

**Title:**

**Adjuvant treatment for *POLE* proofreading domain-mutant cancers: sensitivity to radiotherapy, chemotherapy, and nucleoside analogs**

**Running title:**

**Adjuvant treatment for *POLE*-mutant cancers**

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## Translational relevance

*POLE* proofreading domain-mutant cancers are known for their exceptionally high mutational burden and their excellent prognosis. Here we show that this prognosis cannot be explained by increased sensitivity to adjuvant therapy: this warrants studies exploring omission of adjuvant therapy for early-stage *POLE*-mutant cancer, including endometrial and colorectal cancers, which could decrease overtreatment. Moreover, we found that *POLE* mutations confer increased sensitivity to nucleoside analogs cytarabine and fludarabine. As the optimal therapy for advanced-stage or recurrent *POLE*-mutant cancers is unknown, the possible use of these compounds as targeted treatment option for this patient group, potentially as alternative to or in combination with immune checkpoint inhibition, should be subject of future research. As the prognostic value and therapeutic implications of *POLE* mutations seem generalizable across cancer types, the results of this study support further individualization of treatment for a wide variety of malignancies.

## Abstract

**Purpose:** Pathogenic *POLE* proofreading domain mutations are found in many malignancies where they are associated with ultramutation and favorable prognosis. The extent to which this prognosis depends on their sensitivity to adjuvant treatment is unknown, as is the optimal therapy for advanced-staged or recurrent *POLE*-mutant cancers.

**Experimental design:** We examined the recurrence-free survival of women with *POLE*-mutant and *POLE*-wild-type endometrial cancers (ECs) in the observation arm of the randomized PORTEC-1 EC trial (N=245 patients with stage I EC for analysis). Sensitivity to radiotherapy and selected chemotherapeutics was compared between *Pole*-mutant mouse embryonic stem (mES) cells, generated using CRISPR-Cas9 (*Pole* mutations D275A/E275A, and cancer-associated P286R, S297F, V411L) and isogenic wild-type cell lines.

**Results:** In the observation arm of the PORTEC-1 trial (N=245), women with *POLE*-mutant ECs (N=16) had an improved recurrence-free survival (10yr RFS 100% vs 80.1% for *POLE*-wild-type; HR=0.143, 95% CI=0.001-0.996,  $P=0.049$ ). *Pole* mutations did not increase sensitivity to radiotherapy nor to chemotherapeutics in mES cells. In contrast, *Pole*-mutant cells displayed significantly increased sensitivity to cytarabine and fludarabine (IC<sub>50</sub> *Pole* P286R-mutant vs wild-type: 0.05 vs 0.17  $\mu$ M for cytarabine, 4.62 vs 11.1  $\mu$ M for fludarabine;  $P<0.001$  for both comparisons).

**Conclusions:** The favorable prognosis of *POLE*-mutant cancers cannot be explained by increased sensitivity to currently used adjuvant treatments. These results support studies exploring minimization of adjuvant therapy for early-stage *POLE*-mutant cancers, including endometrial and colorectal cancers. Conversely, *POLE* mutations result in hypersensitivity to nucleoside analogs, suggesting the use of these compounds as a potentially effective targeted treatment for advanced-stage *POLE*-mutant cancers.

## Introduction

Somatic mutations in the proofreading (exonuclease) domain of DNA polymerase  $\epsilon$  (encoded by the *POLE* gene) are found in a wide variety of human malignancies, including 7-12% of endometrial cancers, ~1% of colorectal cancers and sporadically in cancers of the brain, stomach, breast, and pancreas (1-4). During DNA replication, the proofreading activity of polymerase  $\epsilon$  recognizes and excises misincorporated nucleotides, thereby increasing replication fidelity. Pathogenic *POLE* proofreading domain mutations (hereafter referred to as '*POLE* mutations') are associated with an exceptionally high number of single nucleotide variants: cancers with such *POLE* mutations often harbor more than 100 mutations/Mb (2,5). Despite this so-called 'ultramutated' phenotype, patients with *POLE*-mutant cancers have a favorable prognosis. This has been shown in early-stage endometrial cancer, where impact was particularly strong in high-grade endometrioid tumors, stage II/III colorectal cancer, and was suggested for glioblastomas (1,4,6-12). The majority of patients in these studies received additional treatment after surgery. Therefore, we evaluated in an endometrial cancer series and in a cell-based model whether the favorable prognosis of *POLE*-mutant cancers might be caused by increased sensitivity to adjuvant treatment. *POLE* mutations can also be found in advanced-stage disease, where the prognostic impact remains uncertain, and in cancer types with poor prognosis such as glioblastomas. Since these *POLE* mutations reside in the proofreading domain that excises non-matched nucleotides, we investigated the efficacy of nucleoside analogs as potential targeted therapies for advanced-stage *POLE*-mutant cancers.

## Materials and Methods

### *Endometrial cancer patient cohort*

Clinical data of women with endometrial cancer (EC) who did not receive adjuvant treatment (N=369) were collected from the randomized PORTEC-1 trial, which compared external beam radiotherapy (RT) to observation in stage I EC (13). *POLE* mutational status was determined as described previously (7) with the addition of screening *POLE* exon 14 using Sanger sequencing (primers available upon request). For 119 ECs, *POLE* mutational status could not be determined, because screening of exons 9, 13 and 14 failed or because no material was available. Twenty-three cases were designated '*POLE*-wild-type' despite failure of exon 14 sequencing, based on screening of *POLE* exons 9 and 13. Women with ECs harboring a *POLE* mutation but also another relevant molecular alteration (i.e. p53 mutation or mismatch repair deficiency, based on previous analyses (14)) were excluded (*POLE*-mutant & p53-mutant, n=3; *POLE*-mutant, p53-mutant, mismatch repair-deficient, n=2), in accordance with our previous publication (14). This resulted in 245 EC patients for analysis. Details on the specific *POLE* mutations identified in the ECs can be found in Supplementary Table S1.

### *Generation of the cell-based model*

Different *POLE* proofreading domain mutations (P286R, S297F, V411L, and D275A/E277A) were recreated in wild-type mouse-derived embryonic stem (mES) cell lines using CRISPR-Cas9. All cell lines in this study were derived from wild-type primary diploid 129/OLA mouse-derived embryonic stem cell line E14 (15). Cell lines were cultured for three to six weeks from thawing to use in experiments. Cell culture was tested every other month for mycoplasma contamination using MycoAlert Mycoplasma Detection Kit (LT07-318; Lonza, Switzerland).

In order to make these mES cells *Pole*-mutant, a CRISPR guide RNA was designed for each mutation using the web resources available at <http://crispr.mit.edu>. Pairs of these oligonucleotides (Supplementary Table S2) were ordered from Integrated DNA Technologies (Leuven, Belgium), annealed and, after BbsI digestion (New England Biolabs), cloned into a modified expression plasmid pX330 (Addgene #42230; now containing the CRISPR-Cas9 system and a puromycin-resistance marker). Plasmids were isolated using an endotoxin-free Midiprep kit (Qiagen), and gRNA inserts were verified by Sanger sequencing. As template for homologous recombination, PCR fragments (~2.4 kbp) containing the specific *Pole* proofreading domain mutations as well as silent mutations in the PAM sequences were generated as described previously (oligonucleotides in Supplementary Table S3) (16). Introduction of the specific substitutions was confirmed by Sanger sequencing.

Wild-type mES cells were transfected with the mutation-specific Cas9- and CRISPR guideRNA-expressing plasmid and PCR-based repair template using Lipofectamine 2000 (#11668019, Thermo Fisher Scientific). For this transfection, the manufacturer's protocol was followed with minor alterations: use of knock-out DMEM, more Lipofectamine for each transfection (14.4 µl in 125 µl medium), a higher DNA input (3-4 µg), and a 20 minute incubation of diluted DNA in Lipofectamine at room temperature. Puromycin (1.5 µg/ml for two days; A11138-03, Thermo Fisher Scientific) was added to the medium for positive selection of transfected cells. Drug-resistant cells were seeded at a low density. Independent clones were manually picked, expanded and reseeded as single cells. Isolated clones were again manually picked to ensure purity of the resultant cell line. To identify targeted clones, DNA and RNA were isolated from drug-resistant clones and regions were amplified by PCR (oligonucleotides sequences in Supplementary Table S4). *Pole* proofreading domain mutations were designed to yield a restriction site; therefore, PCR products amplified from genomic DNA were subjected to restriction site digestion to verify knock-in (BtgZI for D275A/E277A, BstUI for P286R, MboII for S297F, and AclI for V411L; all New England Biolabs). Amplified genomic DNA and

cDNA products of clones with an introduced restriction site were purified, and mutations were confirmed by Sanger sequencing analysis.

For each *Pole*-mutant mES cell line, metaphase spreads were made, and fluorescence in situ hybridization was performed using biotin-16-dUTP-labelled or digoxigenin-11-dUTP-labeled (#11093070910 and #11093088910 respectively, Roche) bacterial artificial chromosome clones RP24-154A19 and RP24-388K6 (BACPAC Resource Center, Chori). Chromosome numbers were equal for all cell lines used in subsequent experiments, and all cell lines contained two copies of the *Pole* allele (Supplementary Figure S1). Spontaneous mutant frequencies of the *Pole*-mutant mES cell lines were determined at the Hypoxanthine Phosphorybosyl Transferase (*Hprt*) gene as described previously (17). Additional details on these experiments can be found in the Supplementary Methods.

#### *Treatment sensitivity of mES cells*

*Pole* proofreading domain-mutant and wild-type mES cells were treated with 5-fluorouracil (F6627, Sigma Aldrich), methotrexate (S1210, Selleckchem), paclitaxel (S1150, Selleckchem), cytarabine (S1648, Selleckchem), gemcitabine (S1714, Selleckchem), fludarabine (S1229, Selleckchem), cladribine (S1199, Selleckchem), clofarabine (S1218, Selleckchem), doxorubicin, cisplatin, or etoposide. To determine the sensitivity to these compounds, mES cells were seeded in 96-well tissue culture plates (5000 cells/well). The following day, cells were treated with increasing concentrations of each compound and incubated for 24-72 hours (24 hours: cytarabine, gemcitabine, clofarabine, paclitaxel, doxorubicin; 48 hours: cisplatin, cladribine, fludarabine, methotrexate, etoposide; 72 hours: 5-fluorouracil). Seventy-two hours after treatment was initiated, cells were detached with 0.05% trypsin-EDTA and resuspended in PBS containing 2% FCS. Adherent cells were quantified using flow cytometry. Sensitivity to ionizing radiation was determined similarly: mES cells were seeded in 6-well tissue culture plates (50.000 cells/well). The following day, cells were irradiated with a single



dose of up to 8 Gy. Seventy-two hours after irradiation, cells were detached with 0.05% trypsin-EDTA and manually counted. In each experiment, for every cell line, cell counts were normalized to counts of vehicle-treated cells. At least three independent experiments were conducted per cell line for each treatment.

### *Statistical analyses*

For PORTEC-1 participants in the observation arm, recurrence-free survival (RFS) was calculated, defined as the time from randomization to recurrence, with censoring at date of last contact or death in patients without recurrence. RFS was evaluated using the Kaplan-Meier method and compared between *POLE*-mutant and *POLE*-wild-type ECs using a Cox proportional hazards model with Firth's correction, owing to the absence of events in the *POLE*-mutant group (18). To analyze treatment sensitivity in the cell-based model, IC50 values of mES cell lines were calculated for each experiment by non-linear regression analysis (variable slope) of the normalized cell counts. Outliers in IC50 values were identified with Grubbs test for each cell line. For each *Pole*-mutant cell line, IC50s were compared to IC50s of the isogenic wild-type cell line using independent-samples t-tests (with Holm-Bonferroni correction when indicated). Levene's test for equality of variances was used to determine when to assume equal variances. All statistical tests were two-sided, and a *P* value of less than 0.05 was considered statistically significant; this was adjusted downward in case of Holm-Bonferroni correction. All analyses were performed in R (3.3.0 version, package 'coxphf'), Graphpad Prism 7 and SPSS 23.0 software.

## Results

### *Prognosis of patients with POLE-mutant ECs who did not receive adjuvant treatment*

The prognostic significance of *POLE* mutations was evaluated in the observation arm of the randomized PORTEC-1 trial, which included 245 stage I ECs for analysis (Figure 1) (7,13,14). None of the women with *POLE*-mutant ECs (0/16) developed a recurrence, compared to 44 recurrences (19.2%) in 229 patients with *POLE*-wild-type tumors; this difference in RFS was statistically significant (10-year RFS 100% vs 80.1%; HR=0.143, 95% CI=0.001 to 0.996,  $P=0.049$  based on Cox regression with Firth's correction). These results suggest a favorable prognosis of early-stage *POLE*-mutant cancers in the absence of adjuvant treatment, indicating that the increased sensitivity to adjuvant treatment does not explain the good clinical outcome of these tumors.

### *Treatment sensitivity conferred by POLE mutations in a model system*

To support the PORTEC-1 data, we investigated the effect of *POLE* mutations on treatment sensitivity in a model system. To this end, we recreated three common somatic, cancer-associated *POLE* proofreading domain mutations (P286R, S297F, and V411L) (2), as well as a proofreading-deficient positive control (double mutation of exonuclease active sites, D275A/E277A) (19) in isogenic mouse-derived embryonic stem (mES) cell lines using CRISPR-Cas9. For each *Pole* mutation, a minimum of two independent homozygous cell lines was used for subsequent experiments. Consistent with the mutational burden in *POLE*-mutant cancers, these *Pole* mutations gave rise to a mutator phenotype in mES cells with the severity of the phenotype depending on the substitution (P286R ~8x higher than wild-type, D275A/E277A, S297F, V411L ~4x; Supplementary Figure S2).

Sensitivity to adjuvant treatments used for cancer types that harbor *POLE* mutations was determined in the mES cells. *Pole* mutations did not result in increased sensitivity to ionizing

radiation or to selected chemotherapeutic agents (i.e. 5-fluorouracil, cisplatin, paclitaxel, doxorubicin, etoposide and methotrexate; Table 1, Figures 2-3, Supplementary Table S5).

Based on the role of the polymerase  $\epsilon$  proofreading domain in the recognition and excision of mispaired nucleotides, we investigated nucleoside analogs as potential novel targeted therapies for *POLE*-mutant cancers. Indeed, *Pole* mutations resulted in increased sensitivity to cytarabine and fludarabine: sensitivity to both compounds was significantly increased for the *Pole* D275A/E277A-mutant cell lines, but was even more pronounced in cells with somatic *Pole* hotspot mutations (Figure 4, Table 1, Supplementary Tables S5&S6). Because somatic *POLE* mutations in cancers are heterozygous, sensitivity to cytarabine and fludarabine was also assessed in a heterozygous *Pole* S297F-mutant cell line. This resulted in sensitivity lower than in the homozygous *Pole*-mutant lines, but higher than in the wild-type, reaching significance for cytarabine (mean IC<sub>50</sub> 0.14 $\mu$ M vs 0.17 $\mu$ M;  $P=0.025$  for cytarabine; mean IC<sub>50</sub> 7.58 $\mu$ M vs 11.1 $\mu$ M;  $P=0.169$  for fludarabine; Supplementary Figure S3).

## Discussion

In this study, we present two independent lines of evidence to show that the prognostic benefit of *POLE* proofreading domain mutations is independent of adjuvant treatment. First, we provide clinical data from the PORTEC-1 EC trial to show that the favorable outcome associated with *POLE* mutations is also observed in women who did not receive adjuvant therapy. Second, in an *in vitro* model we demonstrate that *POLE* mutations confer increased sensitivity neither to ionizing radiation, nor to commonly used chemotherapeutics. We also show that *Pole* mutations result in significantly increased sensitivity to specific nucleoside analogs currently used to treat hematological malignancies.

This study is limited by the composition of the endometrial cancer cohort: the relatively favorable outcome of stage I disease made it more challenging to confirm the prognostic effect of *POLE* mutations. Its composition also precluded the joined examination of *POLE* mutational status and other prognostically relevant, clinical factors: this should be addressed in future research. Because *POLE*-mutant cancers are characterized by an antitumor immune response (20-22), it should be noted that in the cell-based model, an interaction between the immune system and radiotherapy or chemotherapy could not be assessed. However, the results of the endometrial cancer cohort and the *in vitro* findings are consistent regarding the prognosis of *POLE*-mutant tumors and its independence of adjuvant therapy. These results are supported by previous studies on endometrial and colorectal cancer. McConechy *et al.* observed no EC-related deaths or evidence of recurrent or progressive disease among 14 patients with *POLE*-mutant cancers who did not receive adjuvant treatment (9). Moreover, among 23 patients with stage II *POLE*-mutant colorectal cancers, no recurrences were found irrespective of adjuvant chemotherapy (4). Our *in vitro* findings are in line with another preclinical study in which no significant difference in sensitivity to paclitaxel was found between *POLE*-mutant and *POLE*-wild-type endometrial cancer cell lines (22). This study reported increased

resistance to carboplatin in the *POLE*-mutant cell lines. However, the cell lines used in this study were, contrary to those in our study, non-isogenic, and one carried a *POLE* polymerase domain variant of unknown pathogenicity. Finally, considering the mechanisms of action of the commonly used anti-cancer treatments and the presumed lack of a role herein of DNA polymerase epsilon's proofreading activity, a *POLE* mutation is unlikely to alter sensitivity to these therapies.

We are the first to study cancer-associated *POLE* proofreading domain mutations in mammalian cells. Remarkably, sensitivity to nucleoside analogs cytarabine and fludarabine was much greater in the cancer-associated *Pole* mutants than in the *Pole* proofreading-deficient cell lines (23), supporting the presence of defects besides proofreading alone (2,5). In contrast, these *POLE* mutations resulted in only a four- to eight-fold increase in the spontaneous mutation frequency at the *Hprt* gene, which seems low compared with the ultramutated phenotype found in *POLE*-mutant cancers (2,5). Our mutation data is supported by forward mutation assays with mammalian proteins (5). The mutation frequencies in this study were, however, lower than those previously observed in yeast (24), which may be due to different mechanisms of mutability between yeast and mammals. Future studies are needed to unravel the mechanisms behind the ultramutated phenotype of *POLE*-mutant cancers.

In conclusion, these findings suggest that the favorable prognosis of *POLE*-mutant cancers cannot be explained by increased sensitivity to adjuvant therapy but could be due to increased immunogenicity as described in previous studies or to the accumulation of deleterious mutations ('error catastrophe') (20-22,25). These results support clinical trials such as the PORTEC-4A trial, which prospectively evaluates observation alone for early-stage *POLE*-mutant cancers; this may provide an opportunity to decrease overtreatment and may spare these patients unnecessary side effects (26). In patients with advanced-stage or aggressive tumors, *POLE* mutations identify candidates for targeted therapies. Recent results with immune checkpoint inhibitors for *POLE*-mutant cancers are promising (27,28). This study proposes nucleoside analogs cytarabine and fludarabine as targeted

treatment option for *POLE*-mutant cancers, potentially as an alternative to or in combination with immune checkpoint inhibition, which should be the subject of future research. As the prognostic value and therapeutic implications of *POLE* mutations seem generalizable across cancer types, the results of this study support further individualization of treatment for a wide variety of malignancies.

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## Figure Legends

### **Figure 1. Recurrence-free survival of endometrial cancer patients in the observation arm of the PORTEC-1 study according to *POLE* mutational status.**

Among endometrial cancer patients who did not receive adjuvant treatment, none of the women with *POLE*-mutant endometrial cancers developed a recurrence (0/16, 10-years RFS 100%), as compared to 44 recurrences in 229 women with *POLE*-wild-type tumors (19.2%, 10-year RFS 80.1%). This difference in recurrence-free survival was statistically significant (HR=0.143, 95% CI=0.001 to 0.996,  $P=0.049$ ).

### **Figure 2. Sensitivity to ionizing radiation in *Pole*-mutant and *Pole*-wild-type cell lines.**

Non-linear regression of relative survival after ionizing radiation is shown for homozygous *Pole*-mutant and *Pole*-wild-type mouse embryonic stem cells. The *Pole* D275A/E277A double mutation results in proofreading deficiency (19). The *Pole* P286R, S297F, and V411L mutations are somatic *Pole* hotspot mutations (2). As a radiosensitive positive control cell line, a DNA ligase IV knock-out mouse embryonic stem cell line (DNA ligase IV  $\Delta/\Delta$ ) was used. Sensitivity to ionizing radiation did not significantly differ between *Pole*-mutant and *Pole*-wild-type cells (for details on these comparisons see also Table 1). Dots and error bars indicate mean and standard error of the mean respectively.

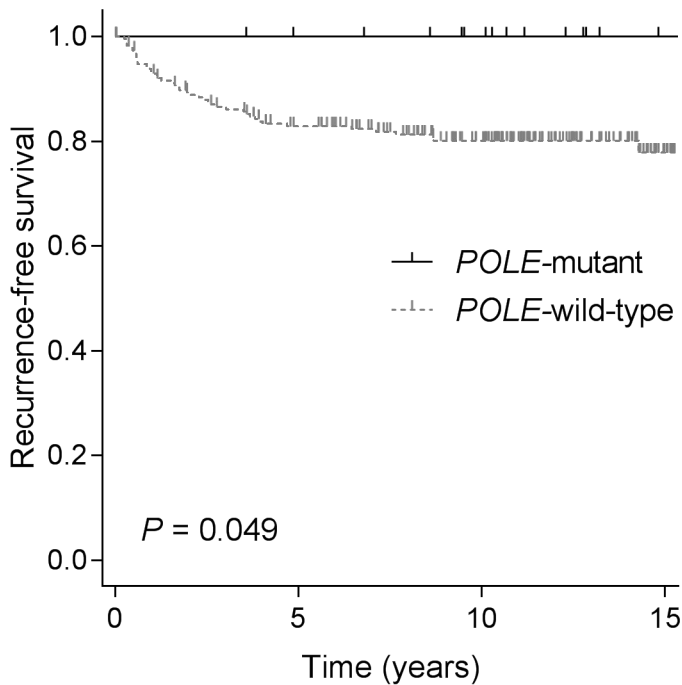
### **Figure 3. Sensitivity to different chemotherapeutic agents in *Pole*-mutant and *Pole*-wild-type cell lines.**

Non-linear regression of relative survival after treatment with chemotherapeutic agents is shown for homozygous *Pole*-mutant and *Pole*-wild-type mouse embryonic stem cells. The *Pole* D275A/E277A double mutation results in proofreading deficiency. The *Pole* P286R, S297F, and V411L mutations are somatic *Pole* hotspot mutations. Sensitivity to 5-fluorouracil, cisplatin, doxorubicin, and etoposide did not significantly differ between *Pole*-mutant and *Pole*-wild-type cells. For methotrexate, a

significant difference was found between IC50s of the *Pole* P286R-mutant cell line and the *Pole*-wild-type cell line. For paclitaxel, a significant difference was found between IC50s of the *Pole* S297F-mutant and the *Pole*-wild-type cell lines. These differences in both methotrexate and paclitaxel did not remain significant after correction for multiple comparisons (for details on these comparisons, see also Table 1). Dots and error bars indicate mean and standard error of the mean, respectively.

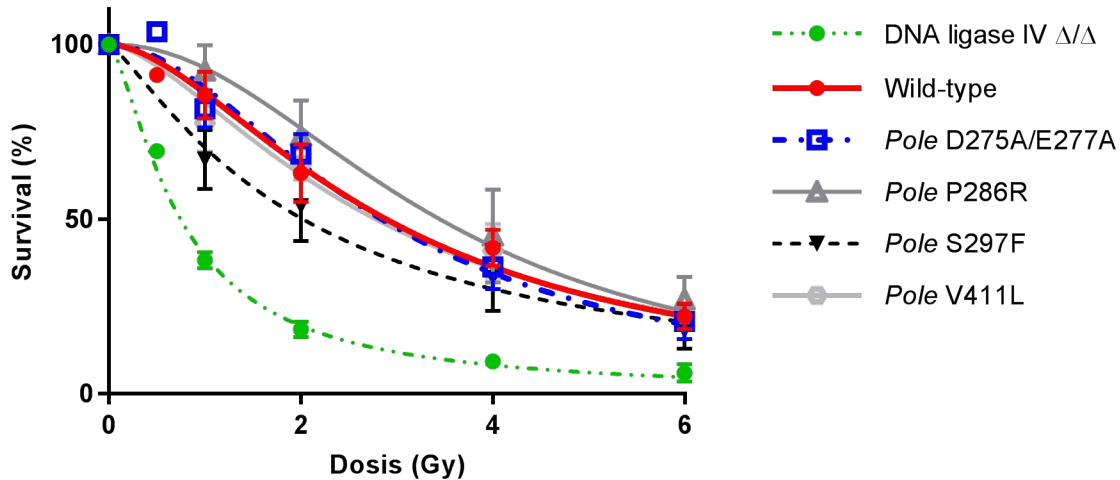
**Figure 4. Cytarabine and fludarabine sensitivity in *Pole*-mutant and *Pole*-wild-type cell lines.**

Non-linear regression of relative survival after nucleoside analog treatment is shown for *Pole*-mutant and *Pole*-wild-type mouse embryonic stem cells. The *Pole* D275A/E277A double mutation results in proofreading deficiency (19). The *Pole* P286R, S297F, and V411L mutations are somatic *Pole* hotspot mutations (2). All homozygous *Pole*-mutant cell lines were significantly more sensitive to cytarabine and fludarabine than the isogenic *Pole*-wild-type cell line (also after multiple comparisons correction, see Table 1). Dots and error bars indicate mean and standard error of the mean, respectively.

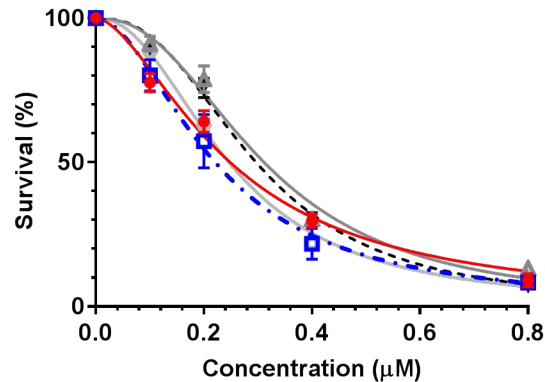


**Number at risk**

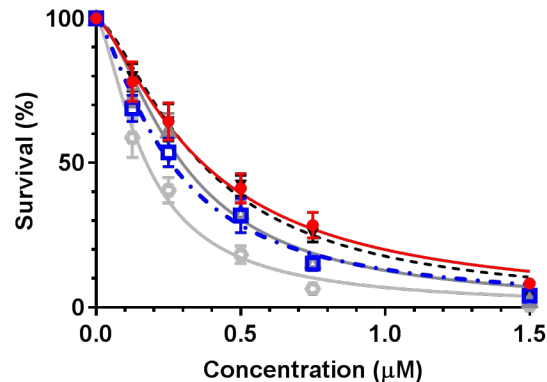
<i>POLE</i> -mutant	16	14	10	1
<i>POLE</i> -wild-type	229	172	122	22



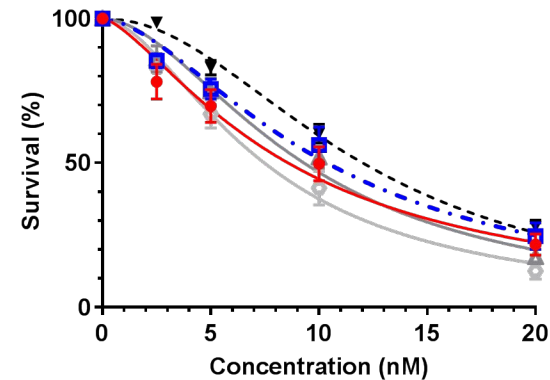
5-Fluorouracil



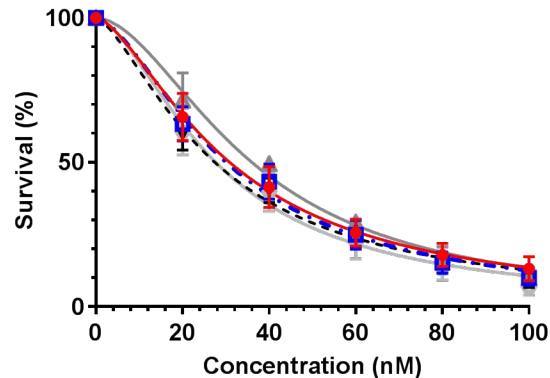
Cisplatin



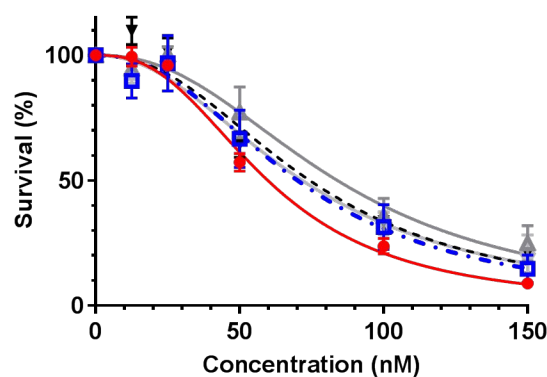
Doxorubicin



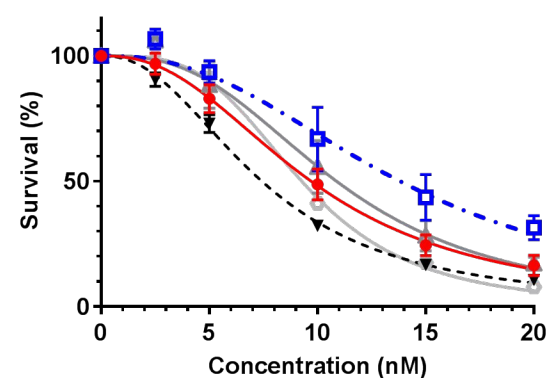
Etoposide



Methotrexate

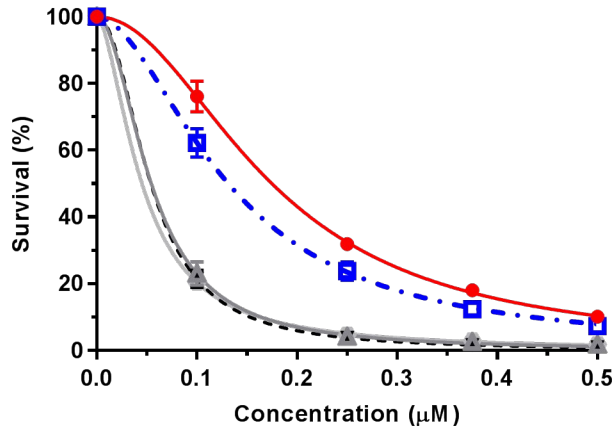


Paclitaxel

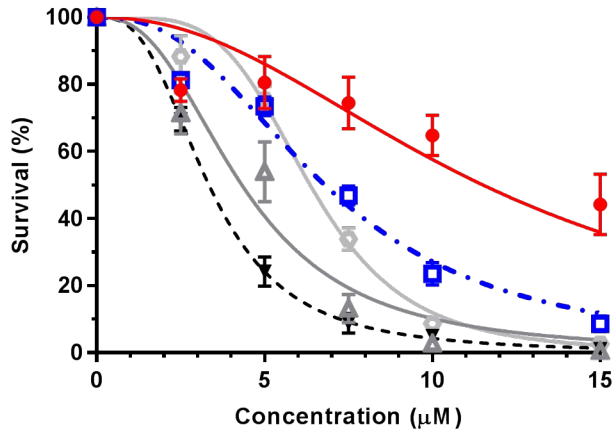


—●— Wild-type    - -■- - *Pole* D275A/E277A    —▲— *Pole* P286R    - -▼- - *Pole* S297F    —◻— *Pole* V411L

## Cytarabine



## Fludarabine



—●— Wild-type  
 -·-■-·- *Pole* D275A/E277A

—▲— *Pole* P286R  
 ---▼--- *Pole* S297F

—◻— *Pole* V411L

**Table 1. Sensitivity to adjuvant treatment strategies conferred by *POLE* proofreading domain mutations in a cell-based model**

Treatment	Wild-type	<i>Pole</i> D275A/E277A <sup>a</sup>		<i>Pole</i> P286R <sup>a</sup>		<i>Pole</i> S297F <sup>a</sup>		<i>Pole</i> V411L <sup>a</sup>	
	IC50 (mean ± SD)	IC50 (mean ± SD)	<i>P</i> (95% CI)	IC50 (mean ± SD)	<i>P</i> (95% CI)	IC50 (mean ± SD)	<i>P</i> (95% CI)	IC50 (mean ± SD)	<i>P</i> (95% CI)
Ionizing radiation (Gy)	2.96 ± 1.28	2.94 ± 1.02	0.976 (-1.63, 1.67)	3.51 ± 1.36	0.553 (-2.53, 1.45)	2.19 ± 1.21	0.390 (-1.16, 2.71)	2.82 ± 0.893	0.866 (-1.70, 1.98)
5-fluorouracil (μM)	0.25 ± 0.04	0.23 ± 0.07	0.513 (-0.06, 0.11)	0.31 ± 0.02	0.057 (-0.12, 0.002)	0.30 ± 0.01	0.109 (-0.11, 0.01)	0.25 ± 0.01	0.895 (-0.06, 0.06)
Cisplatin (μM)	0.39 ± 0.17	0.28 ± 0.09	0.265 (-0.10, 0.32)	0.31 ± 0.07	0.403 (-0.12, 0.28)	0.36 ± 0.09	0.825 (-0.22, 0.27)	0.19 ± 0.06	0.051 (-0.001, 0.40)
Doxorubicin (nM)	8.92 ± 4.04	10.7 ± 2.92	0.468 (-7.01, 3.49)	9.21 ± 0.23	0.909 (-5.85, 5.28)	12.0 ± 1.11	0.237 (-8.76, 2.51)	7.70 ± 1.73	0.586 (-3.66, 6.11)
Etoposide (nM)	27.2 ± 15.7	32.7 ± 7.09	0.515 (-23.0, 12.0)	32.8 ± 7.39	0.513 (-23.1, 12.0)	27.5 ± 7.05	0.971 (-15.9, 15.4)	26.6 ± 10.6	0.937 (-15.6, 16.9)
Methotrexate (nM)	60.1 ± 8.48	70.7 ± 28.1	0.401 (-38.0, 16.9)	85.6 ± 24.6	0.043 (-50.1, -0.97)	74.2 ± 11.1	0.069 (-29.6, 1.46)	75.7 ± 28.2	0.228 (-43.1, 12.0)
Paclitaxel (nM)	9.57 ± 2.94	13.7 ± 3.41	0.084 (-9.04, 0.70)	10.9 ± 3.00	0.490 (-5.53, 2.86)	6.64 ± 0.59	0.039 (0.19, 5.68)	9.14 ± 0.70	0.719 (-2.30, 3.15)
Cytarabine (μM)	0.17 ± 0.02	0.13 ± 0.03	<b>0.027<sup>b</sup></b> (0.006, 0.07)	0.05 ± 0.01	<b>&lt;0.001<sup>b</sup></b> (0.09, 0.15)	0.05 ± 0.01	<b>&lt;0.001<sup>b</sup></b> (0.09, 0.15)	0.04 ± 0.004	<b>&lt;0.001<sup>b</sup></b> (0.10, 0.16)
Fludarabine (μM)	11.1 ± 3.98	6.82 ± 0.69	<b>0.012<sup>b</sup></b> (1.23, 7.40)	4.62 ± 1.50	<b>0.001<sup>b</sup></b> (3.35, 9.68)	3.40 ± 0.51	<b>&lt;0.001<sup>b</sup></b> (4.66, 10.8)	6.29 ± 0.43	<b>0.006<sup>b</sup></b> (1.77, 7.92)

<sup>a</sup>Sensitivity to treatment of mouse embryonic stem cell lines with different homozygous *Pole* proofreading domain mutations (D275A/E277A, P286R, S297F, V411L, respectively) is shown. For each *Pole* mutation, mean IC50 and standard deviation (SD) of one cell line are provided (results of additional cell lines in Supplementary Table S5). IC50s of the *Pole*-mutant cell line are compared to IC50s of the *Pole*-wild-type cell line; the corresponding *P*-values and 95% confidence intervals (CI) are shown.

<sup>b</sup>Difference remains significant after Holm-Bonferroni correction for multiple comparisons.