(isoquinolin-1-yl)-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-f]-[1,2,4]triazine-4,6-dione (22). The title compound was prepared via general procedure 1 steps e–f, using INT 2

and 1-bromoisoquinoline in step e. m/z ES⁺ [M + H]⁺ 413.2; ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.13–8.04 (m, 2H), 7.97–7.88 (m, 1H), 7.82–7.56 (m, 4H), 6.89–6.59 (m, 5H), 6.28–6.19 (m, 1H), 5.58 (q, *J* = 7.2 Hz, 1H), 5.42 (d, *J* = 13.5 Hz, 1H), 4.99 (d, *J* = 13.5 Hz, 1H), 1.34–1.05 (m, 3H).

(S)-5-Hydroxy-1-(1-methyl-1H-pyrrolo[3,2-*c*]pyridin-4-yl)-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-*f*][1,2,4]triazine-4,6-dione (23). The title compound was prepared via general procedure 1 steps e–f, using INT 2 and 4-chloro-1-methyl-1H-pyrrolo[3,2-*c*]pyridine (CAS: 27382-01-0) in step e. m/z ES⁺ [M + H]⁺ 416.2; ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.79 (d, *J* = 5.7 Hz, 1H), 7.69 (d, *J* = 7.5 Hz, 1H), 7.29–7.21 (m, 2H), 7.07–7.00 (m, 1H), 7.00–6.88 (m, 2H), 6.86–6.74 (m, 2H), 6.30 (d, *J* = 7.5 Hz, 1H), 6.18 (d, *J* = 3.3 Hz, 1H), 5.54 (q, *J* = 7.2 Hz, 1H), 5.46 (d, *J* = 13.5 Hz, 1H), 5.19 (d, *J* = 13.5 Hz, 1H), 3.78 (s, 3H), 1.39–1.15 (m, 3H).

(S)-5-Hydroxy-1-(1-methyl-1H-pyrrolo[2,3-*c*]pyridin-7-yl)-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-*f*][1,2,4]triazine-4,6-dione (24). The title compound was prepared via general procedure 1 steps e–f, using INT 2 and 7-chloro-1-methyl-1H-pyrrolo[2,3-*c*]pyridine (CAS: 875340-57-1) in step e. m/z ES⁺ [M + H]⁺ 416.2; ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.82 (d, *J* = 7.5 Hz, 1H), 7.74 (d, *J* = 5.4 Hz, 1H), 7.55–7.45 (m, 1H), 7.43 (d, *J* = 5.4 Hz, 1H), 7.08–6.72 (m, 5H), 6.55–6.48 (m, 1H), 6.18 (d, *J* = 7.5 Hz, 1H), 5.56 (q, *J* = 7.2 Hz, 1H), 5.36–5.13 (m, 1H), 4.94–4.71 (m, 1H), 4.01 (s, 3H), 1.38–1.04 (m, 3H).

(S)-5-Hydroxy-1-(1,6-naphthyridin-5-yl)-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-*f*][1,2,4]triazine-4,6-dione (25). The title compound was prepared via general procedure 1 steps e–f, using INT 2 and 5-chloro-1,6-naphthyridine (CAS: 23616-32-2) in step e. m/z ES⁺ [M + H]⁺ 414.2; ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.07 (s, 1H), 8.49–8.43 (m, 1H), 8.34–8.26 (m, 1H), 7.75–7.67 (m, 2H), 7.62–7.53 (m, 1H), 6.87–6.54 (m, 5H), 6.29–6.20 (m, 1H), 5.64–5.56 (m, 1H), 5.48–5.43 (m, 1H), 4.98–4.87 (m, 1H), 1.39–1.17 (m, 3H).

(S)-5-Hydroxy-1-(2,6-naphthyridin-1-yl)-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-*f*][1,2,4]triazine-4,6-dione (26). The title compound was prepared via general procedure 1 steps e–f, using INT 2 and 1-chloro-2,6-naphthyridine (CAS: 80935-78-0) in step e. m/z ES⁺ [M + H]⁺ 414.2; ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.33 (s, 1H), 8.66–8.58 (m, 1H), 8.27 (d, *J* = 5.7 Hz, 1H), 7.91–7.83 (m, 1H), 7.80 (d, *J* = 5.7 Hz, 1H), 7.69 (d, *J* = 7.5 Hz, 1H), 6.85–6.56 (m, 5H), 6.23 (d, *J* = 7.5 Hz, 1H), 5.60 (q, *J* = 7.2 Hz, 1H), 5.50 (d, *J* = 13.8 Hz, 1H), 5.00 (d, *J* = 13.8 Hz, 1H), 1.42–1.13 (m, 3H).

(S)-5-Hydroxy-1-(2,7-naphthyridin-1-yl)-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-*f*][1,2,4]triazine-4,6-dione (27). The title compound was prepared via general procedure 1 steps e–f, using INT 2 and 1-chloro-2,7-naphthyridine (CAS: 69042-30-4) in step e. m/z ES⁺ [M + H]⁺ 414.2; ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.56–9.17 (m, 1H), 8.80–8.56 (m, 1H), 8.35–8.19 (m, 1H), 7.83–7.57 (m, 3H), 6.74–6.44 (m, 5H), 6.14 (d, *J* = 7.5 Hz, 1H), 5.72–5.51 (m, 2H), 5.03–4.93 (m, 1H), 1.57–1.32 (m, 3H).

(S)-5-Hydroxy-1-(1,7-naphthyridin-8-yl)-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-*f*][1,2,4]triazine-4,6-dione (28). The title compound was prepared via general procedure 1 steps e–f, using INT 2 and 8-chloro-1,7-naphthyridine (CAS: 13058-77-0) in step e. m/z ES⁺ [M + H]⁺ 414.2; ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.78 (br s, 1H), 8.36–8.27 (m, 1H), 8.13 (d, *J* = 5.7 Hz, 1H), 7.76–7.69 (m, 1H), 7.63 (d, *J* = 5.7 Hz, 1H), 7.57 (d, *J* = 7.8 Hz, 1H), 6.94–6.53 (m, 5H), 6.16 (d, *J* = 7.8 Hz, 1H), 5.89–5.52 (m, 3H), 1.50–1.15 (m, 3H).

(S)-1-(4-Chloro-2,7-naphthyridin-1-yl)-5-hydroxy-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-*f*][1,2,4]triazine-4,6-dione (29, ART5537). 4-Chloro-2,7-naphthyridin-1(2H)-one. NCS (2.7 g, 20.5 mmol) was added to a solution of 2,7-naphthyridin-1(2H)-one (CAS: 67988-50-5, 2.5 g, 17.1 mmol) in DMA (50 mL) at 0 °C. The resulting solution was warmed to rt and stirred for 14 h. Upon completion, the reaction mixture was poured into ice–water (100 mL) and the resulting precipitate was collected by filtration to give the title compound (2.3 g, 74%) as a light-yellow solid. m/z ES⁺ [M + H]⁺ 181/183; ¹H NMR (300 MHz, DMSO-*d*₆) δ 11.95 (s, 1H), 9.35

(s, 1H), 8.88 (d, *J* = 5.7 Hz, 1H), 7.81–7.74 (m, 1H), 7.68 (d, *J* = 5.7 Hz, 1H).

1,4-Dichloro-2,7-naphthyridine. A mixture of 4-chloro-2,7-naphthyridin-1(2H)-one (2.3 g, 12.7 mmol) and POCl₃ (30 mL) was stirred at 130 °C for 2 h. Upon completion, the reaction mixture was cooled to rt and concentrated in vacuo. Ice–water (10 mL) was added to the residue and the pH of the aq. mixture was adjusted to pH 8 with sat. aq. NaHCO₃. The resulting precipitate was collected by filtration to give the title compound (2.1 g, 83%) as a brown solid. m/z ES⁺ [M + H]⁺ 199/201/203.

(S)-1-(4-Chloro-2,7-naphthyridin-1-yl)-5-hydroxy-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-*f*][1,2,4]triazine-4,6-dione (29, ART5537). The title compound was prepared via general procedure 1 steps e–f, using INT 2 and 1,4-dichloro-2,7-naphthyridine in step e. m/z ES⁺ [M + H]⁺ 448/450; ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.47 (s, 1H), 8.85 (d, *J* = 6.0 Hz, 1H), 8.43 (s, 1H), 7.83 (d, *J* = 6.0 Hz, 1H), 7.75 (d, *J* = 7.8 Hz, 1H), 6.76–6.52 (m, 5H), 6.15 (d, *J* = 7.8 Hz, 1H), 5.70–5.59 (m, 2H), 5.04–5.00 (m, 1H), 1.43 (d, *J* = 7.2 Hz, 3H); Chiral SFC (Cellulose SZ, MeOH/DCM + 0.2% MSA) gave >99% ee.

(R)-1-(4-Chloro-2,7-naphthyridin-1-yl)-5-hydroxy-3-(1-phenylethyl)-2,3-dihydro-1H-pyrido[2,1-*f*][1,2,4]triazine-4,6-dione (30). The title compound was prepared via general procedure 1 steps e–f, using INT 2 and 1,4-dichloro-2,7-naphthyridine in step e. m/z ES⁺ [M + H]⁺ 448/450; ¹H NMR (300 MHz, CD₃OD) δ 9.47 (s, 1H), 8.85 (d, *J* = 6.0 Hz, 1H), 8.43 (s, 1H), 7.84 (d, *J* = 6.0 Hz, 1H), 7.75 (d, *J* = 7.8 Hz, 1H), 6.79–6.49 (m, 5H), 6.15 (d, *J* = 7.8 Hz, 1H), 5.75–5.55 (m, 2H), 5.02 (d, *J* = 13.8 Hz, 1H), 1.43 (d, *J* = 7.2 Hz, 3H); Chiral SFC (Cellulose SZ, MeOH/DCM + 0.2% MSA) gave >98% ee.

Molecular Modeling. In silico molecular modeling of inhibitors and illustrations were performed with the Maestro suite of software available from Schrödinger, Version 13.2, release 2022-2, Schrödinger LLC, New York, NY, 2022.

Protein Expression and Purification. Full-length EXO1, FEN1 and XPG and truncated GEN1 (1–527) were expressed and purified as described previously for use in the biochemical assays.²³

Truncated EXO1 (1–352) fused to a C-terminal TEV protease site followed by a His-tag was expressed and purified by Peak Proteins following an adapted literature protocol for use in protein crystallography.⁴² In summary, protein was expressed in *E. coli* BL21 Gold, induced with 0.1 mM IPTG and grown at 18 °C overnight. After harvesting, lysis and clarification, protein was purified by capturing in an IMAC, followed by tag cleavage with TEV protease, reverse IMAC, heparin and size exclusion chromatography in a buffer containing 20 mM TRIS-HCl pH 7.5, 100 mM NaCl, 1 mM TCEP, 1 mM MgCl₂. The protein was concentrated to 10.2 mg/mL, flash frozen in liquid nitrogen and stored at –80 °C until further use.

For all proteins, fractions were pooled, concentration determined by A280 nm measurement, aliquoted and snap frozen in liquid N₂. Aliquots were stored at –80 °C for long-term storage and until use.

Protein Crystallography. DNA substrate was prepared by mixing oligo 1 and oligo 2 (see Supporting Information for sequence details) in equimolar amounts solubilized in 50 mM Tris-HCl pH 7.5, 3 mM MgCl₂, 100 mM NaCl buffer to a final concentration of 300 μM. Oligos were annealed by heating at 95 °C for 5 min, after which the heating block was switched off and the mixture was allowed to cool to rt. Once cooled, the DNA substrate was aliquoted and stored at –80 °C until further use.

EXO1 (1–352) protein at a concentration of ~10 mg/mL was incubated with DNA substrate at 167 μM for 30 min on ice in a 1:1 ratio. Compounds were then added to the solution at a final concentration of 2.0 mM and incubated for 30 min on ice. Sample was crystallized at 20 °C by mixing it at a 1:1 ratio with reservoir solution containing 20% PEG 3350, 0.2 M sodium formate (compound 20) or 20% PEG 3350, 0.2 M potassium sulfate (ART5537). Crystals were cryo-protected with 20% ethylene glycol, harvested in a fully automated fashion with the CrystalDirect Harvester⁴³ and flash-cooled in liquid nitrogen.

Diffraction data were collected at Soleil Light Source beamline Proxima 2a using the EIGER X 9 M detector at a wavelength of 0.98011 Å. The data set was processed using autoPROC from Global Phasing Ltd., and the structure was solved by molecular replacement in Phaser using the EXO1 coordinates from PDB 5V06 as a search model.

Structure was refined using Buster and model building using Coot. Ligand restraints (.cif file) were generated starting from the SMILES string with grade2. Structure figures were generated in PyMOL.

Biochemical Assays. Biochemical assays for EXO1, FEN1, GEN1 and XPG were carried out as described previously.²³

HR Reporter Assay. The HR reporter assay was carried out as previously described.²⁵ Briefly, $\sim 3 \times 10^6$ HEK293 cells were reverse transfected with nanoluciferase DNA substrate and control Firefly luciferase plasmid (Promega) at a ratio of 6 μ g nanoluciferase substrate: 2 μ g Firefly plasmid using JetPRIME reagent (2 μ L/ μ g DNA). Cells were then seeded into 96-well plates predosed with compounds and DMSO only control, normalized across all wells with DMSO. After 16 h, Firefly and NanoLuc levels were detected using the Nano-Glo Dual-Luciferase Reporter Assay system (Promega) as per the manufacturer's instructions. NanoLuc luminescence was normalized to Firefly luminescence to determine DNA substrate repair. Percentage repair was calculated relative to the DMSO control.

NanoBRET Target Engagement Assay. The NanoBRET target engagement assay was setup and performed by Promega, Madison, USA as described previously.⁴⁴ Briefly, a NanoLuc-EXO1 expression plasmid was transiently transfected into HEK293 cells and 20 μ L of subsequent cell/DNA suspension dispensed into wells of 384-well assay plates. Tracer (final concentration 125 nM) and test compounds (10 step 1:3 dilution from a top concentration of 10 μ M) in 100% DMSO were dispensed via Echo Labcyte (Beckman Coulter) in technical quadruplicates. To induce NanoLuc activity, 10 μ L of 3x detection substrate NanoBRET Nano-GLO Live Cell Solution in OptiMEM with digitonin (final concentration 150 μ g/mL) were added per well. Kinetics were followed by luminescence multimode reader (DonorEm 450 nm/AcceptorEm 610 nm) for 1 h. NanoBRET readings were taken after 30 min incubation with substrate and digitonin, ratios were calculated and resulting mBRET values were plotted vs compound concentrations and fitted to a nonlinear regression curve (four parameters) to determine EC₅₀ values. Synthesis of the tracer is described in the [Supporting Information](#).

CTG Assays. Cells were grown in appropriate media, as specified by the vendor at 37 °C in 5% CO₂. Cells in exponential growth phase with <20 consecutive passages of culture were used to perform viability assays.

Cell viability evaluation was performed using the CellTiter-Glo Luminescent Cell Viability Assay kit (G7570, Promega). Test compounds in eight 3-fold serial dilutions were dispensed using a Tecan dispenser to 96-well white wall flat-bottomed assay plates (3570, Corning). The final concentration of DMSO was 0.1% (v/v) in each well.

Cells were seeded in 175 μ L media per well. The plates were incubated at 37 °C in 5% CO₂ for 7 days. On day 7, the plates were removed from the incubator and equilibrated at rt for 30 min. 50 μ L CellTiter-Glo was added to each assay well. Plates were then shaken for 4 min at 450 rpm and left to stand at rt for 25 min. Luminescence was recorded with a CLARIOstar plate reader.

All data analysis was carried out using Genedata Screener software. EC₅₀ values were calculated using the SmartFit function.

HCT116 Colony Formation Assay. Exponentially growing HCT116 cells were seeded in 12-well plates at a density of 600 cells/well and left to adhere overnight under standard culture conditions (37 °C, 5% CO₂). Cells were then treated with a 9-point 3-fold serial dilution of EXO1i and left to stand for 3 h. Cells in the + IR group were then irradiated with 1 Gy using a CIX2 device (Xstrahl) and cells in the -IR group were left unirradiated. The irradiation procedure was repeated twice after 24 and 48 h to give a total fractionated IR dose of 3×1 Gy. After a total of 6 d of compound treatment, colonies were washed with PBS and subsequently fixed and stained with 0.3% neutral red (AppliChem)

in 70% EtOH for 15 min. Excess neutral red solution was aspirated, colonies were washed with water and dried at rt. Images were acquired and colonies counted using a GelCount Scanner (Oxford Optronix). Colony counts were normalized to DMSO treated cells with or without irradiation and data were fitted with a nonlinear regression curve to determine EC₅₀ values.

Combination Panel Screen. A combination sensitization screen in a panel of 95 cell lines (comprising 93 cancer lines and two representative noncancer lines) with a fixed dose (300 nM) of olaparib versus ART5537 (run in 6-point dose–response) was performed at Onclead. Cells were seeded at a density ensuring exponential growth for the duration of the experiment, treated for 5 d with compounds, then fixed and stained with sulforhodamine B (SRB). Cell viability was quantified colorimetrically. Drug combination effects were calculated using the Bliss independence method.⁴⁵ Bliss synergy was calculated as the average delta of the observed effect E_{OBS} (i.e., the relative reduction of growth rate compared to untreated controls) and the calculated linear combination of the monotherapy treatment effects ($E1 + 2 = E1 + E2 - E1 E2$) for all concentrations used, according to the following formula.

$$\text{Bliss}_{\text{Score}} = \frac{1}{n} \sum_{i=1}^n E_{1+2_i} - E_{\text{OBS}_i}$$

In this formulation, the resulting Bliss score is a continuous value between −1 and 1 and negative values indicate potential synergism, whereas positive values indicate potential antagonism. Full details of the panel composition and synergy scores are shown in the [Supporting Information](#).

Cell line Generation. HCT116 EXO1 KO cell lines cells were generated at Synthego using CRISPR-Cas9 based gene editing via electroporation of ribonucleoprotein particles. Successful homozygous gene knockout for EXO1 was confirmed by Sanger sequencing and Western blot analysis in two independent cell clones.

Western Blot. Cells were washed in PBS and lysed directly in HGNT buffer (50 mM HEPES, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 25 mM NaF) supplemented with protease and phosphatase inhibitor cocktails (Merck KGaA). The protein concentration of the extracts was quantitated using BCA reagent (Thermo Fisher Scientific) against a bovine serum albumin (BSA) standard curve. Samples were prepared with Laemmli Sample buffer (BioRad) supplemented with β -mercaptoethanol and incubated for 5 min at 95 °C. Lysates (30 μ g) were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on NuPAGE 4–12% Bis-Tris gels (Thermo Fisher Scientific) in MOPS running buffer (Thermo Fisher Scientific). Proteins were subsequently transferred onto PVDF membranes (BioRad) using a wet blotting system (transfer buffer made of 48 mM Tris Base, 39 mM glycine, 20% Methanol, 1.3 mM SDS). Membranes were blocked with Intercept PBS blocking solution (LI-COR) and 0.5x blocking solution +0.01% Tween-20 (PBST) was used for antibody incubation steps. Incubation with primary antibodies (anti-EXO1, Bethyl A302–640A, 1:1000 or anti-GAPDH, Cell Signaling 2118, 1:10 000) was performed overnight at 4 °C. Membranes were washed several times with PBST and incubated with fluorescent dye-conjugated secondary antibodies (Thermo Fisher Scientific) for 1 h at rt. After several PBST washes, fluorescent signals were detected on a LI-COR Odyssey Imager.

AlamarBlue Cell Viability Assay. Cells were seeded in 96-well plates at a density of 100 cells/well and left to adhere overnight under standard culture conditions (37 °C, 5% CO₂). Cells were then treated with a 6-point 3-fold serial dilution of EXO1i and olaparib in a 6 $\times$ 6 combination matrix, plus serial dilutions of each agent alone. Seven days post-treatment, viability was assessed using the AlamarBlue reagent (BioRad), which was added in an amount equal to 10% of the volume in each well and left to stand for 4–6 h. Viability was quantified by fluorescence measurement using a plate reader. Fluorescence was normalized to DMSO control for each cell line and data were fitted with a nonlinear regression curve to determine EC₅₀ values. Combination analysis was performed with Genedata

Screening (Compound Synergy Extension) to calculate Synergy scores (Bliss model), using a formulation whereby the $\text{Bliss}_{\text{excess}}$ is a continuous value and values >2 are typically considered synergistic.

Chromatin Retention Assay. U-2 OS Flp-In TF1 tagGFP2-EXO1 cells were seeded at a density of 4000 cells per well in 100 μL of media in collagen-coated 96-well plates (Revvity, #6055700). 16- to 24 h after seeding, compounds were dispensed using a D300e digital dispenser (Tecan) to generate a 9-point 3-fold serial dilution, equalized to final DMSO concentration. After 24 h, cells were washed with PBS and pre-extracted with 50 μL of ice-cold cytoskeletal (CSK) buffer (10 mM PIPES pH 6.8, 100 mM NaCl, 300 mM sucrose, 1.5 mM MgCl_2 , 5 mM EDTA, 0.4% Triton X-1), for 3 min on ice. Each plate also contained DMSO treated wells that were not pre-extracted to serve as a control. Cells were then washed with PBS and fixed in 4% paraformaldehyde for 10 min at rt. Cells were washed twice with PBS/0.1% Triton X-100 (PBS-TX) and imaged in PBS that contained 4',6-diamidino-2-phenylindole (DAPI 1:10,000) to counterstain DNA using confocal microscopy (Operetta CLS, Revvity) using a long working distance 20 $\times$ objective, capturing 6 fields of view per well with 5 Z-slices and a Z-step of 1.0 μm .

ADME/DMPK Assays and Profiling. All in vitro ADME/DMPK studies were performed at WuXi AppTec or Pharmaron, using standard procedures and assays. Mouse PK studies were performed at Nuvisan GmbH (Berlin), in AAALAC-accredited facilities operating under European Directive 2010/63/EU, German Animal Welfare Act (SOP: 3226).

■ ASSOCIATED CONTENT

Data Availability Statement

X-ray crystallography data was deposited in the Protein Data Bank (PDB) under accession codes: 9SEB (EXO1-DNA-20) and 9SMO (EXO1-DNA-ART5537). Authors will release the atomic coordinates and experimental data upon article publication.

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jmedchem.5c02593>.

Full details for the synthesis and analytical spectra for final compounds, X-ray diffraction data and refinement statistics, extended biochemical and cell assay data and protocols, and SPR data (PDF)

Molecular formula strings (CSV)

■ AUTHOR INFORMATION

Corresponding Author

Sam E. Mann – Artios Pharma Ltd., Cambridge CB22 3FH, U.K.; orcid.org/0009-0007-7333-6196; Email: smann@artios.com

Authors

Owen A. Davis – Artios Pharma Ltd., Cambridge CB22 3FH, U.K.; orcid.org/0000-0002-7053-7079

Jörg Bomke – Merck Healthcare KGaA, 64293 Darmstadt, Germany

Irina Cornaciu – ALPX, 38000 Grenoble, France

Elias Elinati – Artios Pharma Ltd., Cambridge CB22 3FH, U.K.

Bruce Follows – EMD Serono Research & Development Institute, Billerica, Massachusetts 018-21-3936, United States

Alessandro Galbiati – Artios Pharma Ltd., Cambridge CB22 3FH, U.K.

Lerin Geo – Artios Pharma Ltd., Cambridge CB22 3FH, U.K.

Diego Grande – Artios Pharma Ltd., Cambridge CB22 3FH, U.K.; orcid.org/0000-0001-6770-9274

Catherine Jorand-Lebrun – EMD Serono Research & Development Institute, Billerica, Massachusetts 018-21-3936, United States

Claudio A. Lademann – Merck Healthcare KGaA, 64293 Darmstadt, Germany

Julien Lefranc – Merck Healthcare KGaA, 64293 Darmstadt, Germany

Birgitta Leuthner – Merck Healthcare KGaA, 64293 Darmstadt, Germany

Bethany Mason – Artios Pharma Ltd., Cambridge CB22 3FH, U.K.

Claire L. McWhirter – Artios Pharma Ltd., Cambridge CB22 3FH, U.K.; orcid.org/0000-0003-3316-205X

Djordje Musil – Merck Healthcare KGaA, 64293 Darmstadt, Germany

Ulrich Pehl – Merck Healthcare KGaA, 64293 Darmstadt, Germany

Carl Petersson – Merck Healthcare KGaA, 64293 Darmstadt, Germany

Andrea Pica – ALPX, 38000 Grenoble, France

Maria Filipa Pinto – Artios Pharma Ltd., Cambridge CB22 3FH, U.K.

Eeson Rajendra – Artios Pharma Ltd., Cambridge CB22 3FH, U.K.; orcid.org/0000-0002-5301-054X

Christin Rakers – Merck Healthcare KGaA, 64293 Darmstadt, Germany

Ana Toste Rêgo – Artios Pharma Ltd., Cambridge CB22 3FH, U.K.

Helen M. R. Robinson – Artios Pharma Ltd., Cambridge CB22 3FH, U.K.

Daniel Schwarz – Merck Healthcare KGaA, 64293 Darmstadt, Germany

Graeme C. M. Smith – Artios Pharma Ltd., Cambridge CB22 3FH, U.K.

Fiona J. Sorrell – Merck Healthcare KGaA, 64293 Darmstadt, Germany

Frank T. Zenke – Merck Healthcare KGaA, 64293 Darmstadt, Germany

Robert A. Heald – Artios Pharma Ltd., Cambridge CB22 3FH, U.K.; orcid.org/0000-0003-3425-7130

Lars T. Burgdorf – Merck Healthcare KGaA, 64293 Darmstadt, Germany

Complete contact information is available at:

<https://pubs.acs.org/doi/10.1021/acs.jmedchem.5c02593>

Author Contributions

The core Artios-Merck project team, who contributed the vast majority of the work described herein, consisted of J.B., O.D., C.L., C.J.-L., J.L., C.McW., U.P., C.P., E.R., C.R., A.T.R., and F.S. and was jointly led by L.B. and S.M. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. O.D., E.E., L.G., R.H., S.M., B.M., C.McW., A.T.R., H.R., and G.S. are employees of Artios Pharma Ltd.; J.B., L.B., C.L., J.L., B.L., D.M., U.P., C.P., C.R., F.S., and D.S. are employees of Merck Healthcare KGaA; I.C. and A.P. are employees of ALPX; A.G., D.G., M.F.P., and E.R. are former employees of Artios Pharma Ltd.; F.Z. is a former employee of Merck Healthcare KGaA; and B.F. and C.J.-L. are former employees of EMD Serono.

Notes

The authors declare the following competing financial interest(s): O.D., E.E., L.G., R.H., S.M., B.M., C.McW.,

A.T.R., H.R. and G.S. are employees of Artios Pharma Ltd; J.B., L.B., C.L., J.L., B.L., D.M., U.P., C.P., C.R., F.S. and D.S. are employees of Merck Healthcare KGaA; I.C. and A.P. are employees of ALPX.

■ ACKNOWLEDGMENTS

The authors would like to acknowledge colleagues at Pharmaron (Xi'An, China) and o2h (Ahmedabad, India) for assistance with the synthesis of compounds, Proteros Biostructures GmbH (Munich, Germany) for crystallography services, and Peak Proteins (Macclesfield, UK) for protein production services. The authors would specifically like to acknowledge Quamrul Hassan (EMD Serono, Billerica, MA) for supporting NanoBRET measurements, Yvonne Bischoff (Merck Healthcare KGaA, Germany) for supporting SPR measurements, Michaela Schymalla (Merck Healthcare KGaA, Germany) for supporting cell viability assays, and Roch Boivin, Xiaoling Chen, and Lizbeth DeSelm (EMD Serono, Billerica, MA) for supporting compound synthesis. Finally, the authors would like to extend a special acknowledgment to Lumir Krejčí and Kamil Paruch (Masaryk University) and Calum MacLeod, John Maclean, Kevin Foote, and Mike Rowley (currently and formerly of Pharmaron, UK) for helpful early discussions.

■ ABBREVIATIONS

ATR, ataxia telangiectasia and Rad3-related; d, day(s); BSA, bovine serum albumin; CSK, cytoskeletal buffer; DAPI, 4',6-diamidino-2-phenylindole; DCM, dichloromethane; DDR, DNA damage response; DEA, diethylamine; DFT, density functional theory; DIPEA, N,N-diisopropylethylamine; DMA, dimethylacetamide; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DSB, double strand break; dsDNA, double stranded DNA; EDTA, ethylenediaminetetraacetic acid; equiv., equivalent(s); EtOH, ethanol; EXO1, exonuclease 1; FA, formic acid; FEN1, flap endonuclease 1; GFP, green fluorescent protein; GEN1, flap endonuclease GEN homologue 1; h, hour(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HR, homologous recombination; HRD, homologous recombination deficient; HPLC, high-performance liquid chromatography; IR, ionizing radiation; KO, knockout; LE, ligand efficiency; LLE, lipophilic ligand efficiency; MeOH, methanol; min, minute(s); MMEJ, microhomology-mediated end joining; MMR, mismatch repair; NCS, N-chlorosuccinimide; NER, nucleotide excision repair; NHEJ, nonhomologous end-joining; PARP, poly-ADP ribose polymerase; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; rt, room temperature; RPA, replication protein A; SEM, 2-(trimethylsilyl)ethoxymethyl; SPR, surface plasmon resonance; SRB, sulforhodamine B; SSA, single strand annealing; ssDNA, single stranded DNA; T3P, propanephosphonic acid anhydride; TLS, trans-lesion synthesis; WT, wild type; XPG, DNA excision repair protein ERCC-5

■ REFERENCES

- (1) Keijzers, G.; Bakula, D.; Petr, M. A.; Madsen, N. G. K.; Teklu, A.; Mkrtchyan, G.; Osborne, B.; Scheibye-Knudsen, M. Human Exonuclease 1 (EXO1) Regulatory Functions in DNA Replication with Putative Roles in Cancer. *Int. J. Mol. Sci.* **2019**, *20* (1), 74.
- (2) Tran, P. T.; Erdeniz, N.; Symington, L. S.; Liskay, R. M. EXO1-A Multi-Tasking Eukaryotic Nuclease. *DNA Repair (Amst)* **2004**, *3* (12), 1549–1559.
- (3) Bolderson, E.; Tomimatsu, N.; Richard, D. J.; Boucher, D.; Kumar, R.; Pandita, T. K.; Burma, S.; Khanna, K. K. Phosphorylation of Exo1 Modulates Homologous Recombination Repair of DNA Double-Strand Breaks. *Nucleic Acids Res.* **2010**, *38* (6), 1821–1831.
- (4) Qiu, J.; Qian, Y.; Chen, V.; Guan, M. X.; Shen, B. Human Exonuclease 1 Functionally Complements Its Yeast Homologues in DNA Recombination, RNA Primer Removal, and Mutation Avoidance. *J. Biol. Chem.* **1999**, *274* (25), 17893–17900.
- (5) Nimonkar, A. V.; Genschel, J.; Kinoshita, E.; Polaczek, P.; Campbell, J. L.; Wyman, C.; Modrich, P.; Kowalczykowski, S. C. BLM–DNA2–RPA–MRN and EXO1–BLM–RPA–MRN Constitute Two DNA End Resection Machineries for Human DNA Break Repair. *Genes Dev.* **2011**, *25* (4), 350–362.
- (6) Chen, C.-C.; Avdievich, E.; Zhang, Y.; Zhang, Y.; Wei, K.; Lee, K.; Edelmann, W.; Jasin, M.; LaRocque, J. R. EXO1 Suppresses Double-Strand Break Induced Homologous Recombination between Diverged Sequences in Mammalian Cells. *DNA Repair (Amst)* **2017**, *57*, 98–106.
- (7) Jäger, A. C.; Rasmussen, M.; Bisgaard, H. C.; Singh, K. K.; Nielsen, F. C.; Rasmussen, L. J. HNPCC Mutations in the Human DNA Mismatch Repair Gene HMLH1 Influence Assembly of HMutAlpha and HMLH1-EXO1 Complexes. *Oncogene* **2001**, *20* (27), 3590–3595.
- (8) Schmutte, C.; Sadoff, M. M.; Shim, K. S.; Acharya, S.; Fishel, R. The Interaction of DNA Mismatch Repair Proteins with Human Exonuclease I. *J. Biol. Chem.* **2001**, *276* (35), 33011–33018.
- (9) Giannattasio, M.; Follonier, C.; Tourrière, H.; Puddu, F.; Lazzaro, F.; Pasero, P.; Lopes, M.; Plevani, P.; Muzi-Falconi, M. Exo1 Competes with Repair Synthesis, Converts NER Intermediates to Long SsDNA Gaps, and Promotes Checkpoint Activation. *Mol. Cell* **2010**, *40* (1), 50–62.
- (10) Sertic, S.; Mollica, A.; Campus, I.; Roma, S.; Tumini, E.; Aguilera, A.; Muzi-Falconi, M. Coordinated Activity of Y Family TLS Polymerases and EXO1 Protects Non-S Phase Cells from UV-Induced Cytotoxic Lesions. *Mol. Cell* **2018**, *70* (1), 34.e4–47.e4.
- (11) Sertic, S.; Pizzi, S.; Cloney, R.; Lehmann, A. R.; Marini, F.; Plevani, P.; Muzi-Falconi, M. Human Exonuclease 1 Connects Nucleotide Excision Repair (NER) Processing with Checkpoint Activation in Response to UV Irradiation. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108* (33), 13647–13652.
- (12) Giannattasio, M.; Follonier, C.; Tourrière, H.; Puddu, F.; Lazzaro, F.; Pasero, P.; Lopes, M.; Plevani, P.; Muzi-Falconi, M. Exo1 Competes with Repair Synthesis, Converts NER Intermediates to Long SsDNA Gaps, and Promotes Checkpoint Activation. *Mol. Cell* **2010**, *40* (1), 50–62.
- (13) Lee, B. I.; Wilson, D. M. The RAD2 Domain of Human Exonuclease 1 Exhibits 5' to 3' Exonuclease and Flap Structure-Specific Endonuclease Activities. *J. Biol. Chem.* **1999**, *274* (53), 37763–37769.
- (14) Zou, L.; Elledge, S. J. Sensing DNA Damage through ATRIP Recognition of RPA-SsDNA Complexes. *Science* **2003**, *300* (5625), 1542–1548.
- (15) Shi, Y.; Hellinga, H. W.; Beese, L. S. Interplay of Catalysis, Fidelity, Threading, and Processivity in the Exo- and Endonucleolytic Reactions of Human Exonuclease I. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114* (23), 6010–6015.
- (16) Wei, K.; Clark, A. B.; Wong, E.; Kane, M. F.; Mazur, D. J.; Parris, T.; Kolas, N. K.; Russell, R.; Hou, H.; Kneitz, B.; Yang, G.; Kunkel, T. A.; Kolodner, R. D.; Cohen, P. E.; Edelmann, W. Inactivation of Exonuclease 1 in Mice Results in DNA Mismatch Repair Defects, Increased Cancer Susceptibility, and Male and Female Sterility. *Genes Dev.* **2003**, *17* (5), 603–614.
- (17) Sertic, S.; Quadri, R.; Lazzaro, F.; Muzi-Falconi, M. EXO1: A Tightly Regulated Nuclease. *DNA Repair (Amst)* **2020**, *93*, No. 102929.
- (18) Yu, C.; Wu, G. Deciphering the Multifaceted Role of EXO1 in Female-Related Cancers: Implications for Prognosis and Therapeutic Responsiveness. *Front Immunol* **2025**, *16*, No. 1591505.

- (19) Noordermeer, S. M.; van Attikum, H.; Nussenzweig, A.; van Vugt, M. A. T. M.; Luijsterburg, M. S.; Cuppen, E.; Mei, H.; Boom, J.; Callen, E.; Koerse, D.; Everts, M.; San Martin Alonso, M.; van Hoeck, A.; Wendel, T. J.; Uruci, S.; Garzero, V.; Pavani, R.; Schreuder, A.; van de Kooij, B. EXO1 Protects BRCA1-Deficient Cells against Toxic DNA Lesions. *Mol. Cell* **2024**, *84* (4), 659.e7–674.e7.
- (20) Maric, M.; Segura-Bayona, S.; Kuthethur, R.; Takaki, T.; Borel, V.; Stanage, T. H.; Ivanov, M. P.; Parnandi, N.; Hewitt, G.; Millar, R.; Fonseca, C. S.; Patel, H.; Llorian, M.; Warchal, S.; Howell, M.; Chaudhuri, A. R.; Kotsantis, P.; Boulton, S. J. EXO1 as a Therapeutic Target for Fanconi Anaemia, ZRSR2 and BRCA1-A Complex Deficient Cancers. *Nat. Commun.* **2025**, *16* (1), 8476.
- (21) Exell, J. C.; Thompson, M. J.; Finger, L. D.; Shaw, S. J.; Debreczeni, J.; Ward, T. A.; McWhirter, C.; Siöberg, C. L. B.; Molina, D. M.; Abbott, W. M.; Jones, C. D.; Nissink, J. W. M.; Durant, S. T.; Grasby, J. A. Cellularly Active N-Hydroxyurea FEN1 Inhibitors Block Substrate Entry to the Active Site. *Nat. Chem. Biol.* **2016**, *12*, 815–821.
- (22) Wang, Y.; Hess, J. D.; Wang, C.; Ma, L.; Luo, M.; Jossart, J.; Perry, J. J.; Kwon, D.; Wang, Z.; Pei, X.; Shen, C.; Wang, Y.; Zhou, M.; Yin, H.; Horne, D.; Nussenzweig, A.; Zheng, L.; Shen, B. Discovery and Characterization of Small Molecule Inhibitors Targeting Exonuclease 1 for Homologous Recombination-Deficient Cancer Therapy. *ACS Chem. Biol.* **2025**, *20* (6), 1258–1272.
- (23) Mann, S. E.; Lefranc, J.; Alkhatib, O.; Boivin, R.; Bomke, J.; Byrne, R. T.; Cassona, C. P.; Chen, X.; Cornaciu, I.; Costales, P.; Davis, O. A.; DeSelm, L.; Elinati, E.; Follows, B.; Galbiati, A.; Göldner, C.; Hayre, J. K.; Jorand-Lebrun, C.; Lademann, C. A.; Leuthner, B.; Majithiya, J. B.; Malta, C. F.; Mason, B.; McWhirter, C. L.; Neff, B.; Nissink, J. W. M.; Pehl, U.; Petersson, C.; Pica, A.; Pinto, M. F.; Rajendra, E.; Rakers, C.; Toste Rêgo, A.; Robinson, H. M. R.; Sala-Hojman, A.; Schaedler, T. A.; Schürmann, P. J. L.; Silva, D. O.; Smith, G. C. M.; Sorrell, F.; Webster, G. R.; Zenke, F. T.; Heald, R. A.; Burgdorf, L. T. Fragment-Based Discovery and Structure-Led Optimization of MSC778, the First Potent, Selective, and Orally Bioavailable FEN1 Inhibitor. *J. Med. Chem.* **2025**, *68*, 20410.
- (24) Rajendra, E.; Lademann, C.; Mason, B.; Mardin, B.; Armstrong, L.; Peripolli, S.; Kreis, J.; Konstantinou, T.; Pehl, U.; Bomke, J.; et al. Mechanistic insights into the monotherapy and combination potential of FEN1 inhibition in cancer therapy. *Nucleic Acids Research* **2025**, <https://doi.org/10.1093/nar/gkaf1279>.
- (25) Rajendra, E.; Grande, D.; Mason, B.; Marcantonio, D.; Di; Armstrong, L.; Hewitt, G.; Elinati, E.; Galbiati, A.; Boulton, S. J.; Heald, R. A.; Smith, G. C. M.; Robinson, H. M. R. Quantitative, Titratable and High-Throughput Reporter Assays to Measure DNA Double Strand Break Repair Activity in Cells. *Nucleic Acids Res.* **2024**, *52* (4), 1736–1752.
- (26) Miller, R. R.; Madeira, M.; Wood, H. B.; Geissler, W. M.; Raab, C. E.; Martin, I. J. Integrating the Impact of Lipophilicity on Potency and Pharmacokinetic Parameters Enables the Use of Diverse Chemical Space during Small Molecule Drug Optimization. *J. Med. Chem.* **2020**, *63* (21), 12156–12170.
- (27) Casareno, R. L. B.; Cowan, J. A. Magnesium Ns. Manganese Cofactors for Metallonuclease Enzymes. A Critical Evaluation of Thermodynamic Binding Parameters and Stoichiometry. *Chem. Commun.* **1996**, *15*, 1813–1814.
- (28) Goedken, E. R.; Marqusee, S. Co-Crystal of Escherichia Coli RNase HII with Mn²⁺ Ions Reveals Two Divalent Metals Bound in the Active Site. *J. Biol. Chem.* **2001**, *276* (10), 7266–7271.
- (29) Chen, A. Y.; Adamek, R. N.; Dick, B. L.; Credille, C. V.; Morrison, C. N.; Cohen, S. M. Targeting Metalloenzymes for Therapeutic Intervention. *Chem. Rev.* **2019**, *119*, 1323–1455.
- (30) Murai, J.; Huang, S. N.; Das, B. B.; Renaud, A.; Zhang, Y.; Doroshow, J. H.; Ji, J.; Takeda, S.; Pommier, Y. Trapping of PARP1 and PARP2 by Clinical PARP Inhibitors. *Cancer Res.* **2012**, *72* (21), 5588–5599.
- (31) Kötter, A.; Cornils, K.; Borgmann, K.; Dahm-Daphi, J.; Petersen, C.; Dikomey, E.; Mansour, W. Y. Inhibition of PARP1-dependent End-joining Contributes to Olaparib-mediated Radio-sensitization in Tumor Cells. *Mol. Oncol.* **2014**, *8* (8), 1616–1625.
- (32) Pommier, Y.; O'Connor, M. J.; de Bono, J. Laying a Trap to Kill Cancer Cells: PARP Inhibitors and Their Mechanisms of Action. *Sci. Transl. Med.* **2016**, *8* (362), 362ps17.
- (33) Horton, J. K.; Stefanick, D. F.; Naron, J. M.; Kedar, P. S.; Wilson, S. H. Poly(ADP-Ribose) Polymerase Activity Prevents Signaling Pathways for Cell Cycle Arrest after DNA Methylating Agent Exposure. *J. Biol. Chem.* **2005**, *280* (16), 15773–15785.
- (34) Heacock, M. L.; Stefanick, D. F.; Horton, J. K.; Wilson, S. H. Alkylation DNA Damage in Combination with PARP Inhibition Results in Formation of S-Phase-Dependent Double-Strand Breaks. *DNA Repair (Amst)* **2010**, *9* (8), 929–936.
- (35) Helleday, T. The Underlying Mechanism for the PARP and BRCA Synthetic Lethality: Clearing up the Misunderstandings. *Mol. Oncol.* **2011**, *5* (4), 387–393.
- (36) Kedar, P. S.; Stefanick, D. F.; Horton, J. K.; Wilson, S. H. Increased PARP-1 Association with DNA in Alkylation Damaged. PARP-Inhibited Mouse Fibroblasts. *Mol. Cancer Res.* **2012**, *10* (3), 360–368.
- (37) Ciccica, A.; Elledge, S. J. The DNA Damage Response: Making It Safe to Play with Knives. *Mol. Cell* **2010**, *40* (2), 179–204.
- (38) Chapman, J. R.; Taylor, M. R. G.; Boulton, S. J. Playing the End Game: DNA Double-Strand Break Repair Pathway Choice. *Mol. Cell* **2012**, *47* (4), 497–510.
- (39) O'Connor, M. J. Targeting the DNA Damage Response in Cancer. *Mol. Cell* **2015**, *60* (4), 547–560.
- (40) Zhang, H.; Kreis, J.; Schelhorn, S.-E.; Dahmen, H.; Grombacher, T.; Zühlendorf, M.; Zenke, F. T.; Guan, Y. Mapping Combinatorial Drug Effects to DNA Damage Response Kinase Inhibitors. *Nat. Commun.* **2023**, *14* (1), 8310.
- (41) Li, Q.; Qian, W.; Zhang, Y.; Hu, L.; Chen, S.; Xia, Y. A New Wave of Innovations within the DNA Damage Response. *Signal Transduct Target Ther* **2023**, *8* (1), 338.
- (42) Orans, J.; McSweeney, E. A.; Iyer, R. R.; Hast, M. A.; Hellinga, H. W.; Modrich, P.; Beese, L. S. Structures of Human Exonuclease 1 DNA Complexes Suggest a Unified Mechanism for Nuclease Family. *Cell* **2011**, *145* (2), 212–223.
- (43) Zander, U.; Hoffmann, G.; Cornaciu, I.; Marquette, J. P.; Papp, G.; Landret, C.; Seroul, G.; Sinoir, J.; Röwer, M.; Felisaz, F.; Rodriguez-Puente, S.; Mariaule, V.; Murphy, P.; Mathieu, M.; Cipriani, F.; Márquez, J. A. Automated Harvesting and Processing of Protein Crystals through Laser Photoablation. *Acta Crystallogr. D Struct Biol.* **2016**, *72* (4), 454–466.
- (44) Nieman, A. N.; Dunn Hoffman, K. K.; Dominguez, E. R.; Wilkinson, J.; Vasta, J. D.; Robers, M. B.; Lam, N. NanoBRET™ Live-Cell Kinase Selectivity Profiling Adapted for High-Throughput Screening. *Methods Mol. Biol.* **2023**, *2706*, 97–124.
- (45) BLISS, C. I. THE TOXICITY OF POISONS APPLIED JOINTLY. *Annals of Applied Biology* **1939**, *26* (3), 585–615.