WYDZIAŁ BIOLOGII
i OCHRONY ŚRODOWISKA
Uniwersytet Łódzki

Stacjonarne Studia Doktoranckie
Genetyki Molekularnej, Cytogenetyki i Biofizyki Medycznej

Gabriela Barszczewska-Pietraszek

Polimeraza DNA theta jako nowy cel w spersonalizowanej terapii przeciwnowotworowej, na przykładzie nowotworów mózgu i skóry

Polymerase DNA theta as a new target of personalised anticancer therapy, based on glioblastoma and melanoma

Praca doktorska

wykonana w Katedrze Genetyki Molekularnej
Instytutu Biochemii na Wydziale Biologii
i Ochrony Środowiska UŁ

Promotor:

Prof. dr hab. Tomasz Śliwiński
Katedra Genetyki Molekularnej,
Instytut Biochemii, Wydział Biologii
i Ochrony Środowiska,
Uniwersytet Łódzki

Promotor pomocniczy:

Dr n. biol. Piotr Czarny
Zakład Biochemii Medycznej,
Uniwersytet Medyczny w Łodzi

Łódź, 2024

Podziękowania

Chciałam serdecznie podziękować mojemu Promotorowi Panu ***Profesorowi Tomaszowi Śliwińskiemu*** *oraz Promotorowi Pomocniczemu Panu* ***Doktorowi Piotrowi Czarnemu,*** *bez których praca ta nie mogłaby powstać. Dziękuję za opiekę merytoryczną, okazaną mi życzliwość, wyrozumiałość, za poświęcony czas jak i pomoc w przygotowaniu pracy doktorskiej.*

Pragnę podziękować też wszystkim Pracownikom i Kolegom z Katedry Biochemii Medycznej Uniwersytetu Medycznego w Łodzi, a w szczególności Panu ***Profesorowi Januszowi Szemrajowi*** *za współpracę i życzliwość.*

Dziękuję również wszystkim współpracownikom, koleżankom i kolegom z Katedry Genetyki Molekularnej Uniwersytetu Łódzkiego, a w szczególności Doktor Katarzynie Białek i Doktor Małgorzacie Drzewieckiej za ogromne wsparcie w trakcie całego trwania Doktoratu oraz koleżeńską relację.

Szczególne podziękowania kieruję dla wszystkich bliskich, którzy cierpliwie wspierali mnie w tej wymagającej drodze, a przede wszystkim dla mojej ***Mamy, Siostry*** *oraz* ***Męża****. Dziękuję Wam za nieustanne wsparcie i wiarę we mnie.*

Niniejszym to Wam chciałabym dedykować tę pracę.

Spis treści

1. Źródła finansowania

- Badania przeprowadzone w ramach niniejszej rozprawy doktorskiej zostały sfinansowane ze środków Narodowego Centrum Nauki w ramach grantu OPUS 19 nr UMO-2020/37/B/NZ7/00422: *Polimeraza DNA ϑ jako nowy cel w spersonalizowanej terapii przeciwnowotworowej*, którego kierownikiem był prof. dr hab. Tomasz Śliwiński.

NARODOWE CENTRUM NAUKI

2. Współpraca naukowa

- Pracę zrealizowano we współpracy z zespołem prof. dr hab. n. med. Janusza Szemraja, kierownika Zakładu Biochemii Medycznej, Uniwersytetu Medycznego w Łodzi
- Pracę zrealizowano we współpracy z zespołem prof. dr hab. n. med. Macieja Radka kierownika Kliniki Neurochirurgii i Chirurgii Nerwów Obwodowych, Uniwersytetu Medycznego w Łodzi
- Pracę zrealizowano we współpracy z zespołem prof. dr hab. n. med. Janusza Piekarskiego kierownika Kliniki Chirurgii Onkologicznej, Uniwersytetu Medycznego w Łodzi

- Pracę zrealizowano we współpracy z dr n. przyr. Grażyną Hoser z Zakładu Immunologii Translacyjnej i Eksperymentalnej Intensywnej Terapii, Centrum Medyczne Kształcenia Podyplomowego w Warszawie

- Pracę zrealizowano we współpracy z prof. Tomaszem Skorskim z Department of Microbiology and Immunology and Fels Institute for Cancer Research and Molecular Biology Lewis Katz School of Medicine, Temple University, Philadelphia PA, USA

3. Dorobek naukowy

Prace wchodzące w skład rozprawy doktorskiej:

Praca przeglądowa:

1. **Barszczewska-Pietraszek G**, Drzewiecka M, Czarny P, Skorski T, Śliwiński T. Polθ Inhibition: An Anticancer Therapy for HR-Deficient Tumours. Int J Mol Sci. 2022 Dec 24;24(1):319. doi:10.3390/ijms24010319. **IF: 5.6; punkty MEiN: 140 pkt**

Prace doświadczalne – złożone do czasopisma, nieopublikowane:

1. **Barszczewska-Pietraszek G**., Czarny P., Drzewiecka M., Błaszczyk M., Radek M., Synowiec E., Wigner-Jeziorska P., Sitarek P., Szemraj J., Skorski T., Śliwiński T. Polθ inhibitor (ART558) demonstrates synthetic lethal effect with PARP and RAD52 inhibitors in glioblastoma cells., złożona do czasopisma International Journal of Molecular Sciences, **IF: 5.6; punkty MEiN: 140 pkt**

2. **Barszczewska-Pietraszek G**., Czarny P., Hoser G., Jaśniak D., Drzewiecka M., Zaleśna I., Piekarski J., Toma M., Białek K., Wasilewska D., Skorski T., Śliwiński T., Polθ inhibition with simultaneous treatment with PARP or RAD52 inhibitors induces cyto- and genotoxic effect in melanoma cells and reduces tumor growth of human melanoma xenograft, złożona do czasopisma Molecular Cancer Therapeutics, American Association for Cancer Research, **IF: 5.7; punkty MEiN: 140 pkt**

Pozostały dorobek

1. Drzewiecka M, Jaśniak D, **Barszczewska-Pietraszek G**, Czarny P, Kobrzycka A, Wieczorek M, Radek M, Szemraj J, Skorski T, Śliwiński T. Class I HDAC Inhibition Leads to a Downregulation of FANCD2 and RAD51, and the Eradication of Glioblastoma Cells. *J Pers Med*. 2023 Aug 27;13(9):1315. doi: 10.3390/jpm13091315. PMID: 37763083; PMCID: PMC10532614. **IF: 3.4; punkty MEiN: 70 pkt**

2. Drzewiecka M, Gajos-Michniewicz A, Hoser G, Jaśniak D, **Barszczewska-Pietraszek G**, Sitarek P, Czarny P, Piekarski J, Radek M, Czyż M, Skorski T, Śliwiński T. Histone Deacetylases (HDAC) Inhibitor-Valproic Acid Sensitizes Human Melanoma Cells to Dacarbazine and PARP Inhibitor. *Genes (Basel).* 2023 Jun 20;14(6):1295. doi: 10.3390/genes14061295. PMID: 37372475; PMCID: PMC10298302. **IF: 3.5; punkty MEiN: 100 pkt**
3. Drzewiecka M, **Barszczewska-Pietraszek G**, Czarny P, Skorski T, Śliwiński T. Synthe

   tic Lethality Targeting Polθ. *Genes (Basel).* 2022 Jun 20;13(6):1101. doi: 10.3390/genes13061101., **IF: 4.141; punkty MEiN: 100 pkt**
4. Bialek K, Czarny P, Wigner P, Synowiec E, **Barszczewska G**, Bijak M, Szemraj J, Niemczyk M, Tota-Glowczyk K, Papp M, Sliwinski T., Chronic Mild Stress and Venlafaxine Treatment Were Associated with Altered Expression Level and Methylation Status of New Candidate Inflammatory Genes in PBMCs and Brain Structures of Wistar Rats., *Genes (Basel).* 2021 Apr 29;12(5):667. doi: 10.3390/genes12050667. **IF: 4.096; punkty MEiN: 100 pkt**
5. Czarny P, Białek K, Ziółkowska S, Strycharz J, **Barszczewska G**, Sliwinski T. The Importance of Epigenetics in Diagnostics and Treatment of Major Depressive Disorder. *J Pers Med.* 2021 Mar 1;11(3):167. doi: 10.3390/jpm11030167. **IF: 3.508; punkty MEiN: 70 pkt**
6. Wigner P, Synowiec E, Jóźwiak P, Czarny P, Bijak M, **Barszczewska G**, Białek K, Szemraj J, Gruca P, Papp M, Śliwiński T. The Changes of Expression and Methylation of Genes Involved in Oxidative Stress in Course of Chronic Mild Stress and Antidepressant Therapy with Agomelatine. *Genes (Base*l). 2020 Jun 11;11(6):644. doi: 10.3390/genes11060644. **IF: 3.759; punkty MEiN: 100 pkt**
7. Barguilla I, **Barszczewska G**, Annangi B, Domenech J, Velázquez A, Marcos R, Hernández A. MTH1 is involved in the toxic and carcinogenic long-term effects induced by zinc oxide and cobalt nanoparticles. *Arch Toxicol*. 2020 Jun;94(6):1973-1984. doi: 10.1007/s00204-020-02737-y. **IF: 5.153; punkty MEiN: 140 pkt**
8. Blasiak, J.; **Barszczewska, G**.; Gralewska, P.; Kaarniranta, K. Oxidative stress induces mitochondrial dysfunction and autophagy in ARPE-19 cells. *Acta Ophthalmol*. 2019, 97. **IF: 3.362; punkty MEiN: 140 pkt**

Doniesienia konferencyjne krajowe oraz międzynarodowe

1. **Gabriela Barszczewska-Pietraszek**, Piotr Czarny, Tomasz Śliwiński, prezentacja ustna: „Eradication of patient-derived glioblastoma cells by the combined inhibition of DNA polymerase theta with Poly(ADP-ribose) polymerase or Rad52" podczas VIII edycji ogólnopolskiej konferencji genetycznej Genomica na Uniwersytecie Jagielońskim, 17-19.05.2024
2. **Gabriela Barszczewska-Pietraszek**, Piotr Czarny, Tomasz Śliwiński, prezentacja ustna: „Combined inhibition of DNA polymerase theta and Poly(ADP-ribose) polymerase or Rad52 demonstrate anticancer activity on patient-derived melanoma cells" podczas sesji doktoranckiej 20 międzynarodowej i 62 ogólnopolskiej konferencji Juvenes Pro Medicina 9-12.05.2024
3. **Gabriela Barszczewska-Pietraszek**, Tomasz Śliwiński Prezentacja posterowa „Polimeraza Theta jako nowy cel terapii przeciwnowotworowej", V Ogólnopolska Konferecja Doktorantów Nauk o Życiu BioOpen, Uniwersytet Łódzki, Wydział Biologii i Ochrony Środowiska, 7-8.04.2022 r.
4. Małgorzata Drzewiecka, **Gabriela Barszczewska-Pietraszek**, Tomasz Skorski, Tomasz Śliwiński, prezentacja plakatu podczas National Scientific Conference "Knowledge –Key to Success", 6th edition, 22.01.2022 r.,
5. Katarzyna Białek, **Gabriela Barszczewska**, Piotr Czarny, Szemraj Janusz, Papp Mariusz, Tomasz Śliwiński, prezentacja plakatu podczas Intercollegiate Biotechnology Symposium SYMBIOZA, 21-23.05.2021
6. **Gabriela Barszczewska**, Katarzyna Białek, Piotr Czarny, Małgorzata Drzewiecka, Tomasz Śliwiński, Prezentacja plakatu „Analysis of *OGG1* and *MUTYH* gene expression in two brain regions of rats subjected to chronic mild stress and during escitalopram drug intake" podczas Intercollegiate Biotechnology Symposium SYMBIOZA, 21-23.05.2021 r.
7. **Gabriela Barszczewska**, Katarzyna Białek, Tomasz Śliwiński, prezentacja posterowa „Crucial players in gut-brain interaction" podczas IV edycji konferencji on-line "Nauka Okiem Młodego Naukowca", 6.06.2020 r.

8. **Gabriela Barszczewska**, Tomasz Śliwiński, prezentacja posterowa „Wpływ metabolitów mikrobiomu jelita na rozwój neuropsychiatrycznych schorzeń u człowieka”, V Ogólnopolska Konferecja Doktorantów Nauk o Życiu BioOpen, Uniwersytet Łódzki, Wydział Biologii i Ochrony Środowiska, 30 - 31. 05. 2019 r.

Skrócony opis prowadzonych prac wraz z omówieniem wyników

7. Wstęp

Polimeraza DNA theta (Polθ) jest enzymem uczestniczącym w naprawie DNA, głównie pęknięć dwuniciowych (ang. double-strand breaks, DSBs), w szlaku biorącym swą nazwę od tego właśnie białka – z ang. *polymerase theta-mediated end joining* – TMEJ. Polθ, kodowana przez gen *POLQ*, jest wysoce zachowaną ewolucyjnie polimerazą wśród wyższych Eukariontów należącą do rodziny polimeraz A. Charakteryzuje się wysoką tendencją do generowania błędów podczas replikacji nieuszkodzonej matrycy DNA, z częstotliwością rzędu 10^{-3} [1]. Enzym ten składa się z trzech domen: centralnej, polimerazowej na C-końcu i helikalnej na N-końcu, co zdecydowanie wyróżnia ją na tle innych eukariotycznych polimeraz, gdyż jest jedyną posiadającą domenę helikazy [2].

Jak wspomniano powyżej Polθ jest głównym białkiem szlaku naprawy pęknięć dwuniciowych DNA znanym jako TMEJ. TMEJ można uznać za ścieżkę alternatywną dla naprawy przez łączenie niehomologicznych końców DNA (ang. non-homologous end joining, NHEJ) i umieścić ją obok naprawy poprzez łączenie fragmentów mikrohomologicznych (ang. microhomology-mediated end joining - MMEJ) lub alternatywnej naprawy przez łączenie końców (ang. alternative end-joining - a-EJ), ponieważ dzielą one wymóg posiadania fragmentów mikrohomologicznych [2]–[4]. Jednak, niektóre publikacje wyróżniają TMEJ jako odrębny szlak, obok NHEJ, rekombinacji homologicznej (ang. homologous recombination, HR) i SSA, uznając istnienie szlaku a-EJ bez aktywności Polθ. TMEJ jest determinowany przez kilka czynników, w tym: niezależność od Ku, XRCC4 i LIG4, resekcja końców DNA z 3’ jednoniciowymi nawisami, występowanie regionów mikrohomologicznych o długości przynajmniej kilku nukleotydów oraz obecność Polθ [5]. Szlak ten jest wysoce podatny na błędy ze względu na brak zdolności korektorskiej Polθ oraz wadliwe właściwości samego procesu łączenia końców mikrohomologicznych, co prowadzi do akumulacji mutacji [1]. Z drugiej strony, w komórkach nowotworowych z niedoborem HR, w których Polθ ulega zwykle nadekspresji, TMEJ umożliwia ich przeżycie [6]–[11]. W niektórych badaniach zaobserwowano, że TMEJ jest najbardziej kluczowy, gdy HR i NHEJ nie funkcjonują prawidłowo [10], [12]. Istnieją jednak dowody, że jest on aktywny również w komórkach o prawidłowej funkcjonalności HR i NHEJ [13]. Ponadto

udowodniono, że Polθ może uczestniczyć w innych mechanizmach naprawczych i procesach komórkowych, takich jak naprawa przez wycięcie zasady (BER), naprawa wiązań krzyżowych, naprawa pęknięć DNA związanych z replikacją czy odwrotna transkrypcja i TLS (translesion synthesis) [1], [2], [14], [15].

Wszystkie wymienione aktywności białka Polθ składają się na całościową jego rolę w utrzymywaniu stabilności genomu, a także podkreślają ich złożoność. Z jednej strony Polθ funkcjonuje jako polimeraza TLS, kontynuująca replikację pomimo uszkodzeń DNA, a z drugiej pełni rolę głównego białka szlaku mutagennego TMEJ [16], [17]. Również, komórki po inaktywacji Polθ są bardziej wrażliwe na czynniki chemiczne i promieniowanie indukujące uszkodzenia DNA, co często prowadzi do ich śmierci. Natomiast, mutagenna charakterystyka TMEJ łączy się z udziałem Polθ w procesie nowotworzenia i metastazy [4], [18]–[20]. Co więcej, warto zaznaczyć, że zwiększony poziom ekspresji *POLQ* w tkankach zmienionych nowotworowo, często wiąże się ze złym rokowaniem dla pacjentów [8], [18], [21], [22].

Te wszystkie doniesienia podkreślaj niepodważalne znaczenie polimerazy theta w organizmach żywych i przyczyniają się do zwiększonego zainteresowania Polθ w badaniach, dotyczących przede wszystkim jego struktury i funkcji, a także potencjalnej roli jako celu terapeutycznego. Również, wskazują na potrzebę prowadzenia dalszych badań w tym obszarze [23], [24]. Rozwój i progresja nowotworów są często napędzane przez zmiany w różnych genach (np. mutacje, zwiększenie lub zmniejszenie ekspresji danego genu), które współpracują, aby zapewnić komórkom nowotworowym przewagę wzrostową. Często musi dojść do kilku zmian jednocześnie, aby komórki osiągnęły tę przewagę. W tym kontekście szlaki komórkowe odpowiedzialne za naprawę pęknięć dwuniciowych DNA odgrywają kluczową rolę we wzroście komórek i rozwoju nowotworu. W szczególności komórki nowotworowe z niedoborem naprawy DNA mają selektywną przewagę wzrostu, co prowadzi do niestabilności genetycznej i promowania rozwoju guza. W takiej sytuacji często stają się one zależne od ścieżek alternatywnych, co stanowi ich słaby punkt, a więc może zostać wykorzystane w terapii przeciwnowotworowej[1], [25]. Wiele nowotworów posiada mutacje w kanonicznych szlakach naprawy DNA (NHEJ i HR) i kompensuje to przez aktywację zapasowych szlaków przetrwania. Zahamowanie tych szlaków może prowadzić do selektywnej eliminacji komórek nowotworowych minimalizując skutki uboczne w komórkach prawidłowych. Koncepcja ta leży u podstaw syntetycznej letalnościm (SL), rewolucyjnego podejścia do opracowywania nowych środków przeciwnowotworowych dla precyzyjnej

onkologii[19], [26]. Syntetyczna letalność występuje, gdy jednoczesne upośledzenie dwóch genów powoduje śmierć komórki, podczas gdy zahamowanie któregokolwiek z nich nie jest śmiertelne. Koncepcja ta została z powodzeniem zastosowana w terapii nowotworów, zwłaszcza w przypadku inhibitorów polimerazy poli(ADP-rybozy) (PARP)(PARPi) w nowotworach z mutacjami genu *BRCA*[27]–[29]. Opierając się na tej zasadzie, postawiliśmy hipotezę, że celowanie w Polθ, w połączeniu z hamowaniem PARP1 lub RAD52 wywoła syntetyczną letalność i tym samym zwiększy skuteczność terapeutyczną przeciwko glejakowi i czerniakowi względem inhibicji tylko jednym inhibitorem.

Spersonalizowane terapie celowane są uważane obecnie za jedne z najbardziej zaawansowanych strategii przeciwnowotworowych. Podejście to opiera się na wyborze odpowiedniego celu terapeutycznego, poprzedzonego określeniem konstytucji genetycznej nowotworu za pomocą technik molekularnych. W ramach medycyny precyzyjnej stosuje się inhibitory białek naprawczych DSBs, które indukują śmierć komórki w oparciu o syntetyczną letalność. Od czasu sukcesu inhibitorów PARP w zwalczaniu komórek nowotworowych z mutacją BRCA wzrosło zainteresowanie identyfikacją potencjalnych celów SL, a odkrycie, że nowotwory mogą nabyć w toku terapii oporność na PARPi stworzyło niezaspokojoną jak do tej pory potrzebę poszerzania badań w tej dziedzinie [19], [30]–[32].

8. Cel pracy

Podstawowym założeniem badań było poznanie znaczenia polimerazy DNA theta jako głównego celu terapeutycznego i co za tym idzie przeanalizowanie działania inhibitorów tego białka na pierwotne linie komórkowe wyizolowane z nowotworów mózgu i skóry, *in vitro* oraz na uzyskanych w myszach NSG ksenograftach z ludzkiej linii czerniaka, samodzielnie jak i w połączeniu z inhibitorami białek PARP1 lub RAD52 oraz związkami cytotoksycznymi temozolamidem i dekarbazyną, w zależności od rodzaju tkanki oraz ocena zastosowania tego typu związków jako potencjalnych leków przeciwnowotworowych.

Powyższy cel był realizowany poprzez następujące cele szczegółowe:

1. Wyprowadzanie linii pierwotnych do hodowli *in vitro* z guzów litych od pacjentów.
2. Oznaczenie poziomu ekspresji 28 genów zaangażowanych w szlaki naprawy pęknięć dwuniciowych DNA, tj., HR, NHEJ i TMEJ oraz ich korelacja z ekspresją w komórkach prawidłowych w celu wyznaczenia potencjalnych deficytów naprawy DNA prowadzących do syntetycznej letalności lub podwójnej syntetycznej letalności.
3. Ocenę działania cytotoksycznego na komórki glejaka, czerniaka oraz prawidłowe zastosowanych związków i ich kombinacji za pomocą pomiaru żywotności, apoptozy, proliferacji i rozkładu faz cyklu komórkowego oraz ich efektu genotoksycznego poprzez pomiar poziomu fosforylacji histonu H2AX (marker DSBs)
4. Ocenę zastosowania związków i ich kombinacji na komórki glejaka, czerniaka oraz prawidłowe w terapii skojarzonej z promieniowaniem gamma poprzez pomiar DSBs w neutralnym teście kometowym.
5. Ocenę zahamowania wzrostu guza w uzyskanych w myszach NSG ksenograftach z ludzkiej linii czerniaka pod wpływem stosowania inhibitora Polθ samodzielnie lub w kombinacji z inhibitorami białek PARP1 lub RAD52 i związkami alkilującymi.

9. Materiały i metody badawcze

Materiał badawczy wykorzystany w eksperymentach do niniejszej rozprawy doktorskiej stanowiły linie pierwotne wyprowadzone z fragmentów guzów litych mózgu i skóry - glejaka wielopostaciowego (linia GBM21) oraz czerniaka (linia MLN21), pochodzących od pacjentów Kliniki Neurochirurgii i Chirurgii Nerwów Obwodowych Uniwersyteckiego Szpitala Klinicznego im. Wojskowej Akademii Medycznej - Centralny Szpital Weteranów oraz Kliniki Chirurgii Onkologicznej Wojewódzkiego Wielospecjalistycznego Centrum Onkologii i Traumatologii im. M. Kopernika w Łodzi. Dodatkowo wyprowadzone linie były identyfikowane przy użyciu markerów powierzchniowych - CD133 oraz MCSP (ang. melanoma-associated chondroitin sulfate proteoglycan). Kontrole do eksperymentów stanowiły komercyjnie dostępne linie komórek prawidłowych astrocytów (NHA - ang. Normal Human Astrocytes) i melanocytów (NHEM - ang. Normal Human Epidermal Melanocytes) pozyskane z firmy Lonza. Wszystkie linie komórkowe hodowane były w standardowych warunkach w inkubatorze zawierającym 37°C i 5% CO2 z dedykowanymi, najwyższej jakości mediami hodowlanymi.

Materiał do części badań na modelu zwierzęcym stanowiły ksenografty otrzymane z ludzkiej linii komórek czerniaka MLN21, wszczepionych podskórnie do myszy NOD SCID γ.

Badania uzyskały zgodę Komisji Bioetycznej Uniwersytetu Medycznego w Łodzi (nr RNN/23/22/KE)

Związki użyte w badaniach:

-inhibitory Polθ (Polθi):

- ART558 (MedChem Express) – stosowany *in vitro,*

to pierwszy syntetyczny inhibitor Polθ opisany w literaturze. Jako pierwsi opisali go Zatreanu i wsp. (2021), którzy przeprowadzili badania przesiewowe około 165 000 inhibitorów aktywności polimerazy białka Polθ. Na podstawie tego testu wybrano ART558, małocząsteczkowy inhibitor, z najbardziej odpowiednimi wynikami wartości IC50 (7,9 nM), rozpuszczalności i LogD. Jego mechanizm inhibicji opiera się na celowaniu w domenę

polimerazy Polθ, łączy się z allosterycznym miejscem wiązania podjednostki katalitycznej i zwiększa jej stabilność termiczną w obecności DNA [28].

- RP-6658 (MedChem Express) – stosowany w badaniach na ksenograftach

Pierwszy raz badania na temat tego związku zostały opublikowane przez Bubenik i wsp. (2022). Autorzy odkryli, zsyntetyzowali i scharakteryzowali, za pomocą wielu metod biofizycznych, silny, selektywny i biodostępny doustnie inhibitor domeny polimerazy Polθ, podobnie jak ART558. Związek w testach *in vitro* i *in vivo* na komórkach nowotworowych i mysich modelach ksenograficznych, również z niedoborem HR, daje obiecujące wyniki [33]

-inhibitor PARP1 (PARPi):

- talazoparib (BMN673) (Selleckchem)

to lek przeciwnowotworowy stosowany powszechnie w leczeniu raka piersi i prostaty. Wykazano, że wykazuje on działanie cytotoksyczne na komórki nowotworowe poprzez dwa mechanizmy: hamowanie aktywności katalitycznej PARP i blokadę PARP w miejscu uszkodzenia DNA, tym samym zatrzymując dalszą naprawę DNA i prowadząc do apoptozy i/lub śmierci komórki [34].

-inhibiotry RAD52 (RAD52i):

- L-OH-DOPA (Sigma Aldrich) – stosowany *in vitro,*

to dobrze znany inhibitor RAD52, który działa na zasadzie dysocjacji nadstruktury pierścienia RAD52, przenosząc ją do dimerów, co powoduje represję funkcji białka [35].

- D-IO3 (Selleckchem) – stosowany w badaniach na ksenograftach

to związek chemiczny stosowanym jako inhibitor RAD52, który specyficznie hamuje zależne od RAD52 wyżarzanie jednoniciowe (ang. single-strand annealing - SSA)[36]

-związki alkilujące stosowane obecnie w terapiach przeciwnowotworowych:

- temozolomid (TMZ) (Selleck Chem) - glejaka
- dakarbazyna (DTIC) (Sigma Aldrich) – czerniaka,

są to leki cytotoksyczne, przez długi czas stosowane jako główne chemioterapeutyki w leczeniu glejaka i czerniaka. Oba działają poprzez indukcję metylacji zasad purynowych w DNA, prowadząc do uszkodzenia DNA i śmierci komórek[37].

Oba nowotwory podlegały takiemu samemu traktowaniu, z wyjątkiem związków alkilujących dedykowanych konkretnemu typowi raka. Schemat traktowania związkami trwał 120h z dawką przypominającą po 48h i w następujących kombinacjach: Polθi, PARPi lub RAD52i, TMZ lub DTIC samodzielnie; Polθi + PARPi/RAD52i; Polθi + TMZ/DTIC, PARPi + TMZ/DTIC; RAD52i + TMZ/DTIC; Polθi + PARPi/RAD52i + TMZ; Polθi + PARPi/RAD52i + DTIC. Po traktowaniu komórek samodzielnymi związkami i odpowiednimi kombinacjami leków następujące parametry zostały ocenione z użyciem wymienionych metod:

In vitro:

- Cytometryczna ocena przeżywalności oraz ścieżki śmierci komórkowej z wykorzystaniem znakowania jodkiem propidyny i aneksyną V,
- Ocena uszkodzeń DNA, w szczególności pęknięć dwuniciowych poprzez pomiar fosforylacji histonu H2AX oraz neutralną wersję testu kometowego, po dodatkowym zastosowaniu promieniowania gamma,
- Cytometryczna analiza cyklu komórek utrwalonych 70% EtOH i wybarwionych jodkiem propidyny z RNazą,
- Ocena proliferacji i inwazyjności komórek za pomocą testu klonogennego
- Analiza ekspresji genów kodujących białka zaangażowane w naprawę DSBs poprzez RealTime PCR
- Wizualizacja zmian morfologicznych komórek za pomocą podwójnego barwienia kalceiną AM i jodkiem propidyny.

Eksperyment z udziałem zwierząt:

- Pomiar inhibicji wzrostu guza na podstawie zmian w objętości tkanki nowotworowej.

10. Wyniki

Otrzymane wyniki były podzielone między dwa oryginalne manuskrypty względem linii nowotworowej, na której zostały przeprowadzone eksperymenty, odpowiednio glejaka i czerniaka. Profil ekspresji genów w liniach komórkowych glejaka oraz czerniaka nie wykazał obniżenia ekspresji żadnego z badanych genów względem komórek prawidłowych. Natomiast w obu liniach nowotworowych GBM21 i MLN21 zaobserwowano nadekspresję *POLQ* oraz relatywnie wysoką ekspresję genów naprawy HR. Ich jednoczesna wzmożona ekspresja mogła przyczyniać się do zwiększonej efektywności podwójnej inhibicji białek Polθ z PARP1 lub RAD52, względem ich samodzielnej inhibicji.

Żywotność komórek zarówno glejaka jak i czerniaka została obniżona o ok. 50% względem kontroli po samodzielnym zastosowaniu wyżej wymienionych związków. Dalszy spadek żywotności był odnotowany dla podwójnej kombinacji inhibitorów, Polθ z PARP1 lub RAD52. Natomiast, najsilniejszy efekt, z redukcją żywotności do poziomu poniżej 20% był zaobserwowany po zastosowaniu kombinacji trzech związków, znacząco różniący się od samodzielnej inhibicji i połączenia inhibitorów. W tym wypadku zaobserwowano eliminacyjne działanie tej kombinacji również w odniesieniu do komórek prawidłowych, aczkolwiek nie spadała poniżej 40%. W związku z tym, suplementacja związkami alkilującymi terapii opartej na podwójnej inhibicji jest ważnym aspektem do rozważenia w przypadku rozwoju tej strategii terapeutycznej.

W obu przypadkach komórki po traktowaniu, nowotworowe i prawidłowe, zostały zwizualizowane za pomocą podwójnego barwienia kalceiną AM i jodkiem propidyny. W komórkach nowotworowych zaobserwowano znaczny wzrost martwych komórek (wybarwionych na czerwono przez jodek propidyny) oraz zmiana kształtu komórek, widoczna w charakterystycznych dla procesu apoptozy wybrzuszeniach błony komórkowej, które nie były obserwowane dla komórek prawidłowych. Odpowiadające temu obrazowi wyniki zostały otrzymane w cytometrycznej analizie szlaku śmierci komórek glejaka i czerniaka, pokazując znaczny wzrost populacji komórek w późnej apoptozie zarówno po zastosowaniu inhibitorów samodzielnie, ich kombinacji, jak i podwójnej inhibicji ze związkami cytotoksycznymi. Bardzo mały procent komórek ulegał nekrozie, co wskazywałoby, że komórki nowotworowe pod wpływem stosowania inhibitorów wchodzą na drogę śmierci indukowanej – apoptozy. Również w tym wypadku, potrójna kombinacja leków dając najsilniejszy efekt, prowadziła do

wzrostu populacji komórek prawidłowych w późnej apoptozie, jednak w znacznie mniejszym stopniu niż w przypadku nowotworów.

W teście klonogennym wskazującym na zahamowanie proliferacji oraz inwazyjności komórek nowotworowych, zaobserwowano znaczny spadek ilości kolonii wytworzonych przez oba typy komórek nowotworowych w przypadku wszystkich wariantów traktowań w porównaniu do kontroli. Natomiast, w porównaniu do samodzielnego użycia inhibitorów, ich skojarzone działanie dało znacząco zwiększony efekt, a także ponownie najbardziej skuteczne okazały się potrójne kombinacje leków.

Wyniki korespondujące z przedstawionymi do tej pory, otrzymano również w rozkładzie faz cyklu komórkowego. Zaobserwowano znaczący spadek fazy G0/G1 po traktowaniu kombinacjami trzech leków. Natomiast wzrost populacji komórek w fazie S, wskazywałby na zatrzymanie procesu replikacji, spowodowane prawdopodobnie zbyt dużą akumulacją uszkodzeń DNA w tych komórkach. Podobny efekt widoczny był w komórkach prawidłowych.

Uszkodzenia DNA były mierzone, między innymi, za pomocą fosforylacji histonu H2AX. Poziom fosforylacji histonu wskazujący na zwiększone DSBs był podwyższony po zastosowaniu kombinacji inhibitora Polθ i PARP1 w obu nowotworach, oraz ich połączenia ze związkiem cytotoksycznym.

Bardzo ciekawe wyniki zostały otrzymane w neutralnym teście kometowym po dodatkowym zastosowaniu promieniowania gamma do standardowego traktowania, ukazując, że wszystkie warianty traktowania uwrażliwiają komórki czerniaka i glejaka na promieniowanie radiacyjne. Pokazuje to obiecujący kierunek w kontekście potencjalnego zastosowania takiego leczenia wraz z radioterapią. Natomiast, znaczący wzrost uszkodzeń DNA po skojarzonej inhibicji dwóch białek, również w kombinacji ze związkami alkilującymi był widoczny dla obu nowotworów. Indukcja uszkodzeń DNA była zaobserwowana w astrocytach, głównie po zastosowaniu związku alkilującego TMZ.

Przeprowadzony eksperyment na ksenograftach czerniaka pochodzącego od pacjenta pokazał, że zastosowanie związku RP6685 do inhibicji Polθ hamuje wzrost guza. Również jego połączenie z inhibitorem RAD52 oraz dodatek związku alkilującego daje podobny efekt. Aczkolwiek, sam inhibitor RAD52 z rozpuszczalnikiem RP6685 powoduje nawet silniejsze zahamowanie wzrostu guza w porównaniu do inhibitora RP6685 oraz jego połączenia z RAD52i

i DTIC, co pozostaje do dalszych badań. Natomiast wyniki te pokazują, że inhibitor PARP1 w połączeniu z Polθi i DTIC, oraz sam PARP1 z rozpuszczalnikiem RP6685 nie wstrzymuje wzrostu guza, co może prowadzić do wniosku, że nowotwór ten jest oporny na inhibitor PARP1.

11. Wnioski

Przeprowadzone badania pozwoliły na osiągniecie celu postawionego na początku pracy i zweryfikowanie, że polimeraza DNA θ poprzez jej inhibicję wykazuje potencjał do bycia celem kierowanych terapii przeciw nowotworom mózgu czy skóry.

Wyniki otrzymane podczas realizacji pracy doktorskiej pozwalają na sformułowanie następujących wniosków:

1. Inhibicja polimerazy DNA θ powoduje zmniejszenie żywotności komórek glejaka i czerniaka w około 50%, poprzez indukcję apoptozy, której towarzyszy zmniejszenie proliferacji komórek i ich inwazyjnego charakteru.

2. Efekt przeciwnowotworowy inhibicji polimerazy DNA θ wiąże się ze zwiększeniem uszkodzeń DNA oraz uwrażliwienie komórek nowotworowych na radiację.

3. Skojarzona terapia inhibitorami polimerazy θ oraz PARP1 bądź RAD52 powoduje zwiększenie efektu przeciwnowotworowego wobec glejaka i czerniaka, w porównaniu do pojedynczego zastosowania tych związków, poprzez wywołanie syntetycznej letalności.

4. Dodanie związków alkilujących temozolomidu lub dakarbazyny do podwójnej inhibicji białek naprawy może znacznie zwiększyć skuteczność leczenia.

5. Inhibitory i ich skojarzone zastosowanie wykazywało minimalne działanie toksyczne wobec prawidłowych komórek – melanocytów i astrocytów.

6. Zastosowanie inhibitora polimerazy θ (RP6685) samodzielnie oraz w połączeniu z inhibitorem RAD52 i związkiem alkilującym powoduje zahamowanie wzrostu guza na modelu ksenograftów czerniaka pochodzącego od pacjentów.

12.Literatura

[1] Beagan, K.; Mcvey, M. Linking DNA polymerase theta structure and function in healthand disease. Cell Mol. Life Sci. 2016, 73, 603–615

[2] Chen, X.S.; Pomerantz, R.T. DNA Polymerase θ: A Cancer Drug Target with Reverse Transcriptase Activity. Genes 2021, 12, 1146

[3] Trenner, A.; Sartori, A.A. Harnessing DNA Double-Strand Break Repair for Cancer Treatment. Front. Oncol. 2019, 9, 1–10

[4] Brambati, A.; Barry, R.; Sfeir, A. DNA Polymerase theta (Polθ) – an error-prone polymerase necessary for genome stability. Curr. Opin. Genet. Dev. 2020, 60, 119–126

[5] Ramsden, D.A.; Carvajal-Garcia, J.; Gupta, G.P. Mechanism, cellular functions and cancer roles of polymerase- mediated DNA end joining. Nat. Rev. 2022, 23, 125–140

[6] Ceccaldi, R.; Liu, J.C.; Amunugama, R.; Hajdu, I.; Primack, B.; Petalcorin, M.I.R.; O'Connor, K.W.; Konstantinopoulos, P.A.; Elledge, S.J.; Boulton, S.J.; et al. Homologous recombination-deficient tumors are hyper- dependent on POLQ-mediated repair. Nature 2015, 518, 258–262.

[7] Pan, Q.; Wang, L.; Liu, Y.; Li, M.; Zhang, Y.; Peng, W.; Deng, T.; Peng, M.-L.; Jiang, J.-Q.; Tang, J.; et al. Knockdown of POLQ interferes the development and progression of hepatocellular carcinoma through regulating cell proliferation, apoptosis andmigration. Cancer Cell Int. 2021, 21, 482. cell proliferation , apoptosis and migration," *Cancer Cell Int.*, pp. 1–13, 2021.

[8] Lemée, F.; Bergoglio, V.; Fernandez-Vidal, A.; Machado-Silva, A.; Pillaire, M.J.; Bieth, A.; Gentil, C.; Baker, L.; Martin, A.L.; Leduc, C.; et al. DNA polymerase θ up-regulation is associated with poor survival in breast cancer, perturbs DNA replication, and promotes genetic instability. Proc. Natl. Acad. Sci. USA 2010, 107, 13390–13395

[9] Hwang, T.; Reh, S.; Dunbayev, Y.; Zhong, Y.; Takata, Y.; Shen, J.; McBride, K.M.; Murnane, J.P.; Bhak, J.; Lee, S.; et al. Defining the mutation signatures of DNA polymerase θ in cancer genomes. NAR Cancer 2020, 2, zcaa017

[10] Schaub, J.M.; Soniat, M.M.; Finkelstein, I.J. Polymerase theta-helicase promotes end joining by stripping single-stranded DNA- binding proteins and bridging DNA ends. Nucleic Acids Res. 2022, 50, 3911–3921

[11] Wood, R.D.; Doublié, S. DNA polymerase θ (POLQ), double-strand break repair, and cancer. DNA Repair 2016, 44, 22–32.

[12] Luedeman, M.E.; Stroik, S.; Feng, W.; Luthman, A.J.; Gupta, G.P.; Ramsden, D.A. Poly (ADP) ribose polymerase promotes DNA polymerase theta-mediated end joining by activation of end resection. Nat. Commun. 2022, 13, 4547.

[13] Schimmel, J.; Kool, H.; van Schendel, R.; Tijsterman, M. Mutational signatures of non-homologous and polymerase theta-mediated end-joining in embryonic stem cells. EMBO J. 2017, 36, 3634–3649

[14] Liddiard, K.; Aston-Evans, A.N.; Cleal, K.; Hendrickson, E.A.; Baird, D.M. POLQ suppresses genome instability and alterations in DNA repeat tract lengths. NAR Cancer

2022, 4, zcac020 [15] J. Wilson and J. I. Loizou, “Exploring the genetic space of the DNA damage response for cancer therapy through CRISPR-based screens,” pp. 0–3, 2022.

[16] M. Yoshimura *et al.*, Vertebrate POLQ and POLβ Cooperate in Base Excision Repair of Oxidative Mol Cell. 2006 October 6; 24(1): 115–125.

[17] Ukai A., Maruyama T., Mochizuki S., Ouchida R., Masuda K.. Role of DNA polymerase θ in tolerance of endogenous and exogenous DNA damage in mouse B cells. *Genes to Cells* (2006) 11 , 111–121 2

[18] Ceccaldi, R.; Liu, J.C.; Amunugama, R.; Hajdu, I.; Primack, B.; Petalcorin, M.I.R.; O’Connor, K.W.; Konstantinopoulos, P.A.; Elledge, S.J.; Boulton, S.J.; et al. Homologous recombination-deficient tumors are hyper- dependent on POLQ-mediated repair. Nature 2015, 518, 258–262.

[19] Schrempf, A.; Slyskova, J.; Loizou, J.I. Targeting the DNA Repair Enzyme Polymerase θ in Cancer Therapy. Trends Cancer 2021, 7, 98–111.

[20] Carvajal-Garcia, J.; Cho, J.E.; Carvajal-Garcia, P.; Feng, W.; Wood, R.D.; Sekelsky, J.; Gupta, G.P.; Roberts, S.A.; Ramsden, D.A. Mechanistic basis for microhomology identification and genome scarring by polymerase theta. Proc. Natl. Acad. Sci. USA 2020, 117, 8476–8485

[21] Masuda K. *et al.*, “DNA polymerase θ contributes to the generation of C/G mutations during somatic hypermutation of Ig genes,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 102, no. 39, pp. 13986–13991, 2005.

[22] Li J.*et al.*, “Depletion of dna polymerase theta inhibits tumor growth and promotes genome instability through the cgas-sting-isg pathway in esophageal squamous cell carcinoma,” *Cancers (Basel).*, vol. 13, no. 13, 2021.

[23] Yousefzadeh M.J.and Wood R.d., M review DNA polymerase POLQ and cellular defense against DNA damage. *DNA Repair (Amst).*, vol. 12, no. 1, pp. 1–9, 2013.

[24] Mateos-gomez, P.A.; Kent, T.; Kashkina, E.; Pomerantz, R.T.; Sfeir, A. The helicase domain of Polθ counteracts RPA to promote alt-NHEJ. Nat. Struct. Mol. Biol. 2017, 24, 1116–1123.

[25] Zhou, J.; Gelot, C.; Pantelidou, C.; Li, A.; Yücel, H.; Davis, R.E.; Färkkilä, A.; Kochupurakkal, B.; Syed, A.; Shapiro, G.I.; et al. A first-in-class polymerase theta inhibitor selectively targets homologous-recombination-deficient tumors. Nat. Cancer 2021, 2, 598–610

[26] Jackson S.E. and Chester J.D. Personalised cancer medicine, vol. 266, pp. 262–266, 2015.

[27] Drzewiecka, M.; Barszczewska-Pietraszek, G.; Czarny, P.; Skorski, T.; ´Sliwi ´nski, T. Synthetic Lethality Targeting Polθ. Genes 2022, 13, 1101.

[28] Zatreanau, D.; Robinson, H.M.R.; Alkhatib, O.; Boursier, M.; Finch, H.; Geo, L.; Grande,D.; Grinkevich, V.; Heald, R.A.; Langdon, S.; et al. Polθ inhibitors elicit BRCA-gene synthetic lethality and target PARP inhibitor resistance. Nat. Commun. 2021, 12, 3636.

[29] Mengwasser K., *et al.*, Genetic Screens Reveal FEN1 and APEX2 as BRCA2 Synthetic

Lethal Targets. Mol Cell. 2019 March 07; 73(5): 885–899.e6

[30] Pismataro M.C., Astolfi A., Barreca M.L., Pacetti M., Schenone S., Bandiera T, Carbone A., Massari S. Small Molecules Targeting DNA Polymerase Theta (POL θ) as Promising Synthetic Lethal Agents for Precision Cancer Therapy. *J. Med. Chem*. 2023, 66, 6498−6522

[31] Bedard P. L., Hyman D. M, Davids M. S., Siu L. L., Therapeutics Small molecules , big impact : 20 years of targeted therapy in oncology. *Lancet*, vol. 395, no. 10229, pp. 1078–1088, 2020.

[32] Ashworth, A.; Lord, C.J. Synthetic lethal therapies for cancer: What's next after PARP inhibitors? Nat. Rev. Clin. Oncol. 2018, 15, 564–576. [33] M. Bubenik *et al.*, "Cite This:," 2022.

[34] Lord C.J., Ashworth A. PARP inhibitors: Synthetic lethality in the clinic. Science 2017;355(6330):1152–, doi:10.1126/science.aam7344.

[35] Toma M., Sullivan-Reed K., Sliwinski T., Skorksi T. RAD52 as a Potential Target for Synthetic Lethality-Based Anticancer Therapies. *Cancers* 2019, 11, 1561;

[36] Huang F., *et al.*, Targeting BRCA1-and BRCA2-deficient cells with RAD52 small molecule inhibitors, *Nucleic Acids Res.*, vol. 44, no. 9, pp. 4189–4199, 2016.

[37] Koprowska K, Czyż M. Dakarbazyna jako lek przeciwczerniakowy i referencyjny dla nowych programów terapeutycznych * Dacarbazine , a chemotherapeutic against metastatic melanoma and a reference drug for new treatment modalities. Postepy Hig Med Dosw (online), 2011; 65: 734-751

13.Streszczenie w języku polskim

Polimeraza DNA theta (Polθ) i jej inhibitory (Polθi) zyskały szczególną uwagę w ostatnich latach. Polθ jest polimerazą DNA zaangażowaną w kilka mechanizmów naprawy DNA, ale głównie w theta-mediated end joining (TMEJ) - jeden z alternatywnych szlaków naprawy dwuniciowych pęknięć DNA (DSBs). Destabilizacja naprawy DNA i mutacje genetyczne są cechami charakterystycznymi raka, dzięki czemu możliwe jest zastosowanie podejścia syntetycznej letalności do selektywnego zabijania komórek nowotworowych poprzez hamowanie jednego z białek naprawy DNA. Co więcej, komórki nowotworowe ze zmianami w szlakach HR lub NHEJ często stają się zależne od TMEJ.

Głównym celem badań było określenie wpływu inhibicji Polθ i jej kombinacji z inhibicją PARP lub Rad52 oraz związkami alkilującymi na komórki glejaka i czerniaka, jednocześnie oceniając ich wpływ na normalne komórki. Aby to określić, przeanalizowaliśmy żywotność komórek, apoptozę komórek, proliferację komórek i charakter inwazyjny badanych komórek, poziom uszkodzeń DNA, rozkład cyklu komórkowego i profil ekspresji genów.

Wyniki pokazują, że inhibicja polimerazy DNA θ zmniejsza żywotność komórek glejaka i czerniaka o około 50%, poprzez indukcję apoptozy, której towarzyszy zmniejszenie proliferacji komórek i podwyższone uszkodzenia DNA. Terapia skojarzona inhibitorami polimerazy θ i PARP1 lub RAD52 skutkuje zwiększonym działaniem przeciwnowotworowym przeciwko glejakowi i czerniakowi, w porównaniu z pojedynczym zastosowaniem tych związków, najprawdopodobniej poprzez indukowanie syntetycznej letalności. Dodanie związków alkilujących temozolomidu lub dakarbazyny do skojarzonej inhibicji dwóch białek naprawczych może znacznie zwiększyć skuteczność leczenia, chociaż wiąże się to również ze zwiększoną toksycznością dla normalnych komórek.

Podsumowując, na podstawie uzyskanych wyników przypuszczamy, że hamowanie Polθ z jednoczesnym hamowaniem PARP lub Rad52 przynosi syntetycznie śmiertelny efekt na komórki glejaka i czerniaka, pozostawiając jedynie minimalny wpływa na komórki prawidłowe. Co więcej, dodanie leku alkilującego wzmacnia efekt przeciwnowotworowy.

14.Streszczenie w języku angielskim | Summary

DNA polymerase theta (Polθ) and its inhibitors (Polθi) have gained particular attention in the recent years. Polθ is a DNA polymerase involved in several DNA repair mechanisms, but mainly in theta-mediated end joining (TMEJ) – one of the DNA double-strand breaks (DSBs) repair pathways. The DNA repair destabilization and genetic mutations are the cancer hallmarks, making it possible to use the synthetic lethality approach to selectively kill cancer cells by inhibiting one of the DNA repair proteins. Furthermore, cancer cells with alterations in HR or NHEJ pathways often become dependent on TMEJ.

The main objective of the research was to determine the influence of Polθ inhibition and its combination with PARP or Rad52 inhibition and alkylating agents on glioblastoma and melanoma cells, simultaneously assessing their impact on normal cells. In order to evaluate it, we analyzed cell viability, cell apoptosis, cell proliferation and invasive character, level of DNA damage, cell cycle distribution and gene expression profile.

The results show that inhibition of DNA polymerase θ reduces the viability of glioma and melanoma cells by around 50%, through the induction of apoptosis, accompanied by a reduction in cell proliferation and elevated DNA damage. Combination therapy with θ polymerase inhibitors and PARP1 or RAD52 results in an increased anti-tumor effect against glioma and melanoma, compared to single application of these compounds, most likely by inducing synthetic lethality. The addition of alkylating compounds temozolomide or dacarbazine for dual inhibition of repair proteins can significantly increase treatment efficacy, although it is also associated with increased toxicity to normal cells.

In conclusion, based on the results we presume that inhibition of Polθ with simultaneous inhibition of PARP or Rad52 brings a synthetically lethal effect on glioblastoma and melanoma cells, having a minimal effect on normal cells. Moreover, the addition of alkylating drug strengthens the anticancer effect.

Publikacje będące podstawą rozprawy doktorskiej

International Journal of
Molecular Sciences

MDPI

Review

Polθ Inhibition: An Anticancer Therapy for HR-Deficient Tumours

Gabriela Barszczewska-Pietraszek [1,†], Małgorzata Drzewiecka [1], Piotr Czarny [2,†], Tomasz Skorski [3] and Tomasz Śliwiński [1,*]

[1] Laboratory of Medical Genetics, Faculty of Biology and Environmental Protection, University of Lodz, 90-236 Lodz, Poland
[2] Department of Medical Biochemistry, Medical University of Lodz, 92-216 Lodz, Poland
[3] Fels Cancer Institute for Personalized Medicine, Lewis Katz School of Medicine, Temple University, Philadelphia, PA 19140, USA
* Correspondence: tomasz.sliwinski@biol.uni.lodz.pl; Tel.: +48-42-635-44-86
† These authors contributed equally to this work.

Abstract: DNA polymerase theta (Polθ)-mediated end joining (TMEJ) is, along with homologous recombination (HR) and non-homologous end-joining (NHEJ), one of the most important mechanisms repairing potentially lethal DNA double-strand breaks (DSBs). Polθ is becoming a new target in cancer research because it demonstrates numerous synthetically lethal interactions with other DNA repair mechanisms, e.g., those involving PARP1, BRCA1/2, DNA-PK, ATR. Inhibition of Polθ could be achieved with different methods, such as RNA interference (RNAi), CRISPR/Cas9 technology, or using small molecule inhibitors. In the context of this topic, RNAi and CRISPR/Cas9 are still more often applied in the research itself rather than clinical usage, different than small molecule inhibitors. Several Polθ inhibitors have been already generated, and two of them, novobiocin (NVB) and ART812 derivative, are being tested in clinical trials against HR-deficient tumors. In this review, we describe the significance of Polθ and the Polθ-mediated TMEJ pathway. In addition, we summarize the current state of knowledge about Polθ inhibitors and emphasize the promising role of Polθ as a therapeutic target.

Keywords: Polθ inhibitors; anticancer treatment; DNA double-strand break repair; DNA repair enzyme

Citation: Barszczewska-Pietraszek, G.; Drzewiecka, M.; Czarny, P.; Skorski, T.; Śliwiński, T. Polθ Inhibition: An Anticancer Therapy for HR-Deficient Tumours. *Int. J. Mol. Sci.* **2023**, *24*, 319. https://doi.org/10.3390/ijms24010319

Academic Editors: Olga Lavrik and Konstantin Volcho

Received: 25 November 2022
Revised: 15 December 2022
Accepted: 17 December 2022
Published: 24 December 2022

1. Introduction

One of the hallmarks of cancer cells is their genetic instability, which could lead to an increase of mutations in their genomes [1]. As a consequence, the loss of function mutations may take place in the genes that are crucial for cell survival mechanisms, for example, DNA repair systems. Under such conditions, the survival of cancer cells depends on finding a substitute for the lost pathway [2]. If inactivation of a specific set of genes leads to cell death, whereas inactivation of each of these genes individually does not affect cell functioning and survival, then these genes are considered to exhibit "synthetic lethal" interactions [3]. Targeting alternative pathways using inhibitors against DNA double-strand breaks (DSBs) repair proteins is becoming a feasible strategy that has been gaining increasing interest in recent years. An approach based on synthetic lethality might not only prove to be a selective and effective solution in personalized anticancer therapy, but it is already contributing to expanding the knowledge about genetic interactions occurring in cells [4,5].

DNA polymerase theta (Polθ) is encoded by *POLQ*—a unique multifunctional replication and repair gene that encodes a protein with N-terminal superfamily 2 helicase domain exhibiting ATPase activity and C-terminal A-family polymerase domain [6,7]. The possession of helicase domain is a unique Polθ feature among other eukaryotic DNA polymerases. More detailed information about structure and function of Polθ can be found in another paper of Drzewiecka et al. (2022) [4]. Polθ overexpression has been identified in a number of human cancers and has been linked with a poor clinical outcome for liver cancer and breast

cancer patients with homologous recombination (HR) deficiency [8–10]. To target DNA repair vulnerabilities in cancer, Zatrenau et al. (2021) [11] discovered nanomolar potent, selective, low molecular weight, allosteric inhibitors of Polθ ART558 and ART812, which interact with the polymerase domain. ART558 inhibits the major Polθ-mediated DNA repair process, i.e., Polθ-mediated end joining (TMEJ) without targeting non-homologous end joining (NHEJ) [11]. Recently, another biochemical compound, RP-6685, with potential to inhibit Polθ polymerase domain was discovered [12]. Additionally, an antibiotic, novobiocin (NVB), was identified as the inhibitor of Polθ helicase activity [13].

The application of Polθ Inhibitors (Polθi) in the concept of dual synthetic lethality emerged after initial success of PARP inhibitors (PARPi) when it was found that tumor cells do not respond to one drug treatment and develop resistance [14]. Polθ has a particular importance for the repair of DSBs in cancer cells deficient in the HR function. Polθ inhibition boosts the effect of PARPi by exerting a synthetically lethal action on BRCA1- and BRCA2-mutant cancer cells [3,14]. Deficiencies in genes of other DNA damage response (DDR) pathways, e.g., encoding DNA-PKcs which is a crucial component of the classical NHEJ pathway, can also make Polθ a key factor for cellular survival [15]. Furthermore, knocking out *POLQ* in mouse models and non-cancerous cells had minimal effect [16,17]. Therefore, Polθ shows promising results as an antitumor drug target candidate, principally against HR-deficient tumors. Moreover, Polθ inhibitors not only have clinical potential in targeting BRCA-gene defective cancers but could also be used to target PARPi resistance [11,13,14].

A review of literature focused on the role of polymerase theta in the context of synthetic lethality and potential anticancer therapy was conducted, using PubMed and Google Scholar to search. The authors considered studies performed on animals as well as human subjects (in vivo and in vitro) along with clinical trials. Keywords applied were as follows: DNA polymerase theta, polymerase theta inhibitors, ART558, novobiocin, microhomology-mediated end joining, MMEJ, DNA repair, cancer, polymerase theta-mediated end joining, TMEJ, double strand break repair, homologous recombination repair, HR, non-homologous end joining, NHEJ, siRNA, shRNA, RNA interference, CRISPR/Cas9, anticancer therapy, and synthetic lethality.

2. The Role of Polθ—Mediated TMEJ

Polθ is a main protein of TMEJ which is one of the main pathways of DSB repair [7,18]. TMEJ could be considered a substitute pathway to NHEJ and placed side by side with microhomology-mediated end joining (MMEJ) or alternative end-joining (a-EJ) as they share a requirement for microhomology fragments [14,19,20]. However, some publications differentiate TMEJ as a separate pathway, alongside NHEJ, HR, and SSA, considering the existence of a-EJ pathway without Polθ activity. Therefore, in this review the the term "TMEJ" for Polθ-mediated repair process is used, even though it is often called "a-EJ", "alt-NHEJ", etc. in the literature [4,21–24].

TMEJ is determined by several factors, namely: independence on Ku, XRCC4 and LIG4 proteins, resected DNA ends with 3′ single-stranded overhangs, several nucleotide-long microhomology regions and presence of Polθ [21]. Moreover, this repair is highly error-prone due to the lack of Polθ proofreading ability and deleterious characteristics of microhomology end-joining itself. This results in the accumulation of mutations [6]. On the other hand, in HR-deficient tumor cells, where Polθ is usually overexpressed, TMEJ enables their survival (Figure 1) [8–10,22,25–27]. In some studies, it was observed that TMEJ is most crucial when HR and NHEJ are not working properly [24,25]. However, there is evidence that it is active also in NHEJ-proficient cells [28].

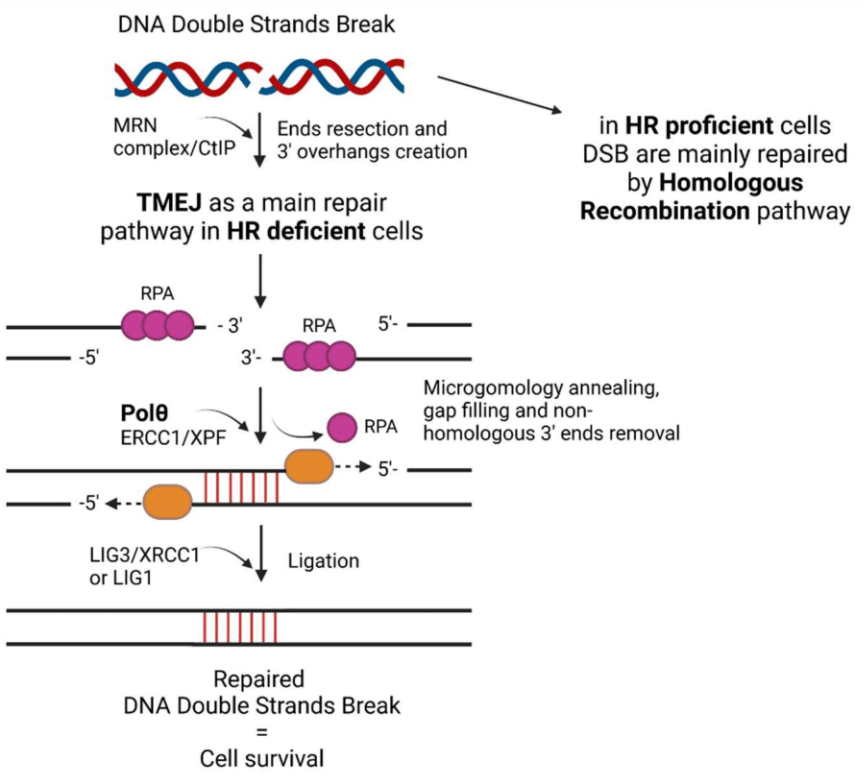


Figure 1. The mechanism of DNA double strand break repair by TMEJ in HR-deficient cells.

Further, going into details of repair mechanism in the first step of the process, the CtIP with MRN complex is needed to initiate end resection and create 3′ overhangs. It is assumed that PARP1 is involved in the recognition of DNA breaks and helps in end resection [19,25,29]. Subsequently, non-homologous 3′ ends are removed by ERCC1/XPF nucleases. Then, Polθ attaches to single-stranded DNA (ssDNA) overhangs and anneals the sequences based on at least 2 bp microhomology [18,29,30]. In this step the helicase domain of Polθ removes RPA from ssDNA tails, while the polymerase domain is responsible for annealing [7,14]. Therefore, both helicase and polymerase domains of Polθ are necessary in cis configuration for TMEJ to function [21]. Eventually, LIG3-XRCC1 complex or LIG1 ligate stabilized DNA ends [18,25,30].

Furthermore, it is believed that Polθ can participate in other repair mechanisms and cell events, such as base excision repair, mismatch repair, replication-associated DNA breaks, or reverse transcription and translation synthesis [6,20,31,32]. However, this is not the subject of this review and further information can be found in the work of Drzewiecka et al. (2022) [4].

3. Different Strategies for Polθ Suppression

The consequences of Polθ inhibition and knockdown in cells have been vastly described in the literature [7,9,28,31,33–36], allowing to evaluate the significance of the protein and its interactions [8]. In the literature, the most used methods include siRNA or shRNA silencing and CRISPR-Cas9 technique, shown by research examples described below. Two other gene editing tools are also described, namely ZFNs (zinc finger nucleases) and TALENs (Transcription activator-like effector nucleases). However, they are considered less efficient and are less frequently used than RNA interference (RNAi) and CRISPR/Cas9, at least in the context of Polθ research [37–39]. In this chapter, the authors will review recent research papers that describe the above-mentioned methods of Polθ inhibition and their consequences.

3.1. RNA Interference Technique—siRNA and shRNA

3.1.1. Description of the Technique

The main objective of RNA interference is to selectively silence a gene via non-coding RNA which targets and triggers degradation of mRNA. Almost 20 years have passed since the first such molecule was discovered, i.e., microRNA (miRNA), further resulting with the Nobel prize for Fire and Mello in 2006 for defining RNA interference and its mechanism [40–42]. Based on this achievement, scientists designed other RNA molecules, and two the most common are siRNA and shRNA [41,43].

Gene silencing can be achieved in two ways: by degradation of the target mRNA induced by small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs) and differently via suppression of specific mRNAs translation induced by miRNA. This paper will focus on the first approach, achieved with siRNA or shRNA. The molecules lead to a similar genetic outcome, however they are different in terms of structure and molecular mechanism, and may have distinct applications [44].

siRNAs are double-stranded RNA molecules which total length is 21–25 nucleotides. Along with piwi-interacting RNAs (piRNAs) and miRNAs, siRNAs are defined as non-coding, small RNAs [45]. Considering the structure, siRNAs have one guiding strand (antisense) and a passenger strand (sense), as well as two 2-nucleotide-long overhangs at 3′ ends. [41,46]. siRNA is formed from long double-stranded RNA, cleaved by Dicer, an enzyme from RNase III family. For the purpose of therapy, siRNAs are synthesized chemically and delivered in various ways to the cytoplasm, i.a.: nanocarriers, aptamers, and antibodies [41,44,47]. In the cytoplasm, siRNA creates the RNA-induced silencing complex (RISC) with proteins Dicer, Argonaute-2 (Ago2), and Trans-activating Response RNA Binding-Protein (TRBP), which later allows siRNA to target mRNA. In this interaction, Ago2 splits the sense strand of the molecule, unwinds the duplex with the use of the Dicer N-helicase domain and leads to the degradation of this strand. Then, the anti-sense strand guides the activated RISC complex to target mRNA with its complementary sequence (Figure 2) [41,46].

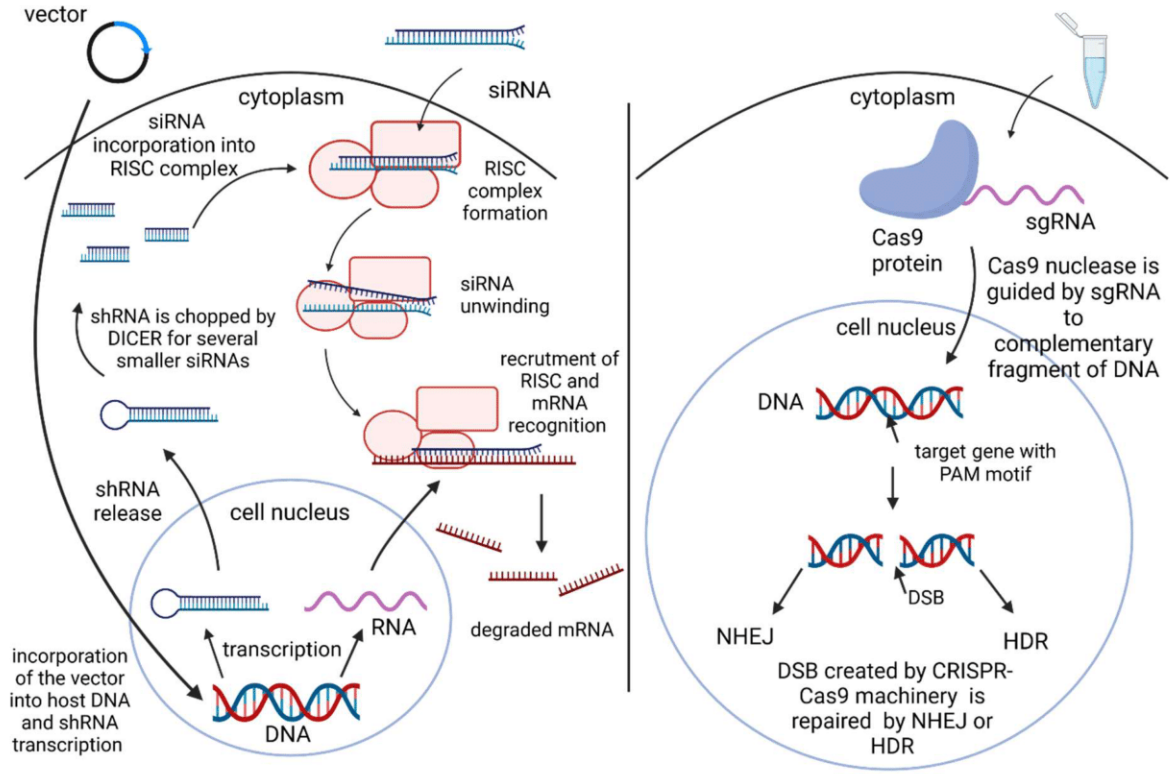


Figure 2. The RNA interference mechanism and CRISPR-Cas9 mechanism in human cell.

Opposite to the siRNA, shRNA needs to be introduced to the cell's nucleus. This could be achieved through a bacterial or viral vector. However, a viral vector is most commonly used, as it is considered more stable and efficient [48]. Usage of a viral vector allows shRNA to be integrated to the genome of host cells and later expressed in the nucleus. Afterwards, the host's protein exportin 5 is responsible for transferring the shRNA outside the nucleus. In the cytoplasm, it forms a complex with Dicer, an RNase III enzyme, which chops up the shRNA into small siRNA duplexes with 20–25 nt of length and 2 nt overhangs at the 3′ end. Then, the siRNAs follow the regular path to degrade desired mRNA (Figure 2) [42,48].

RNAi is a promising technology for the treatment of various diseases such as cancer, viral infections, eye and liver diseases, and some genetic disorders. In many applications, siRNA-based therapies are at the stage of clinical trials [41]. In addition, various studies show that both siRNA and shRNA are effective in vivo with different targets and exhibit potential in personalized therapies [40,44,46]. Moreover, according to Alshaer et al. (2021) [41], siRNA could be a better therapeutic tool than small molecules, since it is highly selective, can reach the target in any location and has only antagonistic effects.

Although it theoretically seems to be a perfect method to perform gene knockdown, there are several drawbacks in practice which should be addressed, such as the way in which RNAi is delivered, off-targets and stabilization of molecules inside cells. Considering these factors, shRNA is regarded to be more efficient than siRNA. When comparing these two molecules, shRNA tends to be more effective inside cells, because it can be synthesized constantly [44]. However, the use of shRNA with a vector could be more complicated and time-consuming [48]. Researchers are still working on improving this method, for example, by means of designing bi-functional shRNA that combines two types of shRNAs are cleaved by RISC-dependent and -independent pathways, thereby leading to gene silencing by mRNA degradation and translation inhibition at the same time [44].

3.1.2. Application in Studies

Several studies using siRNA and shRNA to silence *POLQ* show successful Polθ mRNA depletion [8,9,35,36]. The studies of Dai et al. (2016) [35] and Kelso et al. (2019) [36] on cancer cell lines confirm increased sensitivity of cells to cisplatin after *POLQ* silencing with siRNA.

Moreover, in a research study that involved inhibiting Polθ via siRNA, Ceccaldi et al. (2015) [8] presented its correlation with HR repair mechanism. The authors concluded that Polθ inhibits the HR pathway by direct binding to RAD51, therefore affecting its assembly with ssDNA, which is observed in reduced RAD51 foci formation [8]. Also, they demonstrated a synthetic lethal interaction of Polθ and the HR repair pathway in HR-deficient ovarian tumor cells, which revealed that depletion of both Polθ and HR leads to cellular death.

In addition, in the research of Goullet de Rugy et al. (2016) [49], siRNA was used to perform knockout of Polθ and genes encoding enzymes involved in DNA metabolism, i.e., *FANCA, RECQL5, MUTYH, NEIL1,* and *USP22*, to check synthetic lethal interaction between them, in model of colorectal cancer cells. Mentioned genes were selected in the screen, also performed with use of siRNA in the cells with Polθ overexpression. The study did not show significant changes in cells viability after double knockout, versus cells without Polθ depletion. Therefore, it is possible to assume that Polθ does not have synthetic lethal correlation with any of these genes. Although, the scientists treated the Polθ knockout cells with hydroxyurea and cytarabine, drugs suppressing DNA replication fork progression and they exhibited increased sensitivity to the drugs, observed in decreased cell viability in comparison to the control. These results suggest that Polθ is involved in replication fork interruption [49].

Finally, in the studies conducted by Pan et al. (2021) [9], the shRNA mediated Polθ knockout was performed using a lentiviral vector with the purpose of analyzing Polθ significance in liver cancer cells (HCC). Successfully obtained knockdown led to decreased proliferation, migration, and metastasis, as well as increased apoptosis of cancer cells. Such

effects may suggest that Polθ is involved in these processes and its inhibition may disturb the development of cancer cells. These results were also confirmed in vivo [9].

Presented studies show that Polθ depletion by itself also influences tumors survival, however compilation with different cytotoxic drugs or another DNA repair pathway deficiency increases the sensitivity of cancer cells to these agents. Therefore, in authors' opinion, it is a good indication for potential use of Polθ inhibitors in clinics to use it not as a single therapy, especially given that there is a risk that tumors will develop resistance to Polθ inhibitors, similarly to what was observed when administrating PARPi.

3.2. CRISPR/Cas9 Technology

3.2.1. Description of the Technique

The most recent technique to achieve gene modulation is CRISPR/Cas9—Clustered regularly interspaced palindromic repeats/CRISPR associated protein 9. This technique was designed based on a naturally occurring CRISPR/Cas system in prokaryotes, serving as an immune system, and defending them from foreign DNA particles of a viral or plasmid origin [38,39,50,51]. Three components are crucial in the case of CRISPR/Cas9 procedures, i.e., guide RNA, Cas9 nuclease, and target DNA with protospacer adjacent motif (PAM) [38,39]. Guide RNA, also referred to as single guide RNA (sgRNA), is a molecule that combines functions of two RNAs working in natural processes of bacteria, CRISPR-derived RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA). Cas9 protein, derived from *Streptococcus pyogenes*, is guided by RNA and can target complementary fragments of DNA only when there is PAM motif, a short sequence (2–5 nt) on one strand of DNA [38,50,52]. The cooperation of sgRNA and Cas9 enzyme leads to a double strand break in the target sequence, which could be repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR) mechanisms (Figure 2). NHEJ repair occurs when there is no homology between created ends, and it usually generates knockout of the gene. However, HDR works while homology between ends occurs, which gives a chance to introduce an extra sequence to an existing one and create a knock-in [32,38,50]. It is additionally worth noting that the CRISPR/Cas system described above is of type II (out of three discovered types) and it needs only one Cas9 nuclease [38,39].

CRISPR can have various modifications and be used not only with Cas9 endonuclease (SpCas9), but also with other enzymes, e.g., Cas13, SpCas9, Cpf1, Cas12. Depending on the structure, the characteristics and application of the system may vary, for example Cas13 targets RNA instead of DNA [38,39]. CRISPR/Cas technology has a broad range of applications within gene editing, e.g., DNA and RNA editing, genome screening, live-cell imaging, virus and bacteria pathogen detection, inhibition, and killing, and gene therapy. It is also well developed in cancer research, e.g., in discovering the role of mutations in carcinogenesis by removing them from the genome, or creating cancer models by targeting specific cancer suppressor genes which lead to tumor formation, or by removing the genes that in consequence cause cancer cell death [39]. The application in cancer therapy was proven by Lu et al. (2020) [53] in phase I clinical studies where T-cells with *PD-1 gene* silencing done by CRISPR/Cas9 were administrated to the patients bearing non-small-cell lung cancer. The treatment did not cause adverse effect, howeverit did not stop cancer progression [53]. There is evidence that CRISPR/Cas9 could be used to overcome drug resistance in cancer cells [51].

3.2.2. Application in Studies

This technique has a huge therapeutic potential. However, it also raises some ethical controversies due to its ability to change human genome. Apart from its strongest advantages, such as versatility, easiness to reach the target DNA, relatively high efficiency and possibility to target multiple sites at once, CRISPR has its drawbacks [39,54]. The main one is the off-target effect and induction of uncontrolled changes in the genome. Moreover, there are problems with delivery in vivo and editing efficacy [51,54].

In the studies conducted by Schimmel et al. (2017) [28], with the application of CRISPR/Cas9 scientists were able to analyze, among others, TMEJ activity in mouse embryonic stem cells. The CRISPR technology was used to obtain knockouts of *POLQ, Ku80, LIG4* genes and double knockouts, respectively. Moreover, the site-specific blunt DSBs were introduced in marker gene *HPRT*. This allowed to measure how the mentioned knockouts affect the frequency of mutations in this gene, which are the result of mutation-prone repair, such as TMEJ. The results presented a decreased frequency of mutations in *POLQ* knockout cells compared to wild type cells, while *Ku80* and *LIG4* knockouts did not give any significant change in the mutation frequency compared to control cells. Moreover, in double knockout of *Ku80* and *POLQ*, the mutation frequency was even lower than in *POLQ* depleted cells alone. Furthermore, the authors measured sensitivity of the knockout cells to ionizing radiation. Both TMEJ- and NHEJ-depleted cells exhibited increased sensitivity, compared to wild-type, even though the changes in the mutation frequency were not observed in NHEJ knockouts. This analysis performed in the research made it possible to conclude that TMEJ, next to NHEJ, contributes to error-prone repair of DSBs in mouse embryonic stem cells. Secondly, in the case of an absence of the NHEJ repair mechanism, TMEJ can replace it completely, however not in reverse. Finally, TMEJ repairs DSBs with blunt ends and almost always requires microhomology near DNA break ends, which was measured in the presence of simple deletions induced by Cas9-WT in exon 2 and 3 of *HPRT* gene [28].

In their studies, Ferreira da Silva et al. (2019) [33] examined the role of NHEJ and Polθ-mediated a-EJ by inducing DNA breaks and knockouts with CRISPR-Cas9. The research showed that NHEJ is the main repair pathway to repair Cas9-induced DSBs. This study also confirmed that Polθ-mediated repair can substitute NHEJ when it is not present in cells [33].

Further studies carried out by Mateos-Gomez et al. (2017) [7] showed the application of CRISPR/Cas9 as well as shRNA in gene editing in mouse embryonic stem cells. Due to the CRISPR/Cas9 knockout of the helicase and the polymerase domains of Polθ separately, the scientists could prove their role in DSB repair, highlighting that the helicase domain favors Polθ-mediated repair by removing RPA. One of the measured parameters was frequency of chromosomal translocation, which is assumed to be caused by Polθ activity. Lower frequency of translocation was reported when the helicase or polymerase domain was depleted, which could lead to a conclusion that both are important for DNA ends joining. Next, the authors observed increased accumulation of IR-induced RAD51 foci regardless of which domain was depleted in cells. Moreover, with use of the CRISPR/Cas9 they performed HR-mediated gene targeting assay, revealing that both domains interact in HR suppression. In addition, the usage of shRNA allowed to achieve additional *BRCA1* gene knockout in the studied cells. In comparison to wild type cells, both types of double-knockout cell lines, i.e., without either helicase or polymerase domain and *BRCA1*, exhibit decreased growth. This experiment shows that helicase and polymerase activity of Polθ is necessary for HR-deficient cells. In these assays, cells lacking a central domain which interacts with RAD51 did not differ from wild type cells phenotype [7].

Moreover, Zhou et al. (2021) [13] used the CRISPR-Cas9 technology to knockout Polθ to compare it with novobiocin effects on human cells. The results of this study are further described below in Section 4.1.

Nevertheless, gene silencing is the most common application of CRISPR. The presented studies demonstrates that CRISPR-Cas9 is a powerful and versatile tool, which often brings better results than other methods of gene editing [38,55].

4. PolQ Inhibitors

The topic of Polθ inhibitors is still relatively new and not profoundly described in the literature. Within the last two years, few studies have indicated three potential candidates for Polθ inhibitors: novobiocin (NVB), ART588 with its isomers and RP-6685 [11–13].

4.1. Novobiocin

A coumarin antibiotic referred to as novobiocin, derived from *Streptomyces*, has been used to cure bacterial infections by attaching to the Bergerat fold present in the DNA Gyrase B's ATP-binding site [56,57]. Novobiocin was introduced to cancer studies because of the similarity between DNA Gyrase and Heat shock protein 90 (Hsp90) structure [56]. Hsp90 is an evolutionarily conserved molecular chaperon responsible for maintaining over 300 client proteins, involved in crucial cell processes. Those proteins are also linked with ten hallmarks of cancer. Therefore, Hsp90 was placed as a target of anticancer therapy using NVB as its agent [57–59].

On the contrary to what was initially hypothesized, NVB was found to bind Hsp90 at C-terminal region and inhibit it allosterically, instead of Bergerat fold located at N-terminal ATP-binding site like in case of DNA Gyrase Moreover, it became the first C-terminal Hsp90 inhibitor that did not cause the heat shock response [56,60]. However, research disqualified NVB from antitumor activity due to its high half maximal inhibitory concentration (IC50) value of approximately 700 μM. Nevertheless, based on those results, several derivative compounds that could block the Hsp90 protein were discovered and synthesized [57,58,60,61]. Furthermore, the researchers tried to involve NVB and its derivatives in many other applications, e.g., neurological studies, as a treatment for neurodegenerative disease. Together, these discoveries could give some perspective for the studies on novobiocin as Polθ inhibitor, especially that Polθ share a similar structure to Hsp90 protein considering the helicase domain with ATPase activity.

The studies on NVB targeting Polθ are performed independent on previous once and so far, three original papers about NVB as Polθ inhibitor have been published. The following section will summarize the most important findings of those studies [13,31,62]. To our knowledge Zhou et al. (2021) [13] were the first to introduce NVB to Polθ inhibition. They performed a broad-spectrum analysis (small-molecule screening, secondary screening in the presence of ssDNA, P-based radiometric ATPase assay, dose–response and binding capacity experiment, thermal shift assays, molecular docking), which revealed that novobiocin as a specific inhibitor that binds directly to the helicase domain with ATPase activity, in vitro. Moreover, referring to the previous application of NVB, the scientists excluded its off-target activity on HSP90 and TOP2, a eukaryotic homolog of DNA Gyrase, suspected of being responsible for the cytotoxic effect of NVB in HR-deficient cells. Research proves that NVB particularly targets Polθ in human cells, which was examined by creating Polθ-knockout cells with the CRISPR-Cas9 technology. These cells were more resistant to NVB treatment than wild type cells [13].

NVB binds to the helicase domain of purified Polθ protein. This domain is crucial when deciding whether DSBs will be repaired by HR or TMEJ. By its ability to dissociate RPA from resected DNA ends, it promotes the annealing of microhomologies, and in consequence the TMEJ pathway. Therefore, it is possible to assume that inhibition of polymerase domain with NVB allows RPA action and leads to increase end resection mediated by BLM/EXO1, which stimulate HR repair and block NHEJ at the same time [7,13]. In cells with nonfunctional HR, excessive end resection may occur, accompanied by RPA accumulation, which can lead to cell death. Additionally, the RAD51 accumulation is predicted to be correlated with redundant DSB end resection, however not in PARPi sensitive cells.

The described mechanism is well visible in studies on tumor xenografts of Zhou et al. (2021) [13]. However, intensified end resection is also visible in PARPi resistant cells with HR-restoration or HR-proficient U2OS, after treatment with NVB. The possible explanation of this mechanism is that with continuous inhibition by NVB, HR may not be efficient enough, so over-resected DNA ends and nonfunctional RAD51 accumulate and become toxic for cells. Therefore, it is assumed that NVB could kill cells stimulating DSB end resection or ssDNA and RAD51 accumulation [13].

Zhou et al. (2021) [13] investigated if NVB has a similar effect on cells as Polθ silencing with other methods, such as siRNA knockdown. However, they demonstrated weaker

RAD51 and H2AX foci formation after ionizing radiation (IR) in NVB treated cells than it was confirmed in the studies of Ceccaldi et al. (2015) [8], where Polθ was knocked down by siRNA. These results indicate that NVB inhibits Polθ, leading to DNA repair impairment. Nevertheless, the effect might be weaker than inhibition achieved by siRNA.

In an animal model of mice with transplanted genetically engineered BRCA1-deficient ($BRCA1^{-/-}$) breast cancer, the animals treated with NVB had significantly smaller tumors and lived almost three times longer than those treated with vehicle. In the next models of mouse xenografts with FANCF-deficient and proficient ovarian cancer cell lines, the group observed NVB effectiveness especially on FANCF-deficient tumors when the vehicle had an impact on any of the cases. RAD51 foci were also generated in NVB-treated tumors [13].

Further, in vitro tests show that NVB significantly decreased the survival of $BRCA1^{-/-}$ and BRCA2-deficient ($BRCA2^{-/-}$) RPE1 cells and generates the apoptosis in comparison to WT cells. Moreover, it induces DNA damage (chromosomal aberrations and radial chromosomes) at a similar level as cytotoxic drug mitomycin C.

What should not be neglected in the context of Polθ inhibitors are PARP inhibitors and PARPi resistance, one of the reasons for the studies on Polθi. Therefore, Zhou et al. (2021) [13] examined the synergic activity of NVB, olaparib and rucaparib in HR-deficient cells. A stronger effect of PARPi together with NVB than alone in HR-deficient cells was demonstrated, and additionally NVB decreases the IC50 value of both PARP inhibitors in $BRCA1^{-/-}$ and FANCF-deficient cells [13].

There are different hypotheses on the mechanism in which cells acquire PARPi resistance. Moreover, it is possible that Polθ is involved in this mechanism and its inhibition could resolve this problem [11,19,63]. Research shows that NVB can deal with not only one PARPi resistance mechanism. Zhou et al. (2021) [13] created clones of $BRCA1^{-/-}$ RPE1 (Human Retinal Pigment Epithelial-1) cells resistant to PARPi in at least two different mechanisms, i.e., replication fork stabilization and HR restoration visible via RAD51 foci accumulation. Interestingly, the BRCA1 re-expression was not observed, which was unexpected since the protein interacts with PALB2 and BRCA2 at DNA damage site, indirectly facilitating RAD51 filament formation [64]. It was revealed that one of the clones exhibit lower expression of Shieldin complex component and the other clone decreased expression of 53BP1. Thus, a possible mechanism of HR repair resumption could emanate from the downregulation of the Shieldin complex and further NHEJ repair downregulation [65,66]. Importantly, all the clones kept comparable responsiveness to NVB as parental $BRCA1^{-/-}$ RPE1, not resistant to PARPi. To prove that the NVB effect on cells comes from Polθ inhibition, the researchers genetically depleted Polθ in those clones and parental cells, as well as BRCA1 wild type cells. This influenced HR-deficient cells, which was visualized in a decreased survival rate but not wild type RPE1 cells, leading to conclusion that Polθ inhibition is the most effective in HR-deficient cells. Similarly, two cancer cell lines derived from patients with PARPi-resistance obtained via two different mechanisms, described above, were sensitive to NVB treatment, while the resistance to olaparib lasted. Moreover, after insertion of wild type BRCA1 cDNA to cells, *POLQ* expression and NVB sensitivity were lower. The results described above may lead to a conclusion that HR-deficient cells do not develop cross-resistance to NVB and PARP inhibitors. Moreover, said PARPi resistance mechanisms are independent on BRCA1 and most probably depend on Shieldin complex functioning [13].

However, Zhou et al. (2021) [13] discovered that NVB cannot omit each mechanism of PARPi resistance, namely, *BRCA2* gene somatic reversion. The BRCA2-deficient cells, with acquired PARPi resistance via this mechanism, did not react either to PARPi or NVB. The mentioned results were reflected also in vivo in patient-derived xenografts. Therefore, there is no clear evidence that Polθ plays a role in PARPi resistance, at least a mechanism is not yet known.

As mentioned above, authors of various studies claim that *POLQ* mRNA expression is upregulated in HR-deficient cancer cells [8–10,26,27]. Research proved that elevated expression of POLQ mRNA and protein is specific for HR-deficient cells such as $BRCA1^{-/-}$

cells, and it is correlated with cell sensitivity to NVB, since BRCA1$^{-/-}$ PARPi resistance cell lines also exhibit higher *POLQ* mRNA expression. These observations were also confirmed in vivo in HR-deficient patient-derived xenograft models. Moreover, the cells, which develop PARPi resistance by *BRCA2* somatic reversion, expressed low levels of Polθ, which could indicate that this protein is not necessary for them. In conclusion, Polθ expression could serve as a biomarker of responsiveness to NVB and could be applied in the treatment for patients in the future [13].

To conclude, the publication of Zhou et al. (2021) [13] is the first to present studies on NVB in the role of Polθi, in vitro and in vivo, establishing NVB IC50 value at the level of 100 μM. It highlights the promising role of NVB in killing HR-deficient cells compared to wild type cells. Moreover, NVB enhances the cytotoxic effect of PARP inhibitors in the said cells. Most importantly, this study assumes that NVB can be used either alone or in combination with PARPi to deal with HR-deficient tumors, even in the case of developed PARPi resistance. The research shows that NVB preferentially kills HR-deficient cells both in vitro and in vivo [13].

Similar results were observed in studies of Patterson-Fortin et al. (2022) [62] who used DNA-PK inhibitor, namely peposertib. Performed CRISPR screening revealed that depletion of *POLQ* sensitizes cells to this inhibitor. Moreover, cancer cells with DNA-PK depletion achived in two ways by knockout or treatment with peposertib show upregulated level of Polθ and consequently revealed hypersensitivity to NVB, showing synthetically lethal interaction between these two repair mechanisms. The inhibition of Polθ by NVB and DNA-PK with peposertib induces a toxic level of DSB end-resection. This effect was shown and confirmed in increased RPA, BrdU, γH2AX foci, and ssDNA fragments. Further analysis, which supports the results provided above, showed enhanced RAD51 foci accumulation, increased DNA damage visualized in comet assay and induction of apoptosis. Therefore, this research presents evidence that the inhibition of both TMEJ and NHEJ repair pathways leads to excessive end resection, and in consequence cell death [62].

Another experiment also proved the convergent effect of NVB, peposertib and *TP53* knockout. The cells with *TP53* knockout revealed increased sensitivity to NVB and, in combination with peposertib, it significantly lowers its possible used dose. This mechanism was correlated with increased Polθ expression. Presented results were confirmed also in patient-derived ovarian cancer organoids with *TP53* mutations, showing a drop in viability due to the toxic level of DSB end-resection. What is more, the combination of the treatment with NVB and peposertib leads in vivo to a decrease of tumor growth in mice. Although NVB influence tumor growth alone, together, the inhibitors demonstrate a stronger effect [62].

In conclusion, all findings demonstrate synthetical lethality between Polθ and DNA-PK, the crucial protein of the NHEJ pathway, as well as potency of their dual inhibition in cells lacking *TP53.* Moreover, cells lacking *BRCA1* and *BRCA2* also exhibited hypersensitivity to the combination of NVB and peposertib, which may suggest that not only Polθ is important for HR-deficient cells survival, but also DNA-PK. This indicates a next step in the development of cancer treatment based on Polθ inhibition, particularly with novobiocin [62].

In other studies, researchers apply NVB to inhibit Polθ in cancer cells HCT116, as well as create Polθ knockout by CRISPR, method that was mentioned above in chapter 3 [31]. They used NVB to investigate additional Polθ activity in intra-chromosomal fusion generated by TALEN. The research proved discriminatory Polθ inhibition and reduction of EJ repair by half with an NVB dose of 100 μM. The same dose of NVB did not impact the viability of Polθ depleted cells. However, it significantly decreased the viability of WT cells. On the other hand, NVB did not reduce the frequency of intra-chromosomal fusion in WT cells, but it did only in the cells with changed *POLQ* expression, both surplus and deficient. Therefore, the authors assume that this process might be regulated by mechanisms dependent and independent on Polθ, leading to the conclusions that NVB may have an influence on cells by targeting not only Polθ [31].

The recruitment of the compounds targeting Polθ helicase domain, similarly to NVB, in oncologic patients with HR-deficiency is reported by Ideaya Bioscience (San Francisco, CA, USA), (https://www.ideayabio.com/pipeline/; accessed on 20 October 2022) [13,67]. The past experience of introducing NVB to clinical trial with rather poor results was not very encouraging. However, then, NVB application was not combined with any DNA repair deficiency. Nevertheless, it paves the way for future research [68].

4.2. ART558

Similarly to NVB, only three original papers using ART558 as Polθ inhibitor have been published so far and only several reviews mention it [4,11,18,21,24,32,62,67]. The first one to report were Zatreanu et al. (2021) [11] carried out screening of around 165,000 inhibitors against Polθ polymerase activity. Based on that assay, ART558, a small molecule inhibitor, was selected with most suitable results of the IC50 value (7.9 nM), solubility, and LogD [11].

It is worth pointing out that ART558 has a different mechanism of action compared to NVB since it targets the polymerase domain of Polθ. It binds to the allosteric binding site of the Polθ polymerase catalytic domain and enhances Polθ thermal stability in the presence of DNA. An isomer of ART558, namely ART615, was also discovered. However, this compound shows poor Polθ inhibition at higher concentration than in case of ART558 (at 12 μM). Therefore, it was used as a control compound to ART558 in the study of Zatreanu et al. (2021) [11]. It was also demonstrated that ART558 is specific to polymerase theta because it does not inhibit other polymerases, such as Polα, Polγ, Polη, and Polν, and any other kinases, including PARP1 and PARP2, even at 10 μM concentration [11].

Polθ inhibition by ART558 exhibits a synthetically lethal effect with HR repair genes, such as BRCA2, similar to the inhibition via siRNA. A model of BRCA2$^{-/-}$, cells resistant to the PARP inhibitor, was used to visualize the mechanism. The cells reveal sensitivity to ART558, but not to ART615. Furthermore, similarly to genetic silencing of Polθ, ART558 treatment with olaparib disturbs BRCA2$^{-/-}$ cells survival, confluency and induces apoptosis much stronger than in wild type BRCA2-proficient cells. In addition, the scientists observed that ART558 induces several events related to DNA damage and its level is higher in the knockout cells versus wild type, including accumulated lasting γH2AX foci elevated micronuclei formation and chromosomal abnormalities [11].

Responsiveness of BRCA2$^{-/-}$ cells was also proven in a model of CAPAN1 (pancreatic ductal adenocarcinoma tumor) cells derived from the tumor with naturally occurring BRCA2 mutation. Based on the application of these cells and their modification with a restored open-reading frame of BRCA2, the research shows significantly lower sensitivity of the cells with restored BRCA2 to ART558 compared to the BRCA2$^{-/-}$ cells. What is more, it was confirmed using genomic databases that CAPAN1 appeared to be one of the most sensitive cell lines to Polθ inhibition among other 249 BRCA-deficient tumors [11].

Along with BRCA2, a dual synthetically lethal effect was demonstrated between Polθ inhibition by ART558 and PARP by olaparib in BRCA1$^{-/-}$ RPE1 cells. Meanwhile, ART558 in the same concentration, which influenced knockout cells, had a minimal effect on normal human mammary epithelial cell lines or *BRCA*-gene wild type triple-negative breast tumor cells. ART558 sensitivity was also confirmed ex vivo in tumor organoid derived from BRCA1-mutant breast cancer, which was also sensitive to olaparib. The sensitivity was observed as decreased surviving fraction compared to BRCA1 wild type organoids. The presented results highlight the synthetically lethal interaction between Polθ and BRCA1 or BRCA2 [11].

In order to recognize other factors that could sensitize cells to mentioned inhibitors, the researchers conducted chemosensitization screens to ART558 and olaparib with the use of siRNAs in BRCA1$^{-/-}$ and wild type RPE1 cells. In case of ART558 in wild type cells, siRNAs targeting the following genes caused sensitivity: *BRCA1, PALB2, POT1* and *POLH*. *BRCA1* and *PALB2* also appeared in olaparib sensitivity screening, which could be expected due to their role in HR. However, the role of *POT1* and *POLH* genes is not clear in this mechanism. *POLH* encodes polymerase η which is involved in translesion synthesis, and

its overexpression is correlated with shorter survival of patients with lung cancer, similarly to Polθ [69]. On the other hand, *POT1* encodes one of Shelterin proteins, responsible for telomeres protection and telomerase regulation, the crucial processes for cell survival [70]. Thus, increased sensitivity to ART558 in absence of POT1 may arise from the mechanism independent on DNA repair. In BRCA1$^{-/-}$ cells, the most important observation was that siRNAs targeting genes encoding proteins from the Shieldin complex induced sensitivity to ART558, which was not reported in cells with BRCA1. Also, genetic screens in mice with *POLQ* knockout revealed such a correlation between components of the Shieldin complex and Polθ depletion. The cited results may suggest that ART558 could be used to overcome PARPi resistance acquired by depletion of Shieldin complex elements in BRCA1$^{-/-}$ cells by a mechanism of dual synthetic lethality, which also agrees with the results obtained by Zhou et al. (2021) [11].

Establishing various cell models of gene knockouts and their compilation, i.a., *BRCA1, 53BP1*, and Shieldin components: *SHLD1/2/3*, with *POLQ*, it was found that it gives a synthetic lethal effect. Moreover, the cell models possessing above mutations separately, reveal sensitivity to ART558, whereas staying resistant to olaparib [11].

The promising results of in vitro studies were shadowed by the fact that ART558 exhibits low in vivo metabolic stability in rats microsomes. Thus, if ART558 is to be introduced in clinical trials, this issue must be resolved. Nevertheless, the authors used another inhibitor, ART812, in the part of in vivo studies. It is important point for improvement for this inhibitor. Further, it was observed that tumors established in the rats with introduced double knockout BRCA1 and SHLD2 breast cancer cells were significantly smaller after treatment with ART812 [11].

Patterson-Fortin et al. (2022) [62], mentioned above in the context of novobiocin, also applied ART558. The results were consistent with the one obtained for NVB and confirmed the synthetically lethal relationship of Polθ and DNA-PK, while demonstrating increased cytotoxicity during the treatment with both inhibitors, ART558 and peposertib [62].

Other research group used ART558 in the treatment of transformed mouse embryonic fibroblast cells in extrachromosomal assay [24]. The intention of the experiment was to evaluate role of PARP1 and its inhibition in TMEJ. The study confirmed that a fully depleted function of PARP1 has a merely moderate effect in TMEJ disruption, which may help to understand why double inhibition of Polθ and PARP has a greater impact in the treatment of HR-deficient cancer cells [24].

The cited studies summarize the application of ART558 as a Polθ inhibitor. ART558 has a potential to be applied in cells resistant to PARP inhibitors and could be used in therapy alone or in combination with PARPi. Moreover, research proved the synthetically lethal interaction between Polθ and HR repair mechanism [11,24]. In addition, the derivative of ART812, ART4215, was introduced to clinical trials by the pharmaceutical company Artios Pharma Ltd. (Cambridge, UK). However, there is still much work that needs to be done to develop a sufficient therapeutic method based on the use of this inhibitor [11,18,32,71].

4.3. RP-6685

Recently, research on a new Polθi, RP-6685, was published by Bubenik et al. (2022) [12]. The authors discovered, synthesized, and characterized, via multiple biopsychical methods, a potent, selective, and orally bioavailable inhibitor of Polθ polymerase domain, similarly to ART558. The compound in in vitro and in vivo tests on cancer cells and mouse xenograft models, also HR-deficient, gives promising results. HEK293 LIG4-deficient cells exhibit decreased activity of Polθ-mediated repair pathway, after the treatment with RP-6685. Moreover, BRCA2$^{-/-}$ HCT116 cells revealed a lower proliferation rate caused by RP-6685 treatment. In addition, the mice model with the BRCA2$^{-/-}$ xenograft showed decrease of the tumor growth after first eight days of inhibitor administration, compared to vehicle. However, this effect did not last till the end of the 21st day of treatment. Nevertheless, the publication is rather focused on physicochemical characterization. Therefore, in our opinion, broader research is necessary for this inhibitor [12].

5. Conclusions and Prospects

Polθ may play a significant role in the human organism and be even more important in tumors. The level of its expression is elevated in cancer cells, while the depletion of Polθ achieved in various ways, genetically or chemically, leads to cancer cell death, especially in HR-deficient cells [8–11,13,62]. It is therefore difficult to distinguish the most efficient strategy to inhibit or knockout Polθ protein. However, with increasing knowledge and development, the CRISPR/Cas9 technology seems to be the most promising genetic method. Nevertheless, due to ethical issues, it is still not available for patient therapies, on the contrary to small-molecule inhibitors [38,39,55].

The discovery of Polθ and its inhibitors is undoubtedly a next chapter in cancer treatment. Nevertheless, Polθi research is in early stages and clinical studies are needed to prove their potency. There are high expectations that Polθi will be introduced into cancer therapies, however it is possible that cancer cells will also develop resistance to Polθi, similarly to the case with PARPi [11,13,24,31,51,62,72,73].

If Polθi eventually become registered anticancer drugs, combining them with inhibitors of other DNA-repair proteins such as PARP1, as well as using them in monotherapy could be tested in patients [11,13].

Attention should also be paid to the identification of biomarkers that could indicate tumors which are sensitive to Polθ inhibition. The level of Polθ expression itself could serve as a biomarker [13,62,73].

To summarize, Polθ inhibitors, such as novobiocin, ART558, and ART812, respectively, lead to the death of cancer cells both in vitro and in vivo, mostly in the case of HR-deficient cells. They induce biomarkers of DNA damage, such as RAD51 and RPA foci, γH2AX foci, or micronuclei formation, which may give satisfying results at the stage of pre-clinical research. Both small molecules, or compounds synthesized based on them, are being introduced in clinical trials. Therefore, many scientists are waiting impatiently for the results of the next steps of studies on NVB and ART558 as well as new candidates for Polθi [11,13,18,32,62,67]. Moreover, a newly discovered inhibitor, RP-6685, shows promising results in vitro and in vivo in reducing cancer development [12].

Polθ inhibitors and new Polθ synthetically lethal interactions are fast developing research topics. Results of research showing a new synthetically lethal interaction between novobiocin and DNA-PK inhibitor peposertib were published in August 2022 [62]. This illustrates the potential of the research in this topic and how much is still to be discovered.

Author Contributions: Visualization, writing—original draft preparation, G.B.-P.; writing—review & editing, M.D. and P.C.; conceptualization, funding acquisition, supervision, writing—review & editing, T.S. and T.Ś. All authors have read and agreed to the published version of the manuscript.

Funding: The APC was funded by Polish National Science Center 2020/37/B/NZ7/00422 [T Sliwinski]. T. Skorski was supported by the grants from NIH/NCI 1R01 CA244179, 1R01 CA247707, 2R01 CA186238, and by the Leukemia and Lymphoma Society TRP 6628-21.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: All figures were created with BioRender.com.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Hanahan, D.; Weinberg, R.A. Hallmarks of cancer: The next generation. *Cell* **2011**, *144*, 646–674. [CrossRef] [PubMed]
2. Kaelin, W.G., Jr. The concept of synthetic lethality in the context of anticancer therapy. *Nat. Rev. Cancer* **2005**, *5*, 689–698. [CrossRef] [PubMed]
3. Feng, W.; Simposon, D.A.; Carvajal-Garcia, J.; Price, B.A.; Kumar, R.J.; Mose, L.E.; Wood, R.D.; Rashid, N.; Purvis, J.E.; Parker, J.S.; et al. Genetic determinants of cellular addiction to DNA polymerase theta. *Nat. Commun.* **2019**, *10*, 4286. [CrossRef]

4. Drzewiecka, M.; Barszczewska-Pietraszek, G.; Czarny, P.; Skorski, T.; Śliwiński, T. Synthetic Lethality Targeting Polθ. *Genes* **2022**, *13*, 1101. [CrossRef]
5. Ashworth, A.; Lord, C.J. Synthetic lethal therapies for cancer: What's next after PARP inhibitors? *Nat. Rev. Clin. Oncol.* **2018**, *15*, 564–576. [CrossRef]
6. Beagan, K.; Mcvey, M. Linking DNA polymerase theta structure and function in healthand disease. *Cell Mol. Life Sci.* **2016**, *73*, 603–615. [CrossRef] [PubMed]
7. Mateos-gomez, P.A.; Kent, T.; Kashkina, E.; Pomerantz, R.T.; Sfeir, A. The helicase domain of Polθ counteracts RPA to promote alt-NHEJ. *Nat. Struct. Mol. Biol.* **2017**, *24*, 1116–1123. [CrossRef] [PubMed]
8. Ceccaldi, R.; Liu, J.C.; Amunugama, R.; Hajdu, I.; Primack, B.; Petalcorin, M.I.R.; O'Connor, K.W.; Konstantinopoulos, P.A.; Elledge, S.J.; Boulton, S.J.; et al. Homologous recombination-deficient tumors are hyper- dependent on POLQ-mediated repair. *Nature* **2015**, *518*, 258–262. [CrossRef]
9. Pan, Q.; Wang, L.; Liu, Y.; Li, M.; Zhang, Y.; Peng, W.; Deng, T.; Peng, M.-L.; Jiang, J.-Q.; Tang, J.; et al. Knockdown of POLQ interferes the development and progression of hepatocellular carcinoma through regulating cell proliferation, apoptosis and migration. *Cancer Cell Int.* **2021**, *21*, 482. [CrossRef]
10. Lemée, F.; Bergoglio, V.; Fernandez-Vidal, A.; Machado-Silva, A.; Pillaire, M.J.; Bieth, A.; Gentil, C.; Baker, L.; Martin, A.L.; Leduc, C.; et al. DNA polymerase θ up-regulation is associated with poor survival in breast cancer, perturbs DNA replication, and promotes genetic instability. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 13390–13395. [CrossRef]
11. Zatreanau, D.; Robinson, H.M.R.; Alkhatib, O.; Boursier, M.; Finch, H.; Geo, L.; Grande, D.; Grinkevich, V.; Heald, R.A.; Langdon, S.; et al. Polθ inhibitors elicit BRCA-gene synthetic lethality and target PARP inhibitor resistance. *Nat. Commun.* **2021**, *12*, 3636. [CrossRef] [PubMed]
12. Bubenik, M.; Mader, P.; Mochirian, P.; Vallée, F.; Clark, J.; Truchon, J.F.; Perryman, A.L.; Pau, V.; Kurinov, I.; Zahn, K.E.; et al. Identification of RP-6685, an Orally Bioavailable Compound that Inhibits the DNA Polymerase Activity of Polθ. *J. Med. Chem.* **2022**, *65*, 13198–13215. [CrossRef] [PubMed]
13. Zhou, J.; Gelot, C.; Pantelidou, C.; Li, A.; Yücel, H.; Davis, R.E.; Färkkilä, A.; Kochupurakkal, B.; Syed, A.; Shapiro, G.I.; et al. A first-in-class polymerase theta inhibitor selectively targets homologous-recombination-deficient tumors. *Nat. Cancer* **2021**, *2*, 598–610. [CrossRef] [PubMed]
14. Trenner, A.; Sartori, A.A. Harnessing DNA Double-Strand Break Repair for Cancer Treatment. *Front. Oncol.* **2019**, *9*, 1–10. [CrossRef]
15. Kumar, R.J.; Chao, H.X.; Simpson, D.A.; Feng, W.; Cho, M.G.; Roberts, V.R.; Sullivan, A.R.; Shah, S.J.; Wozny, A.S.; Fagan-Solis, K.; et al. Dual inhibition of DNA-PK and DNA polymerase theta overcomes radiation resistance induced by p53 deficiency. *NAR Cancer* **2020**, *2*, zcaa038. [CrossRef] [PubMed]
16. Stoklosa, T.; Poplawski, T.; Koptyra, M.; Nieborowska-Skorska, M.; Basak, G.; Slupianek, A.; Rayevskaya, M.; Seferynska, I.; Herrera, L.; Blasiak, J.; et al. BCR/ABL Inhibits Mismatch Repair to Protect from Apoptosis and Induce Point Mutations. *Cancer Res.* **2008**, *68*, 2576–2580. [CrossRef]
17. Slupianek, A.; Schmutte, C.; Tombline, G.; Nieborowska-Skorska, M.; Hoser, G.; Nowicki, M.O.; Pierce, A.J.; Fishel, R.; Skorski, T. BCR/ABL Regulates Mammalian RecA Homologs, Resulting in Drug Resistance. *Mol. Cell* **2001**, *8*, 795–806. [CrossRef]
18. Schrempf, A.; Slyskova, J.; Loizou, J.I. Targeting the DNA Repair Enzyme Polymerase θ in Cancer Therapy. *Trends Cancer* **2021**, *7*, 98–111. [CrossRef]
19. Brambati, A.; Barry, R.; Sfeir, A. DNA Polymerase theta (Polθ) – an error-prone polymerase necessary for genome stability. *Curr. Opin. Genet. Dev.* **2020**, *60*, 119–126. [CrossRef]
20. Chen, X.S.; Pomerantz, R.T. DNA Polymerase θ: A Cancer Drug Target with Reverse Transcriptase Activity. *Genes* **2021**, *12*, 1146. [CrossRef]
21. Ramsden, D.A.; Carvajal-Garcia, J.; Gupta, G.P. Mechanism, cellular functions and cancer roles of polymerase- mediated DNA end joining. *Nat. Rev.* **2022**, *23*, 125–140. [CrossRef] [PubMed]
22. Hwang, T.; Reh, S.; Dunbayev, Y.; Zhong, Y.; Takata, Y.; Shen, J.; McBride, K.M.; Murnane, J.P.; Bhak, J.; Lee, S.; et al. Defining the mutation signatures of DNA polymerase θ in cancer genomes. *NAR Cancer* **2020**, *2*, zcaa017. [CrossRef] [PubMed]
23. Hanscom, T.; Woodward, N.; Batorsky, R.; Brown, A.J.; Roberts, S.A.; Mcvey, M. Characterization of sequence contexts that favor alternative end joining at Cas9-induced double-strand breaks. *Nucleic Acids Res.* **2022**, *50*, 7465–7478. [CrossRef] [PubMed]
24. Luedeman, M.E.; Stroik, S.; Feng, W.; Luthman, A.J.; Gupta, G.P.; Ramsden, D.A. Poly (ADP) ribose polymerase promotes DNA polymerase theta-mediated end joining by activation of end resection. *Nat. Commun.* **2022**, *13*, 4547. [CrossRef] [PubMed]
25. Schaub, J.M.; Soniat, M.M.; Finkelstein, I.J. Polymerase theta-helicase promotes end joining by stripping single-stranded DNA-binding proteins and bridging DNA ends. *Nucleic Acids Res.* **2022**, *50*, 3911–3921. [CrossRef] [PubMed]
26. Wood, R.D.; Doublié, S. DNA polymerase θ (POLQ), double-strand break repair, and cancer. *DNA Repair* **2016**, *44*, 22–32. [CrossRef]
27. Kawamura, K.; Bahar, R.; Seimiya, M.; Chiyo, M.; Wada, A.; Okada, S.; Hatano, M.; Tokuhisa, T.; Kimura, H.; Watanabe, S.; et al. DNA polymerase θ is preferentially expressed in lymphoid tissues and upregulated in human cancers. *Int. J. Cancer* **2004**, *109*, 9–16. [CrossRef]
28. Schimmel, J.; Kool, H.; van Schendel, R.; Tijsterman, M. Mutational signatures of non-homologous and polymerase theta-mediated end-joining in embryonic stem cells. *EMBO J.* **2017**, *36*, 3634–3649. [CrossRef]

29. Caracciolo, D.; Riillo, C.; Di Martino, M.T.; Tagliaferri, P.; Tassone, P. Alternative Non-Homologous End-Joining: Error-Prone DNA Repair as Cancer's Achilles' Heel. *Cancers* **2021**, *13*, 1392. [CrossRef]
30. Carvajal-Garcia, J.; Cho, J.E.; Carvajal-Garcia, P.; Feng, W.; Wood, R.D.; Sekelsky, J.; Gupta, G.P.; Roberts, S.A.; Ramsden, D.A. Mechanistic basis for microhomology identification and genome scarring by polymerase theta. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 8476–8485. [CrossRef]
31. Liddiard, K.; Aston-Evans, A.N.; Cleal, K.; Hendrickson, E.A.; Baird, D.M. POLQ suppresses genome instability and alterations in DNA repeat tract lengths. *NAR Cancer* **2022**, *4*, zcac020. [CrossRef] [PubMed]
32. Wilson, J.; Loizou, J.I. Exploring the genetic space of the DNA damage response for cancer therapy through CRISPR-based screens. *Mol. Oncol.* **2022**, *16*, 3778–3791. [CrossRef] [PubMed]
33. Ferreira da Silva, J.; Salic, S.; Wiedner, M.; Datlinger, P.; Essletzbichler, P.; Hanzl, A.; Superti-Furga, G.; Bock, C.; Winter, G.; Loizou, J.I. Genome-scale CRISPR screens are efficient in non-homologous end-joining deficient cells. *Sci. Rep.* **2019**, *9*, 15751. [CrossRef]
34. Mara, K.; Charlot, F.; Guyon-Debast, A.; Schaefer, D.G.; Collonnier, C.; Grelon, M.; Nogué, F. POLQ plays a key role in the repair of CRISPR/Cas9-induced double-stranded breaks in the moss *Physcomitrella patens*. *New Phytol.* **2019**, *222*, 1380–1391. [CrossRef] [PubMed]
35. Dai, C.H.; Chen, P.; Li, J.; Lan, T.; Chen, Y.C.; Qian, H.; Chen, K.; Li, M.Y. Co-inhibition of pol θ and HR genes efficiently synergize with cisplatin to suppress cisplatin-resistant lung cancer cells survival. *Oncotarget* **2016**, *7*, 65157–65170. [CrossRef] [PubMed]
36. Kelso, A.A.; Lopezcolorado, F.W.; Bhargava, R.; Stark, J.M. Distinct roles of RAD52 and POLQ in chromosomal break repair and replication stress response. *PLoS Genet.* **2019**, *15*, e1008319. [CrossRef] [PubMed]
37. Khan, F.A.; Pandupuspitasari, N.S.; Chun-Jie, H.; Ao, Z.; Jamal, M.; Zohaib, A.; Khan, F.A.; Hakim, M.R.; ShuJun, Z. CRISPR/Cas9 therapeutics: A cure for cancer and other genetic diseases. *Oncotarget* **2016**, *7*, 52541–52552. [CrossRef]
38. Ferreira, P.; Choupina, A.B. CRISPR/Cas9 a simple, inexpensive and effective technique for gene editing. *Mol. Biol. Rep.* **2022**, *49*, 7079–7086. [CrossRef]
39. Wang, Y.; Huang, C.; Zhao, W. Recent advances of the biological and biomedical applications of CRISPR/Cas systems. *Mol. Biol. Rep.* **2022**, *49*, 7087–7100. [CrossRef]
40. Pushparaj, P.N.; Aarthi, J.J.; Manikandan, J.; Kumar, S.D. siRNA, miRNA, and shRNA: In vivo Applications. *JDR* **2008**, *87*, 992–1003. [CrossRef]
41. Alshaer, W.; Zureigat, H.; Al Karaki, A.; Al-Kadash, A.; Gharaibeh, L.; Hatmal, M.M.; Aljabali, A.A.A.; Awidi, A. siRNA: Mechanism of action, challenges, and therapeutic approaches. *Eur. J. Pharmacol.* **2021**, *905*, 174178. [CrossRef] [PubMed]
42. Sliva, K.; Schnierle, B.S. Selective gene silencing by viral delivery of short hairpin RNA. *Virol. J.* **2010**, *7*, 248. [CrossRef] [PubMed]
43. McAnuff, M.A.; Rettig, G.R.; Rice, K.G. Potency of siRNA versus shRNA mediated knockdown in vivo. *J. Pharm. Sci.* **2007**, *96*, 2922–2930. [CrossRef] [PubMed]
44. Rao, D.D.; Vorhies, J.S.; Senzer, N.; Nemunaitis, J. siRNA vs. shRNA: Similarities and differences. *Adv. Drug Deliv. Rev.* **2009**, *61*, 746–759. [CrossRef] [PubMed]
45. Dana, H.; Chalbatani, G.M.; Mahmoodzadeh, H.; Karimloo, R.; Rezaiean, O.; Moradzadeh, A.; Mehmandoost, N.; Moazzen, F.; Mazraeh, A.; Marmari, V.; et al. Molecular Mechanisms and Biological Functions of siRNA. *Int. J. Biomed. Sci.* **2017**, *13*, 48–57.
46. Nikam, R.R.; Gore, K.R. Journey of siRNA: Clinical Developments and Targeted Delivery. *Nucleic Acid. Ther.* **2018**, *28*, 209–224. [CrossRef]
47. Lee, W.C.; Berry, R.; Hohenstein, P.; Davies, J. siRNA as a tool for investigating organogenesis: The pitfalls and the promises. *Organogenesis* **2008**, *4*, 176–181. [CrossRef]
48. Moore, C.B.; Guthrie, E.H.; Huang, M.T.; Taxman, D.J. Short Hairpin RNA (shRNA): Design, Delivery, and Assessment of Gene Knockdown. *Methods Mol. Biol.* **2010**, *629*, 141–158.
49. Goullet de Rugy, T.; Bashkurov, M.; Datti, A.; Betous, R.; Guitton-Sert, L.; Cazaux, C.; Durocher, D.; Hoffmann, J.S. Excess Polθ functions in response to replicative stress in homologous recombination-proficient cancer cells. *Biol. Open.* **2016**, *5*, 1485–1492. [CrossRef]
50. Savić, N.; Schwank, G. Advances in therapeutic CRISPR/Cas9 genome editing. *Transl. Res.* **2016**, *168*, 15–21. [CrossRef]
51. Vaghari-Tabari, M.; Hassanpour, P.; Sadeghsoltani, F.; Malakoti, F.; Alemi, F.; Qujeq, D.; Asemi, Z.; Yousefi, B. CRISPR/Cas9 gene editing: A new approach for overcoming drug resistance in cancer. *Cell. Mol. Biol. Lett.* **2022**, *27*, 49. [CrossRef]
52. Ma, Y.; Zhang, L.; Huang, X. Genome modification by CRISPR/Cas9. *FEBS J.* **2014**, *281*, 5186–5193. [CrossRef]
53. Lu, Y.; Xue, J.; Deng, T.; Zhou, X.; Yu, K.; Deng, L.; Huang, M.; Yi, X.; Liang, M.; Wang, Y.; et al. Safety and feasibility of CRISPR-edited T cells in patients with refractory non-small-cell lung cancer. *Nat. Med.* **2020**, *26*, 732–740. [CrossRef] [PubMed]
54. Redman, M.; King, A.; Watson, C.; King, D. What is CRISPR/Cas9? *Arch. Dis. Child. Educ. Pract. Ed.* **2016**, *101*, 213–215. [CrossRef] [PubMed]
55. Boettcher, M.; Mcmanus, M.T. Choosing the Right Tool for the Job: RNAi, TALEN or CRISPR. *Mol. Cell* **2015**, *58*, 575–585. [CrossRef] [PubMed]
56. Pugh, K.W.; Zhang, Z.; Wang, J.; Xu, X.; Munthali, V.; Zuo, A.; Blagg, B.S.J. From Bacteria to Cancer: A Benzothiazole-Based DNA Gyrase B Inhibitor Redesigned for Hsp90 C-Terminal Inhibition. *ACS Med. Chem. Lett.* **2020**, *11*, 1535–1538. [CrossRef]
57. Hyun, S.Y.; Le, H.T.; Nguyen, C.T.; Yong, Y.S.; Boo, H.J.; Lee, H.J.; Lee, J.S.; Min, H.Y.; Ann, J.; Chen, J.; et al. Development of a novel Hsp90 inhibitor NCT-50 as a potential anticancer agent for the treatment of non-small cell lung cancer. *Sci. Rep.* **2018**, *8*, 13924. [CrossRef]

58. Forsberg, L.K.; Davis, R.E.; Wimalasena, V.; Blagg BS, J. Exploiting Polarity and Chirality to Probe the Hsp90 C-terminus. *Bioorg. Med. Chem.* **2019**, *26*, 3096–3110. [CrossRef]
59. Zhou, X.; Wen, Y.; Tian, Y.; He, M.; Ke, X.; Huang, Z.; He, Y.; Liu, L.; Scharf, A.; Lu, M.; et al. Heat Shock Protein 90α-Dependent B-Cell-2-Associated Transcription Factor 1 Promotes Hepatocellular Carcinoma Proliferation by Regulating MYC Proto-Oncogene c-MYC mRNA Stability. *Hepatology* **2019**, *69*, 1564–1581. [CrossRef]
60. Garg, G.; Forsberg, L.K.; Zhao, H.; Blagg BS, J. Development of Phenyl Cyclohexylcarboxamides as a Novel Class of Hsp90 C-terminal Inhibitors. *Chemistry* **2017**, *23*, 16574–16585. [CrossRef]
61. Forsberg, L.K.; Liu, W.; Holzbeierlein, J.; Blagg, B.S.J. Modified Biphenyl Hsp90 C-terminal Inhibitors for the Treatment of Cancer. *Bioorg. Med. Chem. Lett.* **2017**, *27*, 4514–4519. [CrossRef] [PubMed]
62. Patterson-Fortin, J.; Bose, A.; Tsai, W.C.; Grochala, C.; Nguyen, H.; Zhou, J.; Parmar, K.; Lazaro, J.B.; Liu, J.; McQueen, K.; et al. Targeting DNA Repair with Combined Inhibition of NHEJ and MMEJ Induces Synthetic Lethality in TP53-Mutant Cancers. *Cancer Res.* **2022**, *88*, 3815–3829. [CrossRef] [PubMed]
63. Higgins, G.S.; Boulton, S.J. Beyond PARP-POLθ as an anticancer target. *Science* **2018**, *359*, 1217–1219. [CrossRef] [PubMed]
64. Wu, J.; Lu, L.Y.; Yu, X. The role of BRCA1 in DNA damage response. *Protein Cell* **2010**, *1*, 117–123. [CrossRef] [PubMed]
65. Noordermeer, S.M.; Adam, S.; Setiaputra, D.; Barazas, M.; Pettitt, S.J.; Ling, A.K.; Olivieri, M.; Álvarez-Quilón, A.; Moatti, N.; Zimmermann, M.; et al. The shieldin complex mediates 53BP1-dependent DNA repair. *Nature* **2018**, *560*, 117–121. [CrossRef] [PubMed]
66. Dev, H.; Chiang, T.W.; Lescale, C.; de Krijger, I.; Martin, A.G.; Pilger, D.; Coates, J.; Sczaniecka-Clift, M.; Wei, W.; Ostermaier, M.; et al. Shieldin complex promotes DNA end-joining and counters homologous recombination in BRCA1-null cells. *Nat Cell Biol.* **2018**, *20*, 954–965. [CrossRef] [PubMed]
67. Zahn, K.E.; Jensen, R.B. Polymerase θ Coordinates Multiple Intrinsic Enzymatic Activities during DNA Repair. *Genes* **2021**, *12*, 1310. [CrossRef]
68. Eder, J.P.; Wheeler, C.A.; Teicher, B.A.; Schnipper, L.E. A phase I clinical trial of novobiocin, a modulator of alkylating agent cytotoxicity. *Cancer Res.* **1991**, *51*, 510–513.
69. Makridakis, N.M.; Reichardt, J.K.V. Translesion DNA polymerases and cancer. *Front. Genet.* **2012**, *3*, 174. [CrossRef]
70. de Lange, T. Shelterin: The protein complex that shapes and safeguards human telomeres. *Genes Dev.* **2005**, *19*, 2100–2110. [CrossRef]
71. Available online: https://clinicaltrials.gov/ct2/show/NCT04991480 (accessed on 15 November 2022).
72. Baxter, J.S.; Zatreanu, D.; Pettitt, S.J.; Lord, C.J. Resistance to DNA repair inhibitors in cancer. *Mol. Oncol.* **2022**, *16*, 3811–3827. [CrossRef] [PubMed]
73. Carvajal-Maldonado, D.; Wood, R.D. Regulating Polθ in Breast Cancer. *Cancer Res.* **2021**, *81*, 1441–1442. [CrossRef] [PubMed]

International Journal of *Molecular Sciences*

MDPI

Research article

Polθ inhibitor (ART558) demonstrates synthetic lethal effect with PARP and RAD52 inhibitors in glioblastoma cells.

Gabriela Barszczewska-Pietraszek [1], Piotr Czarny[2], Małgorzata Drzewiecka[1], Maciej Błaszczyk[3], Maciej Radek[3], Ewelina Synowiec[1], Paulina Wigner-Jeziorska[1], Przemysław Sitarek[4], Janusz Szemraj[2], Tomasz Skorski[5], Tomasz Śliwiński [1,*]

[1] Department of Molecular Genetics, Faculty of Biology and Environmental Protection, University of Lodz, 90-236 Lodz, Poland, gabriela.barszczewska.pietraszek@edu.uni.lodz.pl (G.B.-P.)
[2] Department of Medical Biochemistry, Medical University of Lodz, 92-216 Lodz, Poland; piotr.czarny@umed.lodz.pl (P.C.)
[3] Department of Neurosurgery, Surgery of Spine and Peripherial Nerves, Medical University of Lodz, University Hospital WAM-CSW, 90-549, Lodz, Poland
[4] Department of Medical Biology, Medical University of Lodz, 92-151 Lodz, Poland
[5] Fels Cancer Institute for Personalized Medicine, Lewis Katz School of Medicine, Temple University, Philadelphia, PA 19140, USA, tskorski@temple.edu.
* Correspondence: tomasz.sliwinski@biol.uni.lodz.pl

Abstract: DNA repair proteins became the popular targets in research on cancer treatment. In our studies we hypothesized that inhibition of DNA polymerase theta (Polθ) and its combination with Poly (ADP-ribose) polymerase 1 (PARP1) or RAD52 inhibition and alkylating drug temozolomide (TMZ) has anticancer effect on glioblastoma cells (GBM21), whereas low impact on normal human astrocytes (NHA). The effect of the compounds was assessed by analysis of cell viability, apoptosis, proliferation, DNA damage and cell cycle distribution, as well as gene expression. The main results shows that Polθ inhibition causes decrease of glioblastoma cells viability by almost 50%. It induces apoptosis which is accompanied by reduction of cell proliferation and DNA damage. Moreover, the effect is stronger when dual inhibition of Polθ with PARP1 or RAD52 was applied, and it is further enhanced by addition of TMZ . The impact on normal cells is much lower, especially considering cells viability and DNA damage. In conclusion we would like to highlight that Polθ inhibition used in combination with PARP1 or RAD52 inhibition has great potential to kill glioblastoma cells, showing synthetic lethal effect, while sparing normal astrocytes.

Keywords: Polθ; RAD52; PARP1 inhibitors; synthetic lethality; DNA repair, DNA damage; glioblastoma; personalized therapy

Citation: To be added by editorial staff during production.

Academic Editor: Firstname Lastname

Received: date
Revised: date
Accepted: date
Published: date

1. Introduction

Glioblastoma (GBM) previously known as Glioblastoma Multiforme now is recognized as Glioblastoma, *Isocitrate dehydrogenase (IDH)* wild-type according to WHO classification of Tumors of the Central Nervous System from 2021, and is treated separately from Astrocytoma, *IDH*-mutant tumors, differently than in previous WHO classification [1,2]. GBM is an adult-type, diffuse, IV grade glioma considered as a most popular and dangerous brain cancer, the median survival time for diagnosed patient is 15 months [1,3–5]. Primary glioblastoma is formed from glial cells and could be characterized by one of these changes: microvascular proliferation, necrosis, *EGFR* amplification, *TERT* promoter mutation, or combined gain of chromosome 7/loss of chromosome 10 copy number [1,2].

Traditional treatment of GBM includes surgical resection if possible, radiotherapy and chemotherapy from which the first recommendation is the therapy with temozolomide (TMZ), commercially available drug, approved by Food and Drug Administration (FDA) and European Medical Agency (EMA) [3,4,6] . TMZ was found to promote methylation of DNA, thus creation of O6-methylguanine which leads to DNA damage and apoptosis in tumor cells [7-9]. We applied TMZ to our research due to its widespread use in patients treatment. Our objective was to assess its effectiveness with simultaneous inhibition of DNA repair proteins, in context of potential combined therapy and its impact on healthy tissues.

Despite existing possibilities, there are many barriers in the treatment of GBM, such as localization of the tumor, its spreading character, hindered drug delivery, heterogeneity of the tumor tissue and continuous issue with tumor recurrence and drug resistance [2]. Therefore, the need for development of the new more precise and efficient treatment is urgent.

The approach of personalized therapy is currently developing in oncology. Studies analyzing molecular markers, gene expression, whole genome sequencing, and epigenetics enable the identification of tumors with particular genetic modifications [10,11]. Mutations in DNA repair pathways, especially of double-strand breaks (DSBs), are important for cancer growth. The cancer cells with repair alterations develop selective growth dominance, combined with genetic instability and further progression. However, usually it makes cancer dependent on one DNA repair pathway, which creates opportunities to defeat tumor cells [11]. The strategy of synthetic lethality (SL) is applied in such cases. The SL is based on affecting two genes simultaneously to kill cells, while silencing any of these genes separately is not lethal[12]. The examples of such treatment are the small molecules inhibitors targeting proteins involved in DNA DSBs repair mechanisms, such as Polθ, PARP1 and Rad52. The inhibitors could be applied in combination to eliminate activity of two or more repair pathways simultaneously or in tumors with specific deficiencies, e.g. common homologous recombination (HR) defect, *BRCA1/2* mutation. The second approach is particularly important for precision medicine since it allows to spare nonmalignant cells [13–15].

The first to obtain significant success in treatment based on SL interaction were PARP inhibitors targeting BRCA-mutated cancer cells. Poly (ADP-ribose) polymerases (PARPs) are DNA repair proteins, which play important role in various processes including HR, base excision repair (BER), classical and alternative non-homologous end joining (NHEJ), nucleotide excision repair (NER), maintenance of replication fork stability, and mismatch repair (MMR). The main representatives are PARP1 and PARP2 proteins [16,17]. There are five different inhibitors that have been approved in the treatment of ovarian and breast cancer in Europe and the United States, i.e. olaparib, rucaparib, niraparib, veliparib and talazoparib [18,19]. Talazoparib is the one used in our study. It is shown that it exhibits cytotoxic effect on cancer cells via two mechanisms: inhibition of PARP catalytic activity and PARP blockade at the site of DNA damage thereby stopping further DNA repair and leading to apoptosis and/or cell death [20,21].

Since the success of PARP inhibitors against BRCA-mutated cancer cells, interest in identifying potential SL targets, e.g. Polθ, has grown. However, the tumors resistance to PARPi has created yet unmet need to expand research in this area [22].

Polθ is a DNA polymerase involved in theta-mediated end joining (TMEJ) – a DSBs repair pathway distinct from other mechanisms by independence on Ku, XRCC4 and LIG4 proteins, presence of resected DNA ends with 3′ single-stranded overhangs and several nucleotide-long microhomology regions. This is the main activity of Polθ, but not only, the protein is engaged in other molecular mechanisms such as translesion synthesis, base excision repair, mismatch repair, replication-associated DNA breaks, or reverse transcription and interstrand crosslinks repair [23,24]. Several Polθ inhibitors, which gained special interest has been developing in laboratories and clinical studies, i.e. Novobiocin, ART558, ART812, ART4215 and RP-6685 described very precisely by Pismataro et al., 2023 with

four registered clinical studies (NCT05687110, NCT06077877, NCT04991480, NCT05898399; clinicaltrials.gov, accessed on 16 January 2024) [11,25–27]. ART558 was applied in in this study. This is the inhibitor which allosterically anchors to binding site of Polθ polymerase catalytic domain and stabilizes it thermally in DNA presence, together leading to disrupted activity of Polθ [11,25].

The third protein engaged to our study – RAD52 due to its ability to anneal ssDNA plays very important role in various mechanisms, i.e. HR repair and SSA, replication fork stabilization and assembly of a displacement loop (D-loop). A well-known inhibitor of RAD52, 6-OH-DOPA disassociate RAD52 ring superstructure transferring it to dimers what results in repressed protein function [28,29].

The previous studies of our group demonstrated synthetically lethal interaction between PARP inhibitor (BMN) and class I histon deacetylases, as well as PARPi and LIG4 deficiency in glioblastoma cells [30,31]. To our knowledge this is first published studies demonstrating influence of Polθi – ART558 and PARPi – BMN673 or RAD52i – L-OH-DOPA on glioblastoma cells.

2. Methodology

2.1. In vitro cell culture

Glioblastoma cell line derived from surgical specimens, was obtained from the patients of the Department of Neurosurgery, Surgery of Spine and Peripheral Nerves, University Hospital WAM-CSW Lodz. The cell line was established in the Laboratory of Medical Biochemistry Department, Medical University of Lodz and named GBM21. The study was approved by the Ethical Commission of the Medical University of Lodz (RNN/23/22/KE), and informed consent was obtained from all patients. To obtain cell line tissue fragment was washed several times with HBSS buffer (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and minced mechanically with a scalpel in sterile conditions. The shredded tissue fragments were passed through a filter with a pore size of 70 μm, and then centrifuged. If a large number of red blood cells were present in the cell pellet, RBC lysis buffer (Sigma-Aldrich) was used.

Glioblastoma cells were cultured in DMEM/F12 medium (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% FBS (Lonza), 100 IU/ml penicillin, 100 μg/ml streptomycin (Lonza) and gentamycin 50 μg/ml (Lonza) in a humidified atmosphere containing 5% CO2 at 37°C. Once the cells have multiplied they were subjected to positive selection via magnetic-activated cell sorting on magnetic separator MiniMACS™ (Miltenyi Biotec) with use of CD133 magnetic microbeads MACS® (Miltenyi Biotec). The Normal Human Astrocytes – NHA (Lonza) were grown in ABM™ Basal Medium supplemented with AGM™ SingleQuots™ Supplements (Lonza) and cultured according to the protocol provided by manufacturer.

GBM21 cell line was tested for *POLQ* gene expression and it exhibited its overexpression versus NHA cells.

2.2. Drug treatment

In the experiments following compounds were used: inhibitor of Polθ – ART558 (MedChem Express), 56,5 μM; inhibitor of PARP1 – talazoparib (BMN673) (Selleckchem), 110 nM; inhibitor of RAD52 – L-OH-DOPA (Sigma-Aldrich), 62,5 μM and alkylating drug – temozolomide (TMZ), 37 μM. Compounds were dissolved appropriately according to the manufacturer's instructions in distilled water or DMSO to a starting concentration of 10 mM, and then working concentrations were prepared immediately before the experiment in DMEM/F12 culture medium. The treatment scheme was established and proved to work in previous experiments. Shortly, cells were incubated with the compounds for 120 h with the second dose after 48 h [30-33]. Following variants of the treatment were used: ART558, BMN673, L-OH-DOPA and TMZ separately, ART558 +

BMN673, ART558 + L-OH-DOPA, ART558 + TMZ, BMN673 + TMZ, L-OH-DOPA + TMZ, ART558 + BMN673 + TMZ, ART558 + L-OH-DOPA + TMZ.

2.3. Flow cytometric analysis of apoptosis and necrosis

Changes in viability and mechanism of cell death after standard treatment described above were analyzed using staining with propidium iodide and FITC Annexin V. Cells were prepared and analyzed according to the FITC Annexin Apoptosis Detection Kit II (BD Biosciences, Franklin Lakes, New Jersey, USA) by flow cytometry. Annexin V has strong affinity to phosphatidylserine, which appears on the cell's surface during early apoptosis, while propidium iodide binds to DNA by penetrating through the fragmented cell membrane, which is characteristic of necrosis and late stages of apoptosis. Cell viability results were also obtained using this assay.

2.4. Cell morphology visualized by fluorescence microscopy

To visualize the influence of inhibitors on cell viability, normal and cancer cells were subjected to calcein AM and propidium iodide (PI) double staining. Cells were stained after standard treatment and incubated for 30 min at 37°C with the mixture of 2 mM calcein AM and propidium iodide 1 mM (Thermo Fisher Scientific, Waltham, Massachusetts, USA) diluted in PBS. Calcein AM, the acetoxymethyl ester of calcein, freely penetrates the membranes of living cells, where the acetoxymethyl group is degraded, allowing calcium binding to calcein showing strong green fluorescence, when excited. Propidium iodide stains the DNA of dead cells showing low plasma membrane integrity, indicated by red fluorescence signal. The results were observed and pictured in an inverted fluorescence microscope LEICA.

2.5. Clonogenic assay

The cancer cells ability to form colonies were measured by clonogenic assay. Prior the test cells had undergone the standard treatment described above. Then, trypan blue staining was used to assess the viability of the treated cells. Following this, 10^3 cells were resuspended in 700 µl of soft agar 0,4% containing DMEM, FBS and antibiotics and plated on a 12-well plate over 700 µl of solidified agar underlay (0.5% also with DMEM, FBS and antibiotics). The medium was applied over the solidified cell layer and changed weekly. Prepared plates were incubated for 2 weeks, at 37 °C, 5% CO_2. The colonies were stained with 0.005% crystal violet and counted under the microscope. Clonogenic efficiency was expressed as percent of untreated control (no. of colonies after treatment vs no. of colonies in control sample × 100%).

2.6. Cell cycle

To analyze the influence of the compounds on cell cycle distribution of glioblastoma and NHA population, cells fixed with 70% cold ethanol were stained with propidium iodide with addition of RNase (BD Biosciences) and analyzed by flow cytometry.

2.7. Measurement of Histone H2AX phosphorylation

The levels of phosphorylated Histone H2AX, constituting of DNA DSBs, were measured by H2AX Phosphorylation Assay Kit (Merck KGaA, Darmstadt, Germany). After the standard treatment the cells were fixed and permeabilized to facilitate staining and detection. The presence of Histone H2AX phosphorylated at serine 139 was detected using a FITC-conjugated anti-phospho-Histone H2AX antibody. Flow cytometry was employed to quantify the number of cells exhibiting positive staining for phosphorylated Histone H2AX.

2.8. Neutral Comet assay

The level of DNA double-strand breaks generated by the used compounds was studied using the neutral comet assay. Following the standard treatment, cells were exposed to gamma radiation of 8 Gy. After that, cell were resuspended in 0.4% low melting point (LMP) agarose solution and immediately applied to the precoated slide with 0.5% normal melting point (NMP) agarose. Prepared slides were subjected to overnight lysis (2.5 M/L NaCl, 100 mM/L EDTA, 10 mM/L TRIS). Then, the slides were placed in developing buffer (300 mM/L NaOH, 1 mM/L EDTA) for 20 minutes. After that, electrophoresis was carried out in the electrophoretic buffer (300 mM sodium acetate, 100mM TRIS) for 1 hour, at 9V and 100 mA. After finishing electrophoresis, the slides rinsed with water were stained with DAPI solution (100 μg/mL) by applying 50 μL of solution to each slide and incubating the slides for at least 45 minutes, at 4°C. To visualize the results, the slides were observed at 200× magnification in an Eclipse fluorescence microscope (Nikon, Tokyo, Japan) attached to a COHU 4910 video camera (Cohu, Inc., San Diego, CA, USA) equipped with a UV-1 A filter block and connected to a personal computer-based image analysis system Lucia-Comet v. 6.0 (Laboratory Imaging, Praha, Czech Republic), 50 comets were counted from each repetition of the experiment. The % of the DNA in comet tail was taken into account.

2.9. Statistical Analysis

Data from at least three independent experiments were analyzed and presented as mean ± SEM. The results were compared in SigmaPlot, using one-way ANOVA with the Holm-Sidak post hoc test. All graphs were done in GraphPad Prism 10. *P*-values of <0.05 were considered statistically significant.

3. Results

3.1. ART558 inhibitor used alone or in combination with PARP1/RAD52 inhibitors and alkylating agent TMZ leads to cell death via apoptosis in patient-derived glioblastoma cells

3.1.1 Cell viability

Inhibition of Polθ or PARP1 or RAD52 gives significant reduction of glioblastoma cell viability in comparison to untreated control (Fig.1 A,C), what is not observe in case of normal cells NHA (Fig.1 B,D). The combination of Polθ inhibition with either PARP1 or RAD52 enhances the effect significantly in comparison to the control and to inhibition of Polθ alone. Although, double treatment with Polθi and RAD52i decreases NHA cell viability significantly versus the control and Polθi, not in such degree as in cancer cells. The addition of TMZ to Polθ inhibition gives significant reduction of cell viability in GBM21 but not NHA. Interestingly, separate treatment with TMZ gives significant reduction of NHA viability, what shows its negative influence on healthy cells. The strongest effect, and significantly higher than single inhibitions or treatment with TMZ and double combinations, was obtained when three compounds together, either Polθi, PAPRi and TMZ or Polθi, RAD52i and TMZ in both cell lines. However, this effect in GBM21 is around 4 times stronger than in NHA.

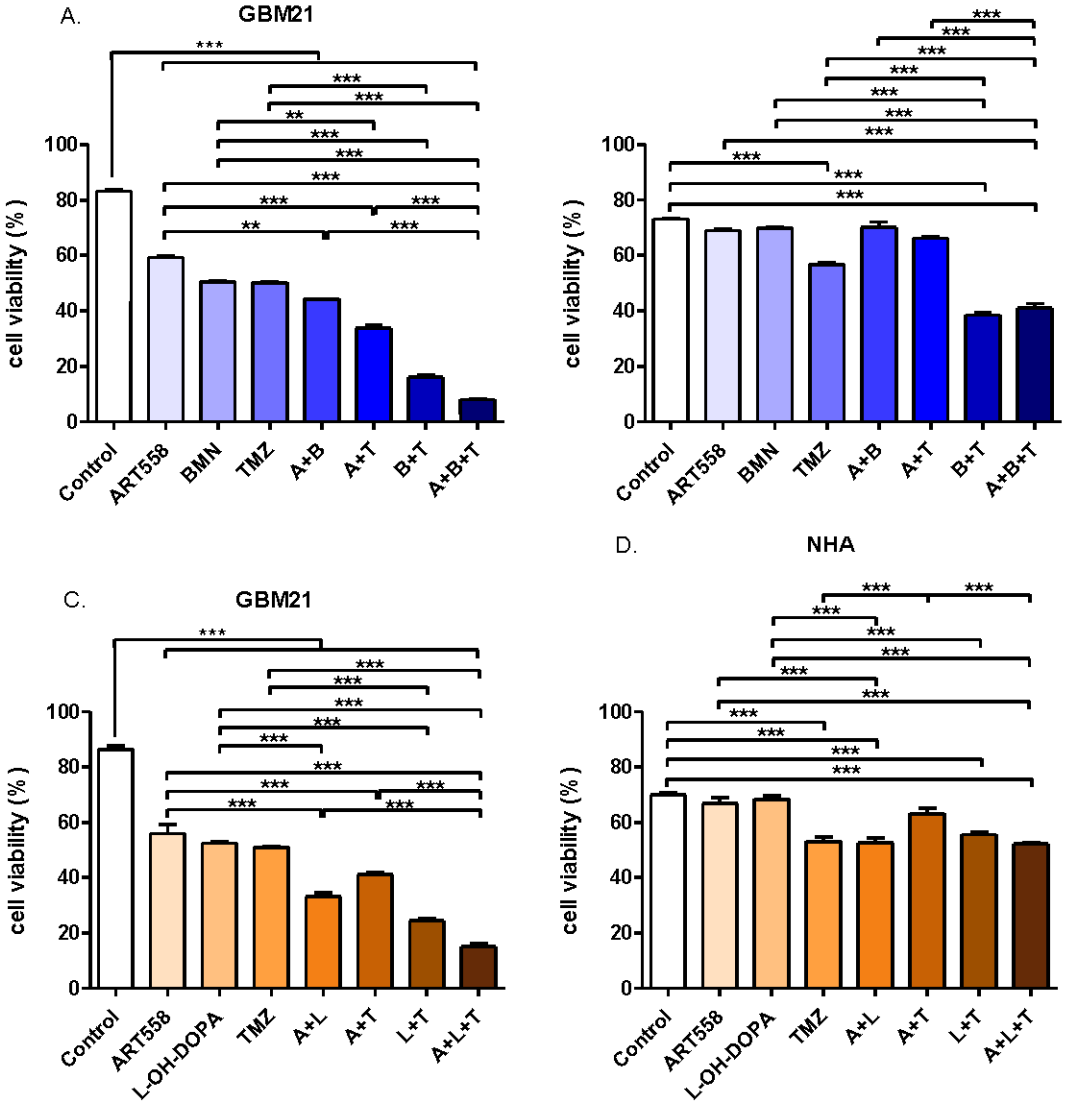


Fig. 1 The effect of DNA repair proteins inhibition and treatment with alkylating agent and their combinations on cancer GBM21 and normal NHA cells viability. Three independent experiments were performed, and the results are shown as the mean ± standard error of the mean (SEM). * $p \leq 0.05$, ** $p \leq 0.01$, *** p-value ≤ 0.001; A., C. In comparison to the control all results are statistically significant with p-value ≤ 0.001; A – ART558, B – BMN673, L – L-OH-DOPA, T – TMZ.

3.1.2 Visualization of morphological changes by double Calcein AM/PI staining

Morphological changes induced by ART558 +/- were assessed by Calcein AM/PI double staining (Figure 2) Cells treated with the inhibitors showed the characteristic hallmarks of cellular homeostasis disorders such as disrupted cellular membrane integrity well visible by penetration of PI. Also, the change in cell size and general appearance of the cells could be observed. These morphology changes were much more noticeable in cancer than in normal cells, where almost no differences were observed. These alterations of cellular morphology were in agreement with the increasing number of dead cells stained with PI especially in samples treated with ART558, TMZ and BMN or L-OH-DOPA. Based on visual assessment the evident differences between treatment groups are observed: separate usage of compounds give around 10 % of dead cells, dual inhibition and Polθ with TMZ up to 50%, and the rest of treatment variants more than 50%.

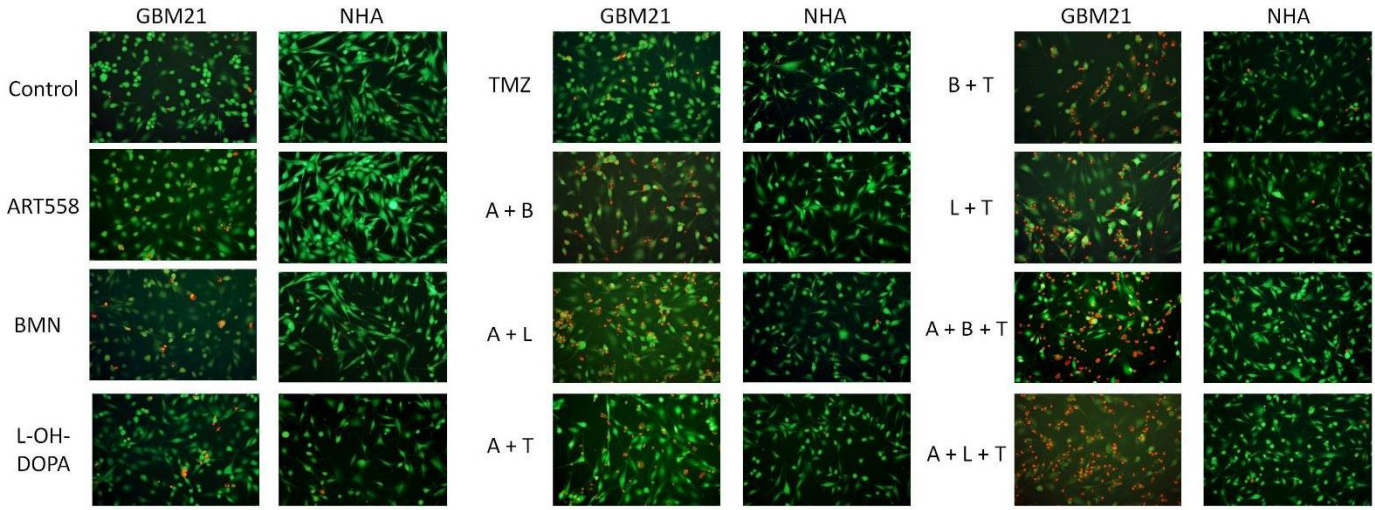


Fig. 2 The effect of DNA repair proteins inhibition and treatment with alkylating agent and their combinations on cancer GBM21 and normal NHA cells, visualized with Calcein AM and PI staining, magnification of the pictures is 10x. A – ART558, B – BMN673, L – L-OH-DOPA, T – TMZ.

3.1.3 Cell death mechanism

Double staining with PI and FITC Annexin V was used to determine whether the compounds induced apoptosis. Most of the cancer cells after treatment are in stage of late apoptosis, some of them in early apoptosis, and little part in necrosis, which indicate that inhibitors and TMZ lead to apoptotic cell death. Percentage of cancer cells in early apoptosis was significantly increased after the treatment with combination of Polθi and PARPi or RAD52i, Polθi and TMZ, and three compounds together in comparison to the control and separate inhibition of Polθ. Similar correlation was observed in cells population in late stage of apoptosis, despite the fact that combined inhibition of Polθ with PARP or RAD52 did not give the significant increase in comparison to separate inhibition of these proteins (Fig. 3 A,C). Interestingly, higher population of necrotic cells were observe in NHA than GBM21, suggesting that normal cells less likely undergone apoptosis. Moreover, dual inhibition of Polθ and PARP with TMZ significantly increased necrotic population of normal cells. In GBM21 differences between variants of treatment and control were

not detectable in case of necrosis. The combined treatment with Polθi and PARPi and TMZ significantly elevated the level of NHA cells in early and late apoptosis and in comparison to the control, and separate inhibition of Polθ and Polθ with PARP in case of late apoptosis (Fig. 3 B). The combined treatment with Polθi and RAD52i with or without TMZ gave significant increase of NHA cells only in early apoptosis, compared to control and Polθ inhibition alone (Fig. 3 D)

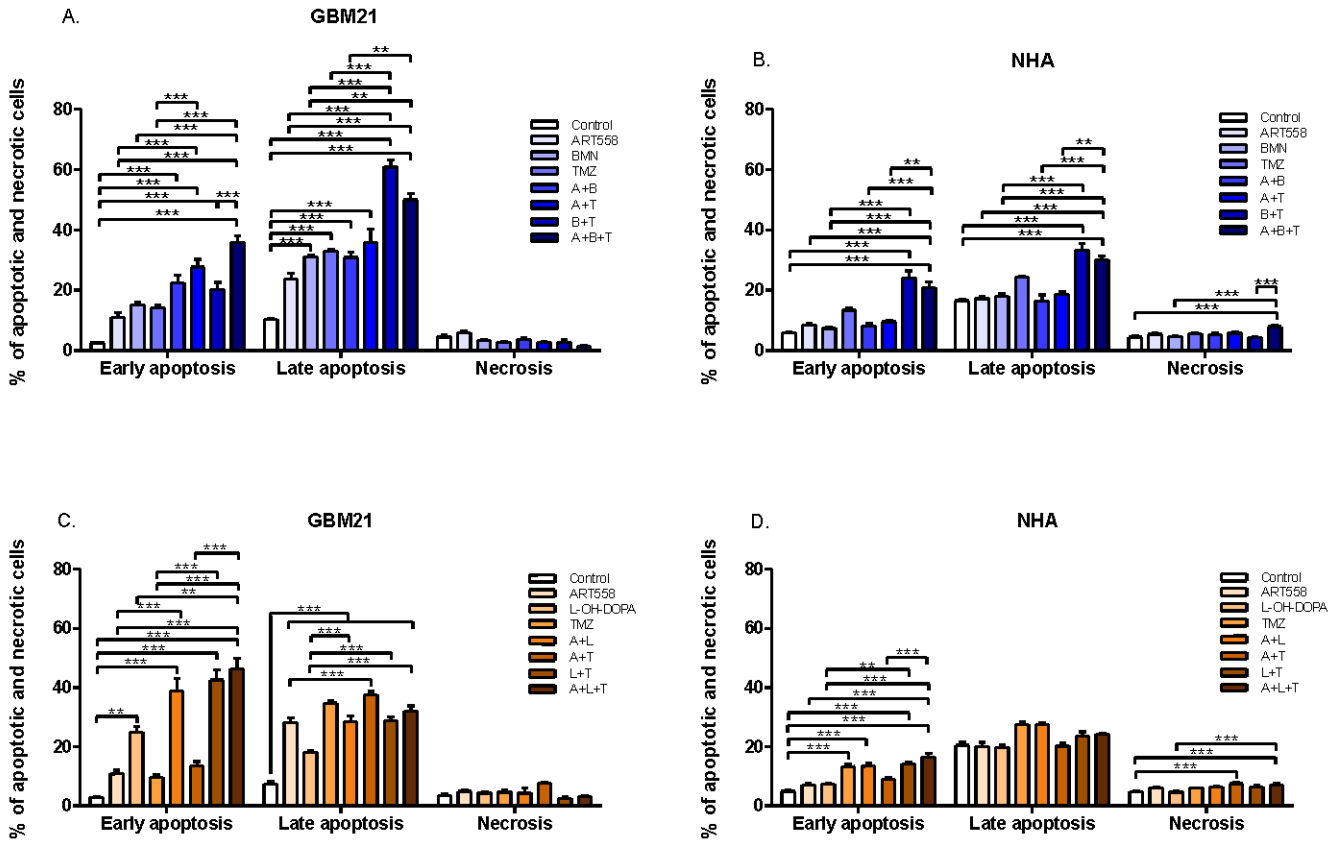
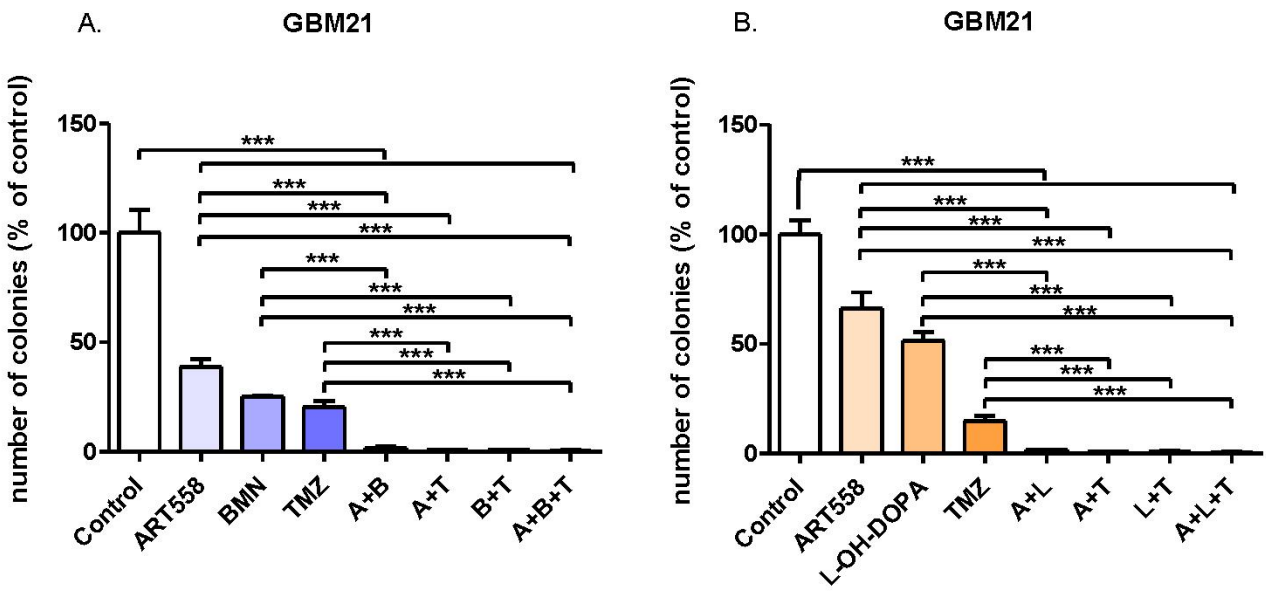


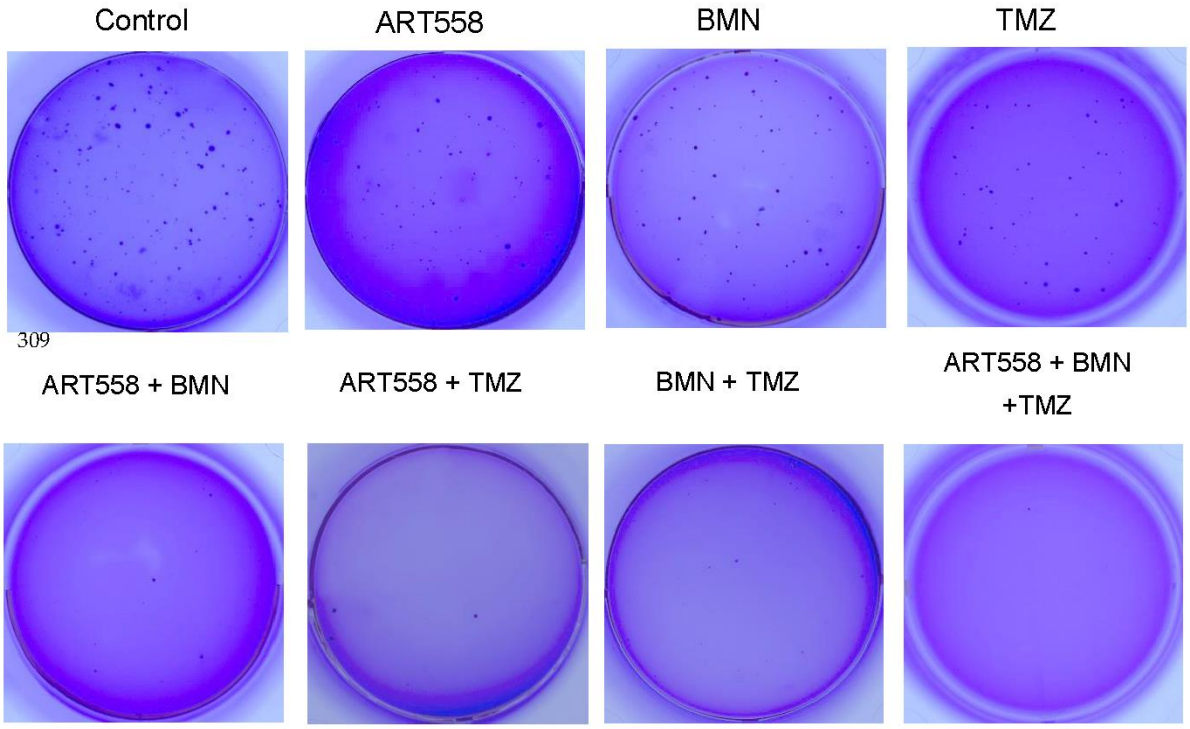
Fig. 3 Proapoptotic effect of DNA repair proteins inhibition and alkiliting agent and their combinations on cancer GBM21 and normal NHA cells. Three independent experiments were performed, and the results are shown as the mean ± standard error of the mean (SEM). * $p \leq 0.05$, ** $p \leq 0.01$, *** p-value ≤ 0.001; C. late apoptosis, in comparison to the control all results are statisticaly significant with p-value ≤ 0.001; A – ART558, B – BMN673, L – L-OH-DOPA, T – TMZ.

3.2. Inhibition of Polθ and coinhibiton with either PARP1 or Rad52 decreases invasive characteristic of the glioblastoma cells

We used clonogenic assay to measure the ability of single cancer cells to proliferate and form colonies after the treatment with the inhibitors and cytotoxic drug. We observed decreased cell proliferation visualised in significant reduction of colony formation between the control and each treatment variant. Also, in comparison to single inhibition of Polθ, the combination of Polθi and PARPi or RAD52i or dual inhibition with TMZ gave statistically significant reduction of colonies number. The results are also presented in the photos (Fig. 4 C, D)


A.
GBM21
number of colonies (% of control)
150
100
50
0
***
Control
ART558
BMN
TMZ
A+B
A+T
B+T
A+B+T
B.
GBM21
number of colonies (% of control)
Control
ART558
L-OH-DOPA
TMZ
A+L
A+T
L+T
A+L+T

C.


Control
ART558
BMN
TMZ
ART558 + BMN
ART558 + TMZ
BMN + TMZ
ART558 + BMN
+TMZ

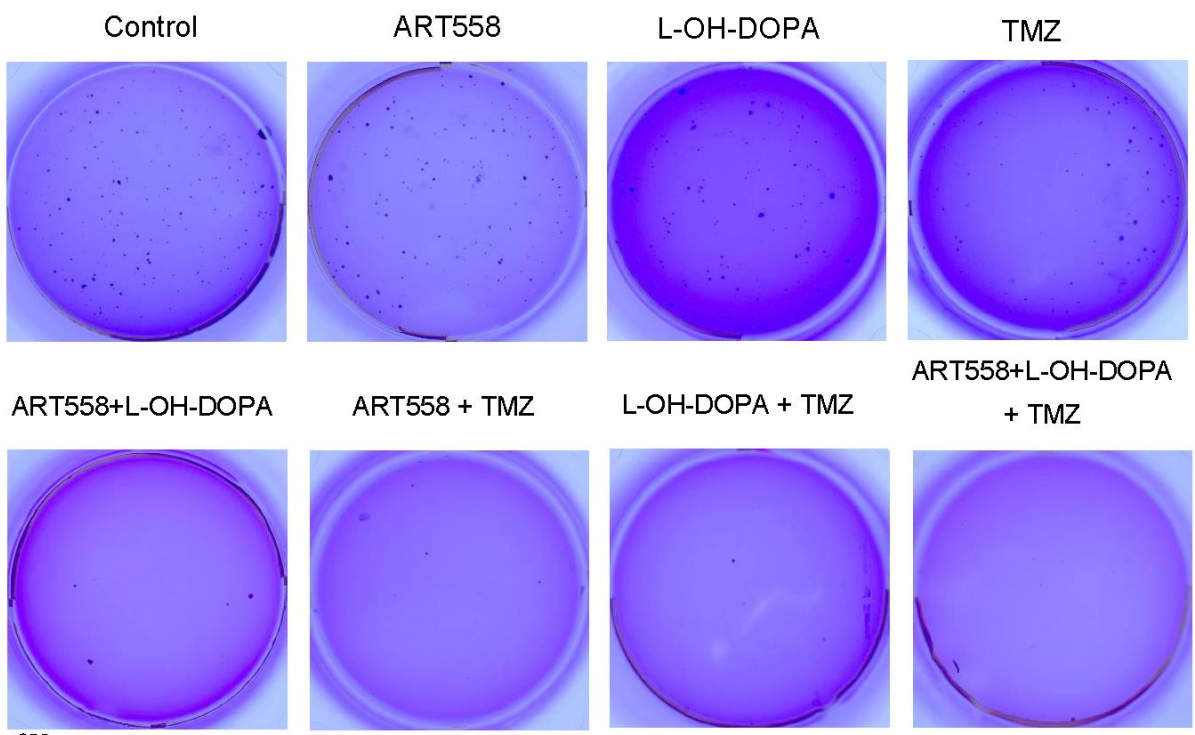


Fig. 4 Reduction of colony formation induced by DNA repair proteins inhibition and alkylating agent and their combinations in GBM21 cells. Three independent experiments were performed, and the results are shown as the mean ± standard error of the mean (SEM). * $p \leq 0.05$, ** $p \leq 0.01$, *** p-value ≤ 0.001; A – ART558, B – BMN673, L – L-OH-DOPA, T – TMZ.

3.3 Combined inhibition of Polθ and either PARP1 or Rad52 and induction of DNA alkylation by TMZ leads to decrease of G0/G1 and arrest of cell populations in S phase, both cancer and normal

In GBM21 the significant effect in cell cycle changes was observed after treatment with RAD52i or PARPi and TMZ, i.e. decrease of G0/G1 cell population in comparison to the control in both cases and single inhibition of PARP, respectively. The addition of Polθi to these treatments gave similar results, significant versus control and PARPi. Additionally, they were significant to single inhibition of Polθ in variant with RAD52i and to dual inhibition of PARP with Polθ in variant of PARPi (Fig. 5 A,C). Also, the combination of Polθi with TMZ caused significant arrest of cell cycle in G2/M and S phases and decrease in G0/G1 in comparison to the control and Polθi (Fig. 5 A,C). Further, the inhibition of Polθ and RAD52 with TMZ induced statistically significant shift of the cells to S phase in comparison to the control and Polθi (Fig. 5 C). Similar results as shown for GBM21 were obtained for NHA cells (Fig. 5 B,D).

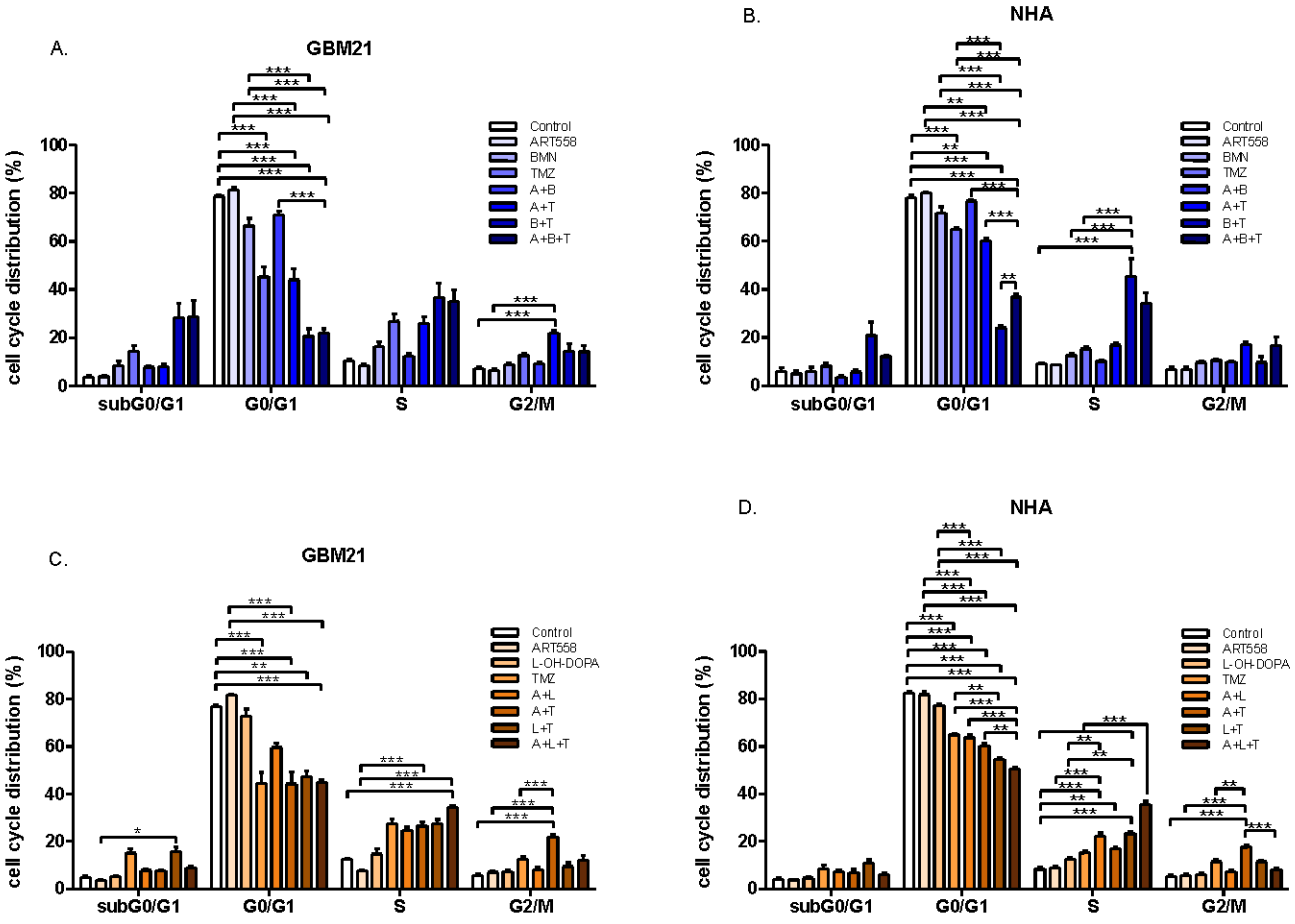


Fig. 5 Distribution of cells through cycle phases after the inhibition of DNA repair proteins and alkiliting agent treatment and their combinations in GBM21 and NHA cells. At least two independent experiments were performed, and the results are shown as the mean ± standard error of the mean (SEM). * $p \leq 0.05$, ** $p \leq 0.01$, *** p-value ≤ 0.001; A – ART558, B – BMN673, L – L-OH-DOPA, T – TMZ.

3.4. Coinhibition of Polθ with PARP1 increases the number of DSBs in the glioblastoma cells

H2AX phosphorylation measurement served as marker of DSBs. The results of this assay showed significant induction of H2AX phosphorylation in GBM21 after the dual inhibition of Polθ and PARP1, treatment and dual inhibition with TMZ in comparison to the control (Fig. 6 A). Also, separate usage of TMZ, as well as with Polθi induced DSBs at very similar level, significantly versus control (Fig. 6 A,C). The NHA exhibited significantly higher level of H2AX phosphorylation after the treatment with TMZ and the combination of Polθi and PARP1i and TMZ in comparison to the control and separate inhibition of Polθ.

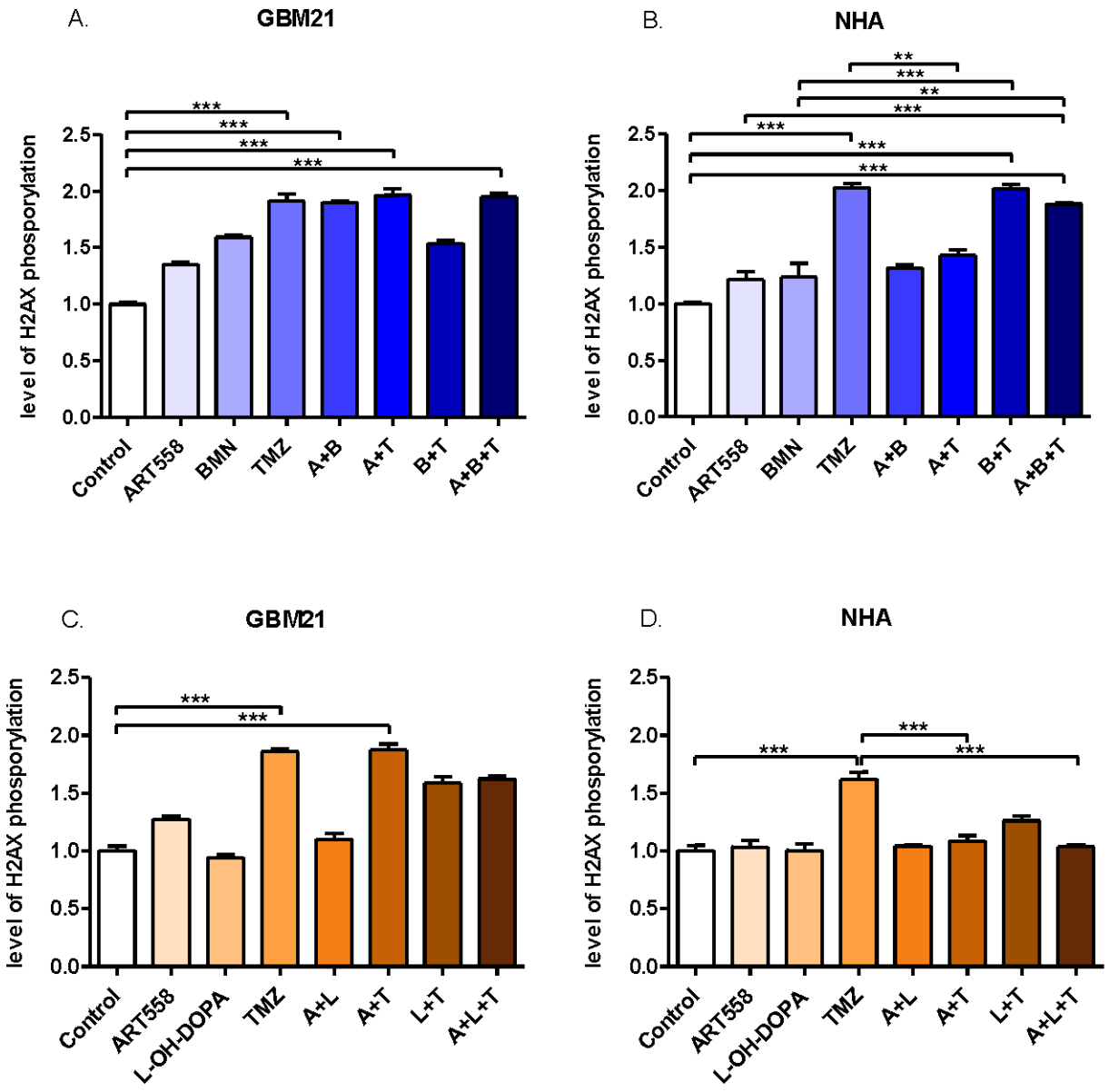


Fig.6 H2AX phosphorylation expressed as level of fluorescence signal relative to the control induced by the inhibition of DNA repair proteins and alkylating agent treatment and their combinations in GBM21 and NHA cells. At least two independent experiments were performed, and the results are shown as the mean ± standard error of the mean (SEM). * $p \leq 0.05$, ** $p \leq 0.01$, *** p-value ≤ 0.001; A – ART558, B – BMN673, L – L-OH-DOPA, T – TMZ.

3.5. Coinhibition of Polθ with PARP1 enhances the genotoxic effect obtained by gamma radiation in the glioblastoma cells, in contrast to normal cells.

The amount of DNA in comet tail is a direct marker of DNA damage, specifically double strand breaks. The exposure of the cells to gamma radiation was applied to simulate radiotherapy and analyse its combination with the used compounds. The percentage of DNA in tail in GBM21 cells was significantly elevated between all variants of the treatment and control in Figure 7A and 7C. Also, this cells exhibited significantly higher level of DNA in tail after the dual inhibition of Polθ and PARP1, and dual inhibition with TMZ

in comparison to separate treatment with Polθi. Similarly, the combination of Polθ and RAD52 inhibition with TMZ gave significant increase of DNA damage in comparison to the separate treatment with these inhibitors. Observing relatively high level of DSBs after the combination of the treatment with radiation, we assume that Polθ inhibition in all variants can sensitise cells to radiotherapy. The percentage of DNA in tail after the treatment in NHA cells was much lower then in cancer cells, showing generally low genotoxic effect of the treatment and ability of the cells to repair DSBs after radiation. Figure 7B shows significant increase of DNA damage only after the treatment with TMZ and the results in Figure 8D do not show statistically significant differences.

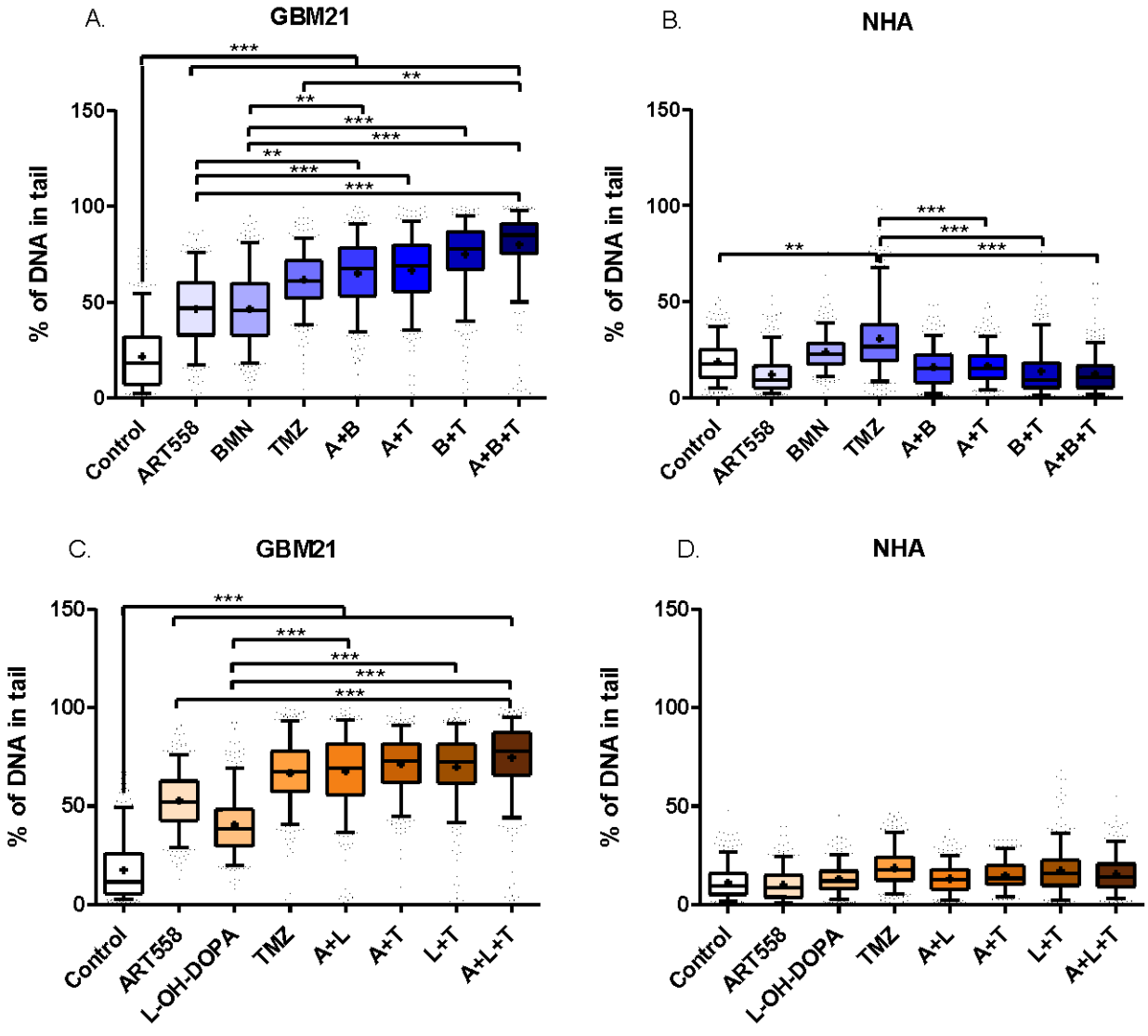


Fig. 7 Double strand breaks induced by the inhibition of DNA repair proteins and alkylating agent treatment and their combinations in GBM21 and NHA cells, visualized as percentage of DNA in comet tails. 50 randomly selected cells in three independent experiments were analysed and are shown in box plots with whiskers representing 5-95 percentile. "+" shows mean value; * $p \leq 0.05$, ** $p \leq 0.01$, *** p-value ≤ 0.001; A. In comparison to the control all results are statistically significant with p-value ≤ 0.001; A – ART558, B – BMN673, L – L-OH-DOPA, T – TMZ.

4. Discussion

The inhibition of Polθ in context of cancer treatment has been broadly investigated for last few years [25,26,34-40]. In this study we determine that Polθ inhibition cause glioblastoma cells viability decrease by almost 50%, driven mainly by apoptosis, which was accompanied by reduction of cell proliferation and DNA damage. Importantly, especially in context of future application, the combined inhibition of Polθ with PARP1 or RAD52 boosts this anticancer effect in most of the presented experiments. Similarly, the simultaneous inhibition of Polθ with PARP1 or RAD52 was performed in the study on HR-deficient leukemias showing strengthened antileukemia effect, in comparison to separate use of Polθi. Authors suggest that targeting two proteins of distinct repair activities, such as Polθ with PARP1 or RAD52, fosters synthetic lethality, which is consistent with our findings. [38].

Further enhancement of this synergistic effect was observed in our study by the supplementation with TMZ. Also, addition of TMZ to Polθi alone gives significant decrease of glioblastoma viability and proliferation, increase of apoptosis and DNA damage in comparison to separate Polθ inhibition and control group. Moreover, the combination of TMZ with Polθi shows protective effect for NHA cells, observed in increased cell viability, decreased population of cells in late apoptosis and lower level of DSBs than after separate use of TMZ. Together this results point next direction for the future development of such treatment.

Importantly, the results show that normal human astrocytes are impacted by the compounds in much lower degree, they still are, especially by TMZ and the combination of three compounds (PARPi or RAD52i variant). Such treatments significantly decreased cell viability and increased rate of apoptosis and H2AX phosphorylation level. The deleterious activity of TMZ is expectable due to its alkylating mechanism, which is not specified for cancer cells, but concerns all cells in organism [7-9]. Interestingly, the cell cycle distribution profile of NHA after all variants of treatment is similar to glioblastoma cells.

Also, it is important to highlight that GBM21 cells have relatively high expression of POLQ when compared to normal cells (Supplementary Fig. S1), while we did not determine a downregulation of any gene encoding DSBs repair proteins. This could be one of the reasons why combined inhibition of Polθ with PARP1 or RAD52 kills cancer cells more effectively than inhibition of Polθ separately, while the effect of the treatment is less profound to normal cells. Concordantly, other research groups demonstrate that HR-deficient cells are more sensitive to Polθ depletion [25,38-40]. Moreover, there are multiple reports that POLQ is upregulated in cancer cells, also those with HR-deficiency, which could correlated with higher sensitivity to Polθ inhibition [25,41,42].

Moreover, in context of future possible therapies, we examined the influence of applied treatment with radiation, showing synergistic genotoxic effect on cancer cells, with much lower impact on normal cells and almost no differences between variants of the treatment. The research of Rodriguez-Berriguete et al., (2023) brings supporting results that ART558 by inhibition of Polθ sensitizes cancer cells, independent on HR-deficiency, to radiotherapy [43]. In addition, other studies show that human and mouse cancer cells with Polθ depletion are more sensitive to gamma radiation [44,45]. These findings could be correlated with involvement of Polθ in genome stability maintenance [46-48]. Thus, it could be other possibility for the development of the therapy based on Polθ inhibition.

The research of Ronson and Starowicz [39,40] identified interesting interaction that during Polθ inhibition in 53BP1-, USP48- deficient cells and Polθ depletion in 53BP1- and BRCA1/2-deficient cells, RAD52 enhances its toxicity by promoting DNA resection, chromosome breaks, DSBs and cell death. The research suggests that moderate inhibition of RAD52 can help cells to survive, while high-concentration inhibition of RAD52 during Polθ inhibition/depletion would be too harmful, confirming synthetic lethality interaction between Polθ and RAD52 [39,40]. The interaction which is triggered by simultaneous inhibition of Polθ and RAD52 also in our studies. However, due to unknown 53BP1 gene expression, we are not able to assess if similar mechanism is the cause of this effect.

Moreover, we cannot forget about drug resistance, which is already known and studied drawback of PARP inhibitors. The hope to overcome this problem is placed in Polθ inhibition [22,25,26,49,50]. Zatreanu et al. (2021) and Zhou et al. (2021) were first to introduce ART558 and Novobiocin, respectively, as the Polθ inhibitors with very promising results, also in PARPi resistant tumors [25,26]. Zatreanu et al. (2021) indicated that Polθ inhibition could be used to overcome PARPi resistance acquired by depletion of Shieldin complex components in BRCA1−/− cells by a mechanism of dual synthetic lethality, which also agrees with the results obtained by Zhou et al. (2021) [25,26]. Apart from mentioned above mutations/depletion of Shieldin complex, PARPi resistance can be caused by: (i) reversion mutations in BRCA1/2 genes, leading to restoration of functional protein and HR pathway; (ii) involvement of mutations in 53BP1 protein; (iii) mutation in PARP itself; (iv) pharmacokinetic changes of the drug. These scenarios are also possible in context of Polθ inhibition, however it is hard to predict them. The solution for this issue, similarly to PARPi resistance, could be the combination of Polθ inhibition with inhibition of other DNA repair proteins [22]. The results of our research show that combination of Polθ inhibition with either PARPi or RAD52i intensifies the cytotoxic and genotoxic effect in glioblastoma, in lower extent than in astrocytes. Thus it may be the way to evade cells resistance, however further research on resistant cell line would be recommended.

Summarizing, the synthetic lethality achieved by simultaneous inhibition of Polθ and PARP or Polθ and RAD52 is a promising approach to eliminate glioblastoma cells. Nevertheless, it must face yet many challenges to overcome such as dosing, toxicity to healthy tissues and cells resistance, before it could be implemented to the patients treatment.

5. Conclusions

Presented and discussed results show that targeting Polθ in cancer treatment has a great potential and should be further explored. The extensive work on research and development of new and more efficient Polθ inhibitor continues, also with use of AI [36,51]. The synthetic lethality achieved by combined inhibition of Polθ with other DNA repair proteins such as PARP1 or RAD52 have even grater effect to defeat glioblastoma, however it needs to be study broader in context of its toxicity. In our opinion the addition of alkylating agent temozolomide could significantly increase efficiency, but also toxicity, of the potential treatment with these inhibitors.

To have the potential for application in real cancer treatment the results should be expanded by in vitro studies in more cell lines and in vivo experiments, followed by clinical studies on humans. It is worth to notice, as a limitation of the study, that it was performed on only one patient-derived cancer cell line, therefore it cannot be directly scaled up, however it points out the role of personalized medicine. Also, more extensive analysis of glioblastoma cells genetic profile is necessary to reveal the correlations which lie beneath antitumor activity of Polθ inhibition.

Supplementary Materials: Figure S1 Glioblastoma gene expression.

Author Contributions: G.B.-P. original draft preparation, performed the cell culturing and drug treatment, flow cytometry analysis, clonogenic assay, gene expression, analysis of the data; E.S. and P.W.-J. performed the comet assay; M.D. performed flow cytometric analysis; M.B, M.R provided with surgical specimens; P.S. performed calcein/propidium iodide double staining, J.S., P.C. reviewed and edited the manuscript, analyzed the data; T. Ś. and T.S. conceived the project, designed the experiments, reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: The APC and the research was funded by the Polish National Science Center UMO-2020/37/B/NZ7/00422 [T Sliwinski]. T. Skorski was supported by the grants from NIH/NCI 1R01 CA244179, CA247707, CA186238, CA244044, and CA2372861.

Institutional Review Board Statement: The study performed on cells derived from surgical specimens was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee the Medical University of Lodz (no RNN/23/22/KE) and informed consent was obtained from all patients.

Informed Consent Statement: Not applicable

Data Availability Statement: The data presented in the study are available upon request.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Louis D.N., Perry A., Wesseling P., Brat D.J., Cree I.A., Figarella-Branger D., Hawkins C., Ng H.K., Pfister S.M., Reifenberger G., Soffietti R., von Deimling A., Ellison D.W. The 2021 WHO classification of tumors of the central nervous system: A summary. *Neuro Oncol*. 2021;23(8):1231–51.
2. Yabo YA, Niclou S.P, Golebiewska A. Cancer cell heterogeneity and plasticity : A paradigm shift in glioblastoma. *Neuro-Oncology* 2022;24:669–82.
3. Fisher J.P., Adamson D.C. Current FDA-Approved Therapies for High-Grade Malignant Gliomas. *Biomedicines* 2021;1–13.
4. Kotecha R., Odia Y., Khosla A.A., Ahluwalia M.S. Key Clinical Principles in the Management of Glioblastoma. *JCO Oncol Pract* 2023; 19(4): 180–189, DOI https://doi. org/10.1200/OP.22. 00476
5. Badkas A., De Landtsheer S., Sauter T. Expanding the Disease Network of Glioblastoma Multiforme via Topological Analysis. Int. J. Mol. Sci. 2023, 24, 3075. https://doi.org/ 10.3390/ijms24043075
6. Kreatsoulas D., Bolyard C., Wu B.X., Cam H., Giglio P., Li Z. Translational landscape of glioblastoma immunotherapy for physicians : guiding clinical practice with basic scientific evidence. *J Hematol Oncol*. 2022;1–30, DOI:https://doi.org/10.1186/s13045-022-01298-0
7. Wick W., Platten M., Weller M. New (alternative) temozolomide regimens for the treatment of glioma. *Neuro-Oncology* 2009; DOI: 10.1215/15228517-2008-078)
8. Neyns B., Tosoni A., Hwu W-J., Reardon D.A. Dose-Dense Temozolomide Regimens Antitumor Activity, Toxicity, and Immunomodulatory Effects.*Cancer* 2010;2868–77, DOI: 10.1002/cncr.25035
9. Lee S.Y. ScienceDirect Temozolomide resistance in glioblastoma multiforme. *Genes & Diseases* 2016 *3*(3), 198–210. https://doi.org/10.1016/j.gendis.2016.04.007
10. Stupp R., Brada M., Van Den Bent M.J., Tonn J., Pentheroudakis G.Clinical practice guidelines High-grade glioma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of Oncology* 2014;25(3):iii93–101. http://dx.doi.org/10.1093/annonc/mdu050
11. Pismataro M.C., Astolfi A., Barreca M.L., Pacetti M., Schenone S., Bandiera T, Carbone A., Massari S. Small Molecules Targeting DNA Polymerase Theta (POL θ) as Promising Synthetic Lethal Agents for Precision Cancer Therapy. *J. Med. Chem.* 2023, 66, 6498–6522, https://doi.org/10.1021/acs.jmedchem.2c02101
12. Huang A., Garraway L.A., Ashworth A., Weber B.. Synthetic lethality as an engine for cancer drug target discovery. *Nat Rev Drug Discov*. 2020;19(1):23–38, http://dx.doi.org/10.1038/s41573-019-0046-z
13. O'Neil N.J., Bailey M.L., Hieter P. Synthetic lethality and cancer. *Nat Rev Genet*. 2017;18(10):613–23., http://dx.doi.org/10.1038/nrg.2017.47
14. Patel P.S., Algouneh A., Hakem R. Exploiting synthetic lethality to target BRCA1 / 2-deficient tumors : where we stand. *Oncogene* 2021;3001–14, http://dx.doi.org/10.1038/s41388-021-01744-2
15. Drzewiecka M., Barszczewska-Pietraszek G., Czarny P., Skorski T. Śliwiński T. Synthetic Lethality Targeting Polθ. *Genes (Basel)* 2022;13(6):1101, https://doi.org/10.3390/ genes13061101
16. Livraghi L., Garber J.E. PARP inhibitors in the management of breast cancer : current data and future prospects. BMC Med. 2015;1–16, http://dx.doi.org/10.1186/s12916-015-0425-1
17. Rajawat J., Shukla N., Mishra D.P.. Therapeutic Targeting of Poly (ADP-Ribose) Polymerase-1 in Cancer : Current Developments , Therapeutic Strategies , and Future. *Med Res Rev*. 2017 1(0):1–30, DOI 10.1002/med.21442
18. Caracciolo D., Riillo C., Teresa M., Martino D. Tagliaferri P. Alternative Non-Homologous End-Joining: Error-Prone DNA Repair as Cancer's Achilles' Heel. *Cancers* 2021; http:// doi.org/10.3390/cancers13061392
19. Huang R., Zhou P.K. DNA damage repair: historical perspectives, mechanistic pathways and clinical translation for targeted cancer therapy. *Signal Transduct Target Ther*. 2021 Dec 1;6(1), https://doi.org/10.1038/s41392-021-00648-7
20. Lord C.J., Ashworth A. PARP inhibitors: Synthetic lethality in the clinic. *Science* 2017;355(6330):1152–, doi:10.1126/science.aam7344.
21. Slade D. PARP and PARG inhibitors in cancer treatment. *Genes Dev*. 2020;34(5):360–94, http://www.genesdev.org/cgi/doi/10.1101/gad.334516.119
22. Baxter J.S., Zatreanu D., Pettitt S.J., Lord C.J. Resistance to DNA repair inhibitors in cancer. *Molecular Oncology* 2022;1–17, doi:10.1002/1878-0261.13224
23. Yousefzadeh M.J., Wood R.D.. Mini review DNA polymerase POLQ and cellular defense against DNA damage. *DNA Repair (Amst)* 2013;12(1):1–9. http://dx.doi.org/10.1016/j.dnarep.2012.10.004
24. Wood R.D., Doublié S. DNA polymerase θ (POLQ), double-strand break repair, and cancer. *DNA Repair (Amst)*. 2016 August ; 44: 22–32, doi:10.1016/j.dnarep.2016.05.003.
25. Zatreanau, D.; Robinson, H.M.R.; Alkhatib, O.; Boursier, M.; Finch, H.; Geo, L.; Grande, D.; Grinkevich, V.; Heald, R.A.; Langdon, S.; et al. Polθ inhibitors elicit BRCA-gene synthetic lethality and target PARP inhibitor resistance. *Nat. Commun*. 2021, 12, 36, http://dx.doi.org/10.1038/s41467-021-23463-8
26. Zhou, J.; Gelot, C.; Pantelidou, C.; Li, A.; Yücel, H.; Davis, R.E.; Färkkilä, A.; Kochupurakkal, B.; Syed, A.; Shapiro, G.I.; et al. A first-in-class polymerase theta inhibitor selectively targets homologous-recombination-deficient tumors. *Nat. Cancer* 2021, 2, 598–610,

27. Bubenik, M.; Mader, P.; Mochirian, P.; Vallée, F.; Clark, J.; Truchon, J.F.; Perryman, A.L.; Pau, V.; Kurinov, I.; Zahn, K.E.; et al. Identification of RP-6685, an Orally Bioavailable Compound that Inhibits the DNA Polymerase Activity of Polθ. *J. Med. Chem*. 2022, 65, 13198–13215.
28. Sullivan K., Cramer-Morales K., Mcelroy D.L., Ostrov D.A., Haas K., Childers W., Hromas E., Skorski T. Identification of a Small Molecule Inhibitor of RAD52 by Structure-Based Selection. *PLoS ONE* 2016 11(1), doi:10.1371/journal.pone.0147230
29. Toma M., Sullivan-Reed K., Sliwinski T., Skorksi T. RAD52 as a Potential Target for Synthetic Lethality-Based Anticancer Therapies. *Cancers* 2019, 11, 1561; doi:10.3390/cancers11101561
30. Drzewiecka M.; Jaśniak D.; Barszczewska-Pietraszek G.; Czarny P.; Kobrzycka A.; Wieczorek M.; Radek,M.; Szemraj J.; Skorski T.; Śliwiński T. Class I HDAC Inhibition Leads to a Downregulation of FANCD2 and RAD51, and the Eradication of Glioblastoma Cells. *J. Pers. Med*. 2023, 13, 1315. https:// doi.org/10.3390/jpm13091315
31. Toma M., Witusik-Perkowska M., Szwed M., Stawski R., Szemraj J., Drzewiecka M., et al. Eradication of LIG4-deficient glioblastoma cells by the combination of PARP inhibitor and alkylating agent. *Oncotarget*. 2018 Dec;9(96):36867–77.
32. Czyż M., Toma M., Gajos-Michniewicz A., Majchrzak K., Hoser G., Szemraj J., et al. PARP1 inhibitor olaparib (Lynparza) exerts synthetic lethal effect against ligase 4-deficient melanomas. *Oncotarget*. 2016 7(46):75551–60.
33. Drzewiecka M., Gajos-Michniewicz A., Hoser G., Jaśniak D., Barszczewska-Pietraszek G., Sitarek P., et al. Histone Deacetylases (HDAC) Inhibitor—Valproic Acid Sensitizes Human Melanoma Cells to Dacarbazine and PARP Inhibitor. *Genes (Basel)*. 2023;14(6):1295.
34. Syed A., Filandr F., Patterson-Fortin J., Bacolla A., Ravindranathan R., Zhou J., et al. Novobiocin blocks nucleic acid binding to Polθ and inhibits stimulation of its ATPase activity. *Nucleic Acids Res*. 2023;51(18):9920–37.
35. Schrempf A., Bernardo S., Arasa Verge E.A., Ramirez Otero M.A., Wilson J., Kirchhofer D., et al. POLθ processes ssDNA gaps and promotes replication fork progression in BRCA1-deficient cells. *Cell Rep*. 2022;41(9):111716, https://doi.org/10.1016/j.celrep.2022.111716
36. Wang Y., Wang C., Liu J., Sun D., Meng F., Zhang M., et al. Discovery of 3-hydroxymethyl-azetidine derivatives as potent polymerase theta inhibitors. *Bioorg Med Chem*. 2024 Apr 1;103:117662.
37. Fang W., Wang J., Ma X., Shao N., Ye K., Zhang D., et al. A Progressively Disassembled DNA Repair Inhibitors Nanosystem for the Treatment of BRCA Wild-Type Triple-Negative Breast Cancer. *International Journal of Nanomedicine* 2023:18 6001–19.
38. Sullivan-Reed K., Toma M.M., Drzewiecka M., Nieborowska-Skorska M., Nejati R., Karami A., et al. Simultaneous Targeting of DNA Polymerase Theta and PARP1 or RAD52 Triggers Dual Synthetic Lethality in Homologous Recombination–Deficient Leukemia Cells. *Mol Cancer Res*. 2023;21(10):1017–22.
39. Starowicz K., Ronson G., Anthony E., Clarke L., Garvin A.J., Beggs A.D., et al. RAD52 underlies the synthetic-lethal relationship between BRCA1/2 and 53BP1 deficiencies and DNA polymerase theta loss. *bioRxiv* 2022; 485027; doi: https://doi.org/10.1101/2022.03.20.485027
40. Ronson G.E., Starowicz K., Anthony E.J., Piberger A.L., Clarke L.C., Garvin A.J., et al. Mechanisms of synthetic lethality between BRCA1/2 and 53BP1 deficiencies and DNA polymerase theta targeting. *Nat Commun*. 2023;14(1).
41. Schrempf A., Slyskova J., Loizou J.I.. Targeting the DNA Repair Enzyme Polymerase θ in Cancer Therapy. *Trends in Cancer* 2021;7(2):98–111, https://doi.org/10.1016/j.trecan.2020.09.007
42. Patterson-Fortin J., Jadhav H., Pantelidou C., Phan T., Grochala C., Mehta A.K., et al. Polymerase θ inhibition activates the cGAS-STING pathway and cooperates with immune checkpoint blockade in models of BRCA-deficient cancer. *Nat Commun*. 2023;14(1):1390, http://www.ncbi.nlm.nih.gov/pubmed/36914658
43. Rodriguez-Berriguete G., Ranzani M., Prevo R., Puliyadi R., Machado N., Bolland H.R., et al. Small-molecule Polθ inhibitors provide safe and effective tumor radiosensitization in preclinical models. *Clin Cancer Res*. 2023;(22):1–12.
44. Higgins G.S., Prevo R., Lee Y., Helleday T., Muschel R.J., Taylor S., et al. A siRNA Screen of Genes Involved in DNA Repair Identifies Tumour Specific Radiosensitisation by POLQ Knockdown. *Cancer Res*. 2010 April 1; 70(7): 2984–2993, doi:10.1158/0008-5472.CAN-09-4040
45. Ukai A., Maruyama T., Mochizuki S., Ouchida R., Masuda K.. Role of DNA polymerase θ in tolerance of endogenous and exogenous DNA damage in mouse B cells. *Genes to Cells* (2006) 11 , 111–121 2, DOI: 10.1111/j.1365-2443.2006.00922.x
46. Brambati A., Barry R., Sfeir A. necessary for genome stability. *Curr Opin Genet Dev*. 2021;(646):119–26, doi:10.1016/j.gde.2020.02.017
47. Lemée F., Bergoglio V., Fernandez-vidal A., Machado-silva A, Pillaire M. DNA polymerase θ up-regulation is associated with poor survival in breast cancer , perturbs DNA replication , and promotes genetic instability. *PNAS* 2010;107(30).
48. Liddiard K., Aston-Evans A.N., Cleal K., Baird D.M., Hendrickson E.A.. POLQ suppresses genome instability and alterations in DNA repeat tract lengths. *NAR Cancer*. 2022;4(3):1–20., https://doi.org/10.1093/narcan/zcac020
49. Lavudi K. Banerjee A., Li N., Yang Y., Cai S., Bai X., et al. ALDH1A1 promotes PARP inhibitor resistance by enhancing retinoic acid receptor-mediated DNA polymerase θ expression. *npj Precis Oncol*. 2023;7(1):1–11, https://doi.org/10.1038/s41698-023-00411-x
50. Oh G., Wang A., Wang L., Li J., Werba G., Weissinger D., et al. POLQ inhibition elicits an immune response in homologous recombination-deficient pancreatic adenocarcinoma via cGAS/STING signaling. *J Clin Invest*. 2023;133(11):1–14., https://doi.org/10.1172/JCI165934

51. Fried W., Tyagi M., Minakhin L., Chandramouly G., Tredinnick T., Ramanjulu M., et al. Discovery of a small-molecule inhibitor that traps Polθ on DNA and synergizes with PARP inhibitors. *Nat Commun.* 2024;15(1):1–15, https://doi.org/10.1038/s41467-024-46593-1

Glioblastoma GBM21 gene expression

Methodology : RNA isolation and gene expression

Analysis of mRNA expression of 28 selected genes, which products are involved in following DSB repair mechanisms: HR, NHEJ, TMEJ and a-NHEJ, began with the isolation of total RNA using RNeasy Mini Kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions. After the detachment from culture bottles, cells were centrifuged and then resuspended in PBS to determine cell density. Approximately $5x10^6$ cells were used for isolation, with a survival rate of 97-99%.

Subsequently, the purity and quantity of RNA were evaluated by spectrophotometric analysis in Picodrop, then 10 ng/µL RNA was converted into complementary DNA (cDNA), using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Real-time PCR was performed with TaqMan® probes dedicated to detect 4 reference and the selected genes. The RT-PCR reactions were conducted on CFX96 ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California, USA) with parameters consisted of an initial step of 95 ◦C for 10 min, followed by 30 cycles of denaturation at 95 ◦C for 15 s and annealing/extension at 60 ◦C for 60 s., using TaqMan™ Universal Master Mix II probes, no UNG (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Each reaction was performed in triplicates. *ACTB* was used as a reference gene after analysis in Geneinvestigator where it was established with the most stable expression between 4 selected reference genes. The results were calculated as fold change of genes expression in cancer versus normal cells ($2^{-\Delta\Delta Ct}$).

Description of the reuslts: To assess genetic profile of cancer cell line and potential deficinency in any of repair pathway we determine expression level of 28 genes, selected due to their activity in DSBs repair pathways. We did not observed a downregulation of any recalled gene versus NHA cells. However, relatively high expression of HR-related genes and *POLQ* of TMEJ, while all genes involved in NHEJ have realtively low expression. Therefore, this may explain higher effectivness of dual inhibition with Polθi and PARPi or RAD52i.

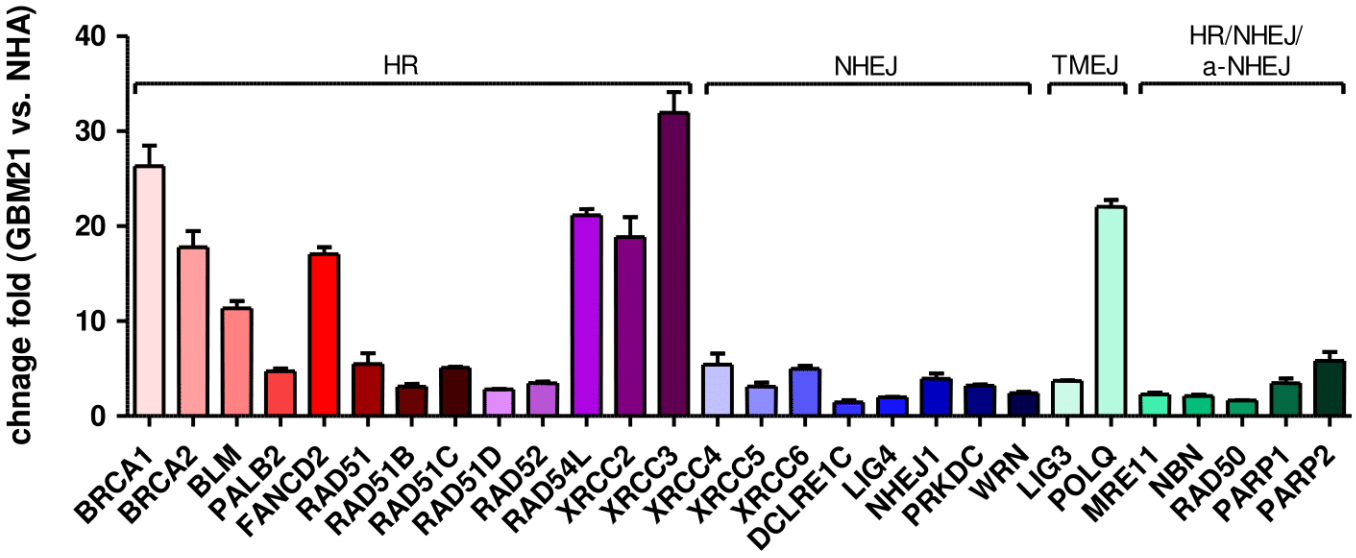


Fig. 1 Expression profile of 28 genes, categorized due to their activity in following DNA double strand breaks repair mechanisms: homologous recombination (HR), non-homologous end joining (NHEJ), theta-mediated end joining (TMEJ), alternative non-homologous end joining (a-NHEJ) in GBM21 glioblastoma cells, presented as a fold change in reference to normal cells NHA. Results represent mean value ± SEM from the experiment performed in triplicate.

[IJMS] Manuscript ID: ijms-3088553 - Co-Authorship Confirmation

susy@mdpi.com <susy@mdpi.com>
on behalf of
IJMS Editorial Office <ijms@mdpi.com>
Tue 18/06/2024 16:31

To:Gabriela Barszczewska-Pietraszek <gabriela.barszczewska.pietraszek@edu.uni.lodz.pl>
Cc:IJMS Editorial Office <ijms@mdpi.com>

Dear Mrs. Barszczewska-Pietraszek,

We are writing to let you know that we have received the below submission to International Journal of Molecular Sciences for which you are listed as a co-author.

Manuscript ID: ijms-3088553
Type of manuscript: Article
Title: Polθ inhibitor (ART558) demonstrates synthetic lethal effect with PARP and RAD52 inhibitors in glioblastoma cells.
Authors: Gabriela Barszczewska-Pietraszek, Piotr Czarny, Małgorzata Drzewiecka, Maciej Błaszczyk, Maciej Radek, Ewelina Synowiec, Paulina Wigner-Jeziorska, Przemysław Sitarek, Janusz Szemraj, Tomasz Skorski, Tomasz Śliwiński *
Received: 18 Jun 2024

In order to confirm your connection to this submission, please click here to confirm your co-authorship:
https://susy.mdpi.com/author/confirm/2205682/eLYWQ2ND

Kind regards,
IJMS Editorial Office

Research article

Polθ inhibition with simultaneous treatment with PARP or RAD52 inhibitors induces cyto- and genotoxic effect in melanoma cells and reduces tumor growth of human melanoma xenograft.

Gabriela Barszczewska-Pietraszek[1], Piotr Czarny [2], Grażyna Hoser[3], Dominika Jaśniak[1], Małgorzata Drzewiecka[1], Izabela Zaleśna[4], Janusz Piekarski[4], Monika Toma[5], Katarzyna Białek[2], Danuta Wasilewska[3], Tomasz Skorski[5], Tomasz Śliwiński[1]*

1 Department of Molecular Genetics, Faculty of Biology and Environmental Protection, University of Lodz, 90-236 Lodz, Poland; gabriela.barszczewska.pietraszek@edu.uni.lodz.pl (G.B.-P.)
2 Department of Medical Biochemistry, Medical University of Lodz, 92-216 Lodz, Poland; piotr.czarny@umed.lodz.pl (P.C.)
3 Department of Flow Cytometry, Medical Center for Postgraduate Education, 01-813 Warsaw, Poland.
4 Department of Surgical Oncology, Medical University in Lodz, 93-513 Lodz, Poland
5 Fels Cancer Institute for Personalized Medicine, Lewis Katz School of Medicine, Temple University, Philadelphia, PA 19140, USA

Running Title : Inhibition of DNA repair proteins for melanoma treatment.

* Corresponding author: Tomasz Sliwinski, tomasz.sliwinski@biol.uni.lodz.pl; University of Lodz, 90-236 Lodz, Poland
* The authors declare no potential conflicts of interest.
* This manuscript includes 3500 words, 7 figures.

Abstract

Polθ inhibitors (Polθi) hold great potential as anticancer treatment due to its ability to target cancer cells specifically. The particular Polθi targets are tumors with DNA repair deficiencies, which often become reliant on theta-mediated end-joining, a DNA repair mechanism facilitated by Polθ. The studies performed on HR-deficient cancer cells show that simultaneous inhibition of Polθ with other DNA repair protein such as PARP or RAD52 leads to synthetic lethal correlation killing cancer cells selectively. In this study, we show that combining Polθi (ART558) with either PARP1 or RAD52 inhibitors enhances anticancer effect on melanoma in comparison to separate treatment with Polθi, especially when paired with dacarbazine (DTIC). In addition, we observed reduced tumor size in human melanoma xenografts after the combined treatment with Polθ inhibitor (RP-6685), RAD52 inhibitor (D-IO3) and DTIC. The treatment's impact on normal cells appears minimal compared to cancer cells, although further research is needed to ensure safety and efficacy. The potential drug resistance poses the greatest challenge, but the promising results of combined Polθ inhibition suggest a new direction for melanoma therapy.

1.Introduction

According to World Health Organization (WHO) skin cancer is the most commonly diagnosed tumor worldwide. Melanoma represents only about 3% of the cases yearly, however this is the type of skin cancer leading to majority of deaths in United States (1). Melanoma is a cancer arisen from skin cells melanocytes. It appears when melanocytes start to grow uncontrollably what is connected with occurring genetic changes, the most often, triggered by UV radiation exposure (2-4). Risk of developing melanoma varies of several factors: age, sex, skin color, amount of melanocystic nevi and family history. The risk is higher among men and the lighter the skin color is (5,6).

The first approach in melanoma treatment is a surgical resection of the cancerous lesion and adequate surrounding tissue (7). While diagnosis at the early stage of the disease is a critical element to increase patients survival, usually melanoma gets to deeper layers of skin and spreads into other parts of body (3,8). Then, other therapeutic methods must be used, i.e. radiation therapy, chemotherapy and immunotherapy (2). Dacarbazine (DTIC) is a cytotoxic drug which has been used for a long time as a main chemotherapeutic for melanoma treatment. It induces methylation of purine bases in DNA, leading to DNA damage and cell death, working analogously to the drug temozolomide (2,4,9). Regardless of its prevalence, treatment with DTIC has several drawbacks, including: low water solubility and short shelf life of the drug; intravenous administration; low patients response rate (10-15%); cells acquiring resistance to the drug and cells ability to repair DNA damage efficiently (2,4). The last opens the possibilities for the use of DTIC in combination with small molecule inhibitors of proteins involved in DNA repair pathways.

DNA polymerase theta (Polθ) and its inhibitors (Polθi) have gained particular attention recently. Polθ is an error-prone translesion synthesis (TLS) polymerase involved in several DNA repair mechanisms, but mainly in theta-mediated end joining (TMEJ) – one of the DNA double-strand breaks (DSBs) repair pathways (10-12). Polθi have been developed as a response to the therapeutic need and initial success of Poly (ADP-ribose) polymerases inhibitors (PARPi) in BRCA-deficient cancers treatment. In case of PARPi, as well as initiated studies with use of Polθi, the approach of synthetic lethality (SL) is used to eliminate cancer cells selectively. The scientists discovered that depletion of repair protein such as Polθ, PARP or RAD52 in the cancer cells with some other repair pathways deficiencies, e.g. DNA-PK (non-homologous end joining - NHEJ) or BRCA1/2 (homologous recombination - HR) leads to cell death whereas disruption of single pathway does not (13-15). The DNA repair destabilization and genetic mutations are the cancer hallmarks, therefore it possible to use SL approach to selectively kill cancer cells (12,15,16). Moreover, cancer cells with alterations in HR or NHEJ pathways often become dependent on TMEJ (17).

The background of our research was the hypothesis that inhibition of Polθ separately, or in combination with either PARP or RAD52 will cause synthetic or dual synthetic lethality of melanoma cells, respectively, depending on the genetic profile of the cell line.

2. Materials and Methods

2.1. In vitro cell culture

Melanoma cell line derived from surgical specimens, was obtained from the patients of the Department of Oncological Surgery, Copernicus Memorial Hospital in Łódź. The cell line was established in the Laboratory of Medical Biochemistry Department, Medical University of Lodz and named MLN21. The study was approved by the Ethical Commission of the Medical University of Lodz (RNN/23/22/KE), and informed consent was obtained from all patients. To obtain cell line, tissue fragment was washed several times with HBSS buffer (Gibco, ThermoFisher) and minced mechanically with a scalpel in sterile conditions. The shredded tissue fragments were then incubated in HBSS buffer with 3 mM calcium chloride and 1 mg/ml collagenase IV, on a shaker at 37°C for about 1h. At the end of the incubation, DNase was added to the solution at a concentration of 10 μg/ml. This solution was passed through a filter with a pore size of 70 μm, and then centrifuged. If a large number of red blood cells were present in the cell pellet, RBC lysis buffer (Sigma-Aldrich) was used.

Melanoma cells were cultured for first weeks in in RPMI medium (Gibco, ThermoFisher) with 10% FBS (Lonza) and antibiotics (100 IU/ ml penicillin, 100 mg/ml streptomycin (Gibco) and then replaced with DMEM/F12 medium (Gibco, ThermoFisher) supplemented with 10% FBS (Lonza), 100 IU/ml penicillin, 100 μg/ml streptomycin (Lonza) and gentamycin 50 μg/ml (Lonza) in a humidified atmosphere containing 5% CO2 at 37°C. Once the cells have multiplied they were subjected to positive selection via magnetic-activated cell sorting on magnetic separator MiniMACS™ (Miltenyi Biotec) with use of anti-melanoma magnetic microbeads MACS® (Miltenyi Biotec). The Normal Human Melanocytes - NHEMs (Lonza) were grown in MBM™ - 4 Basal Medium supplemented with MGM™ - 4 SingleQuots™ Supplements (Lonza) and cultured according to the protocol provided by manufacturer.

MLN21 cell line was tested for *POLQ* gene expression and it exhibited its overexpression versus NHEM cells.

2.2. Drug treatment

In the experiments following compounds were used: Polθi – 55 μM ART558 (MedChem Express); PARP1i – 4 nM talazoparib (BMN673) (Selleckchem); RAD52 inhibitor (RAD52i) – 62,5 μM L-OH-DOPA (Sigma-Aldrich) and cytotoxic drug – 1 mM dacarbazine (DTIC). Compounds were dissolved appropriately according to the manufacturer's instructions in distilled water or DMSO to a starting concentration of 10 mM, and then working concentrations were prepared immediately before the experiment in DMEM/F12 culture medium. The treatment scheme was established and proved to work in previous experiments. Shortly, cells were incubated with the compounds for 120 h with the second dose after 48 h (18,19)(20,21). Following variants of the treatment were used: ART558, BMN673, L-OH-DOPA and DTIC separately, ART558 + BMN673, ART558 + L-OH-DOPA, ART558 + DTIC, BMN673 + DTIC, L-OH-DOPA + DTIC, ART558 + BMN673 + DTIC, ART558 + L-OH-DOPA + DTIC.

2.3. Flow cytometric analysis of apoptosis and necrosis

Changes in viability and mechanism of cell death after standard treatment described above were analyzed using staining with propidium iodide and FITC Annexin V. Cells were prepared and analyzed according to the FITC Annexin Apoptosis Detection Kit II (BD Biosciences, Franklin Lakes, New Jersey, USA) by flow cytometry. Annexin V has strong affinity to phosphatidylserine, which appears on the cell's surface during early apoptosis, while propidium iodide binds to DNA by penetrating through the fragmented cell membrane, which is characteristic for necrosis and late stages of apoptosis. Cell viability results were also obtained using this assay.

2.4. Cell morphology visualized by fluorescence microscopy

To visualize the influence of inhibitors on cells viability, normal and cancer cells were subjected to calcein AM and propidium iodide (PI) double staining. Cells were stained after standard treatment and incubated for 30 min at 37°C with the mixture of 2 mM calcein AM and propidium iodide 1 mM (Thermo Fisher Scientific, Waltham, Massachusetts, USA) diluted in PBS. Calcein AM, the acetoxymethyl ester of calcein, freely penetrates the membranes of living cells, where the acetoxymethyl group is degraded, allowing calcium binding to calcein showing strong green fluorescence, when excited. Propidium iodide stains the DNA of dead cells showing low plasma membrane integrity, indicated by red fluorescence signal. The results were observed and pictured in an inverted fluorescence microscope LEICA.

2.5. Clonogenic assay

The cancer cells ability to form colonies were measured by clonogenic assay. Prior the test cells had undergone the standard treatment described above. Then, trypan blue staining was used to assess the viability of the treated cells. Following this, 10^3 cells were resuspended in 700 µl of soft agar 0,4% containing DMEM, FBS and antibiotics and plated on a 12-well plate over 700 µl of solidified agar underlay (0.5% also with DMEM, FBS and antibiotics). The medium was applied over the solidified cell layer and changed weekly. Prepared plates were incubated for 2 weeks, at 37 °C, 5% CO_2. The colonies were stained with 0.005% crystal violet and counted under the microscope. Clonogenic efficiency was expressed as percent of untreated control (no. of colonies after treatment vs no. of colonies in control sample × 100%).

2.6. Cell cycle

To analyze the influence of the compounds on cell cycle distribution of glioblastoma and NHA population, cells fixed with 70% cold ethanol were stained with propidium iodide with addition of RNase (BD Biosciences) and analyzed by flow cytometry.

2.7. Measurement of Histone H2AX phosphorylation

The levels of phosphorylated Histone H2AX (γH2AX), constituting of DNA DSBs, were measured by H2AX Phosphorylation Assay Kit (Merck KGaA, Darmstadt, Germany). After the standard treatment the cells were fixed and permeabilized to facilitate staining and detection. The presence of Histone H2AX phosphorylated at serine 139 was detected using a FITC-conjugated anti-phospho-Histone H2AX antibody. Flow cytometry was employed to quantify the number of cells exhibiting positive staining for γH2AX.

2.8. Neutral Comet assay

The level of DNA double-strand breaks generated by the used compounds was studied using the neutral comet assay. Following the standard treatment, cells were exposed to gamma radiation of 8 Gy. After that, cell were resuspended in 0.4% low melting point (LMP) agarose solution and immediately applied to the precoated slide with 0.5% normal melting point (NMP) agarose. Prepared slides were subjected to overnight lysis (2.5 M/L NaCl, 100 mM/L EDTA, 10 mM/L TRIS). Then, the slides were placed in developing buffer (300 mM/L NaOH, 1 mM/L EDTA) for 20 minutes. After that, electrophoresis was carried out in the electrophoretic buffer (300 mM sodium acetate, 100mM TRIS) for 1 hour, at 9V and 100 mA. After finishing electrophoresis, the slides rinsed with water were stained with DAPI solution (100 µg/mL) by applying 50 µL of solution to each slide and incubating the slides for at least 45 minutes, at 4°C. To visualize the results, the slides were observed at 200× magnification in an Eclipse fluorescence microscope (Nikon, Tokyo, Japan) attached to a COHU 4910 video camera (Cohu, Inc., San Diego, CA, USA) equipped with a UV-1 A filter block and connected to a personal computer-based image analysis system Lucia-Comet v. 6.0 (Laboratory Imaging, Praha, Czech Republic), 50 comets were counted from each repetition of the experiment. The % of the DNA in comet tail was taken into account.

2.9. Xenograft experiment

NOD SCID γ (NSG) mice (12- to 16-week-old), housed in a sterile environment and allowed free access to food and water, were injected subcutaneously with $2x10^5$ MLN21 melanoma cells. After 15 days when tumors developed mice were randomly assigned into 11 different groups: untreated (n=4), and treated orally either with RP-6685 (MedChem Express) (80 mg/kg bodyweight once a day, diluted in DMSO + vit.E with 1-methyl-2-pyrrolidinone) (n=4); RP-6685 solvent separately (n=4) and BMN673 (0.33 mg/kg bodyweight once a day, diluted in DMSO) with RP-6685 solvent (n=3) or intraperitoneally with D-IO3 (5 mg/kg bodyweight once a day, diluted in DMSO) with RP-6685 solvent (n=3) and DTIC (8 mg/kg bodyweight every second day, diluted in PBS) (n=4) or RP-6685 with BMN673 (n=4); RP-6685 with D-IO3 (n=9); RP-6685 with DTIC (n=4); RP-6685 + BMN673 with DTIC (n=4) and RP-6685 + D-IO3 with DTIC (n=4) (same dosing as in monotherapy) for 16 days. After the end of experiment the mice were slaughtered and tumors were collected and measured. The study was approved by the local Ethical Committee. Tumor growth inhibition (% TGI) was defined as: % TGI = ((TVvehicle/last − Tvvehicle/day0) − (Tvtreated/last − Tvtreated/day0))/(Tvvehicle/last − Tvvehicle/day0) × 100.

2.10. Statistical Analysis

Data from at least three independent experiments were analyzed and presented as mean ± SEM. The results were compared in SigmaPlot, using one-way ANOVA with the Holm-Sidak post hoc test. All graphs were done in GraphPad Prism 10. *P*-values of <0.05 were considered statistically significant.

2.11. Data Availability

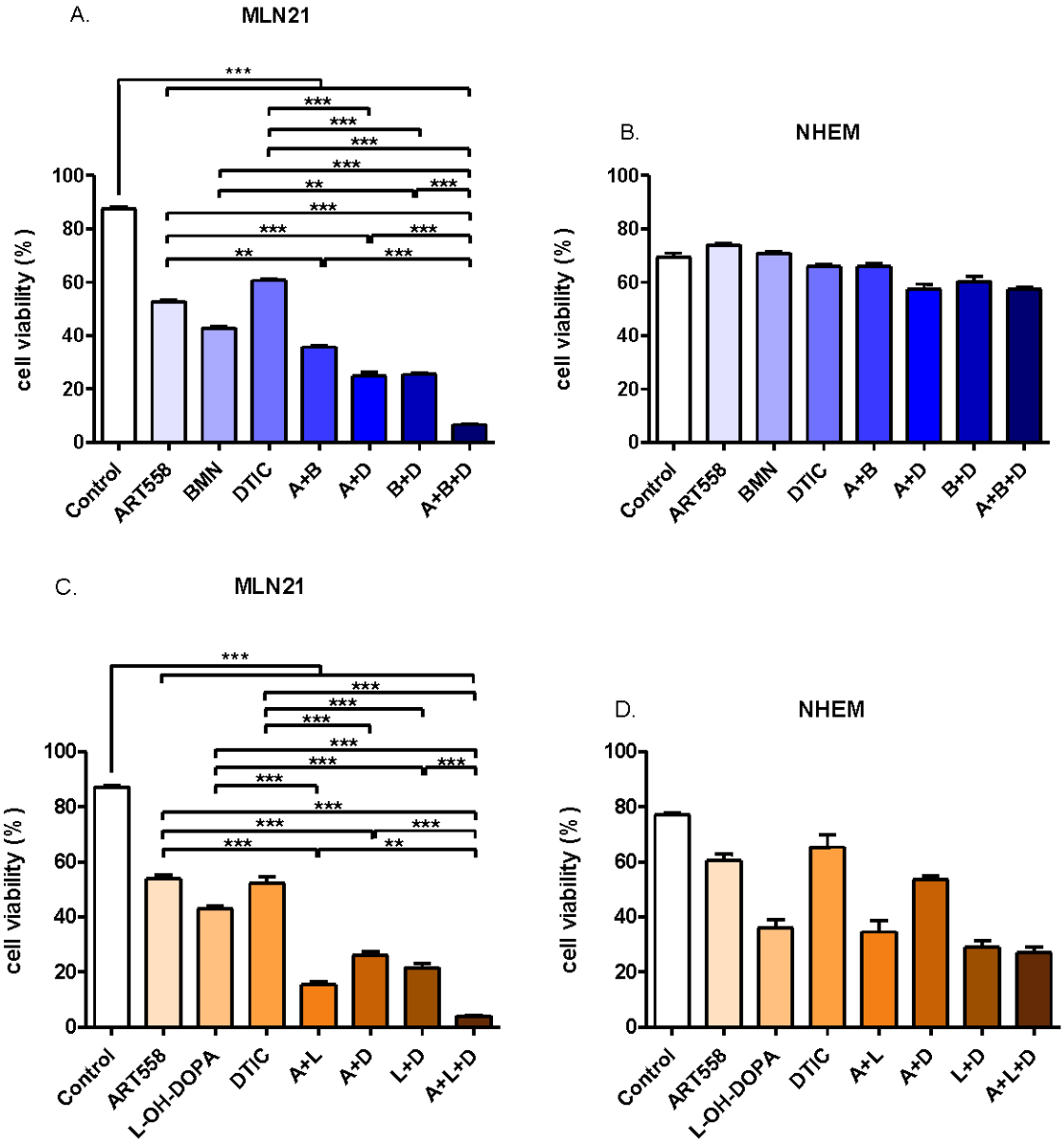
The data generated in this study are available upon request from the corresponding author.

3.Results

3.1. ART558 inhibitor used alone or in combination with PARP1/RAD52 inhibitors and alkylating agent DTIC induced cytotoxic effects in patient-derived melanoma cells

3.1.1. Cell viability

Applied inhibitors and alkylating drug used alone, reduced MLN21 cell viability by around 50% versus control. Polθ inhibition in combination with PARP1i or RAD52i gave significantly higher reduction of MLN21 vibility in comparison to Polθ inhibition itself. Also, addition of DTIC enhanced significantly the anticancer properties of separately used inhibitors: Polθi, PARPi or RAD52i. However, the strongest effect, and significantly higher than all variants of treatment, was observed after addition of DTIC to dual inhibitions, in both PARPi and RAD52i variants (Fig. 1 A,C). The changes in NHEM viability after all variants of treatment did not give statistical significant results (Fig. 1 B,D). The double staining with calcein AM and PI ilustrated the described changes in cell viability between MLN21 and NHEM, untreated and treated with the both combinations of three drugs: Polθi, DTIC and PARPi or RAD52i. The increased amount of dead MLN21 cells (stained in red) and morphorogical changes of alive ones: disrupted cellular membrane integrity and bulks, were visible after treatment. Also, the change in cell size and general appearance of the MLN21 cells could be observed. The changes in NHEM morphology were not present, only few dead cells were marked (Fig. 1 E).



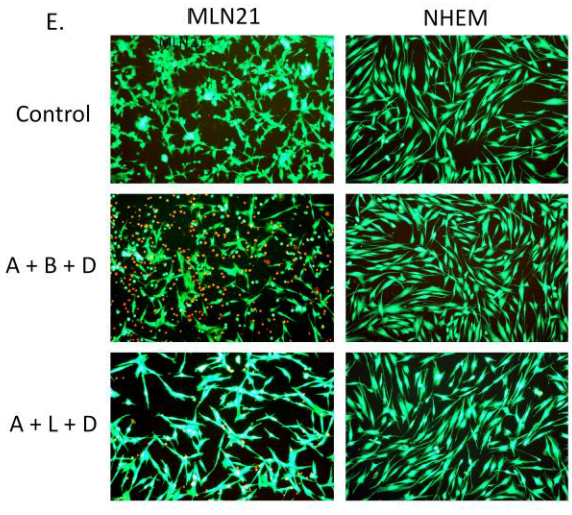


Fig. 1 A.,B.,C.,D. The effect of DNA repair proteins inhibition and treatment with alkiliting agent and their combinations on cancer MLN21 and normal NHEM cells viability. Three independent experiments were performed, and the results are shown as the mean ± standard error of the mean (SEM). * $p \leq 0.05$, ** $p \leq 0.01$, *** p-value ≤ 0.001; E. The pictures visualize morphological changes after combined treatment versus control with either Polθi, PARPi and DTIC or Polθi, RAD52i and DTIC in the MLN21 and NHEM cells, all stained with Calcein AM and propidium iodide, microscope magnitude is 10x. A – ART558, B – BMN673, L – L-OH-DOPA, D – DTIC.

3.1.2. Mechanism of cell death

The highest percentage of the cells after treatment was in late apoptosis, while the lowest in necrosis, which indicate that inhibitors and DTIC lead to cancer cell death via apoptosis (Fig 2 A,C). The notable increase of MLN21 in early and late apoptosis was observed after treatment with Polθi and PARPi with DTIC, compared to the control, separate use of this compounds and dual inhibition of Polθ and PARP (Fig. 2 A). Moreover, late stage of apoptosis was induced significantly in comaprison to the control after all variants of treatment (Fig 2 A,C). Regarding the combination of Polθi with RAD52i, it gave significant increase of early and late apoptosis in MLN21 versus the control, Polθi and RAD52i. The addition of DTIC to this combination caused similarly significant elevation of early apoptosis, and notable induction of late apoptosis, versus dual inhibition of Polθ and RAD52, as well as single inhibitions (Fig. 2 C). Further, the combination of Polθi with DTIC gave significant increase of MLN21 population in early and late apoptosis in comaprison to the control, and Polθi in case of early apoptosis (Fig 2 A,C).

Differences in all varianats of treatment were almost not detectable in case of necrosis for both cell lines, besides the significant increase of necrosis for MLN21 cells after Polθi and Polθi with PARPi versus the control (Fig. 2 A). The distribution of the NHEM cells through the mechanisms of cell death was similar as for MLN21, however the differences between variants of treatments were not statisticly significant. The exception was a significant increase of NHEM cell percentage in late apoptosis after RAD52i with DTIC in comparison to the control and this combination with Polθi in comparison to control, separate treatment with Polθi and DTIC, and their simultaneous application (Fig. 2 D). Considering, especially, the variant with PARPi, the treatemnt has lower impact on normal cells (Fig 2 B).

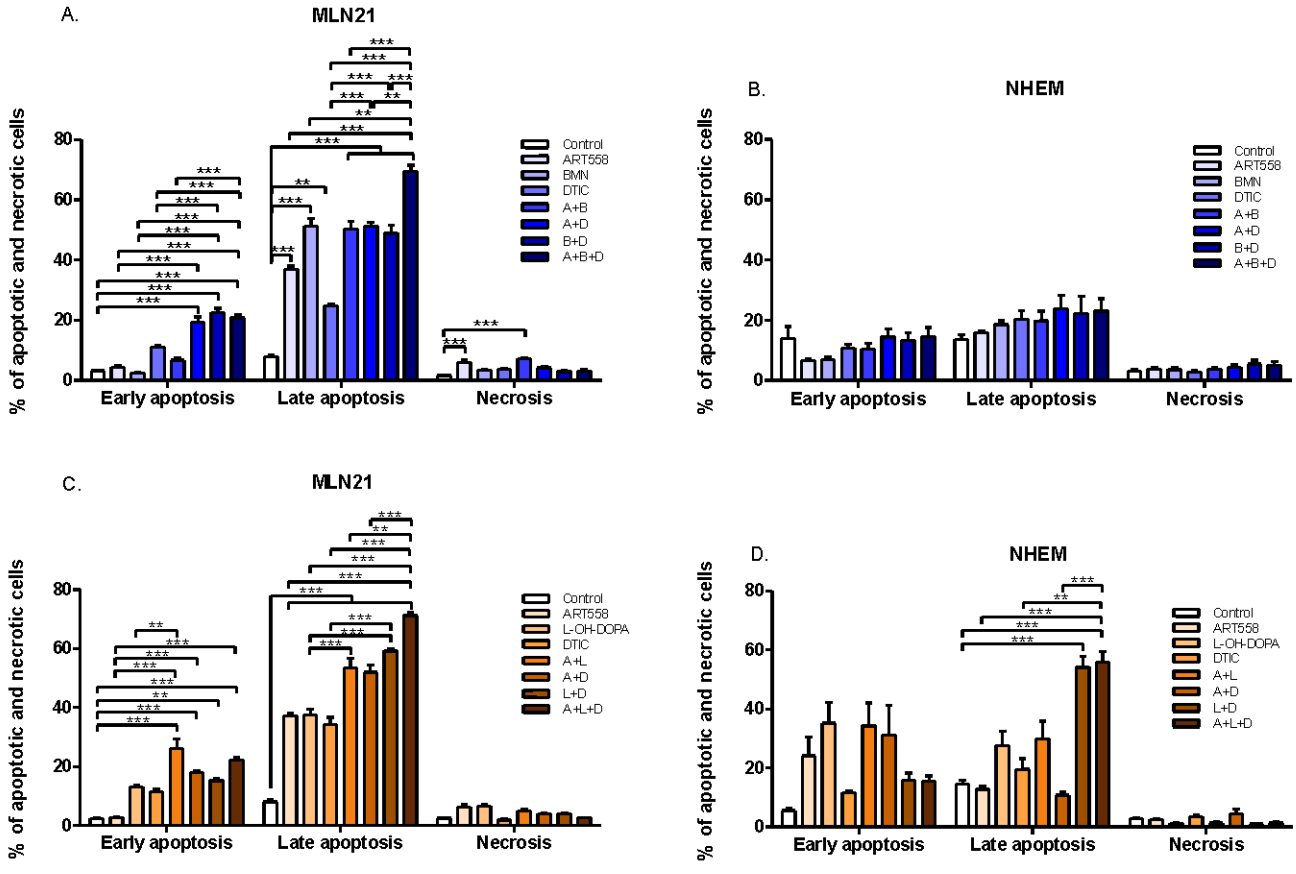
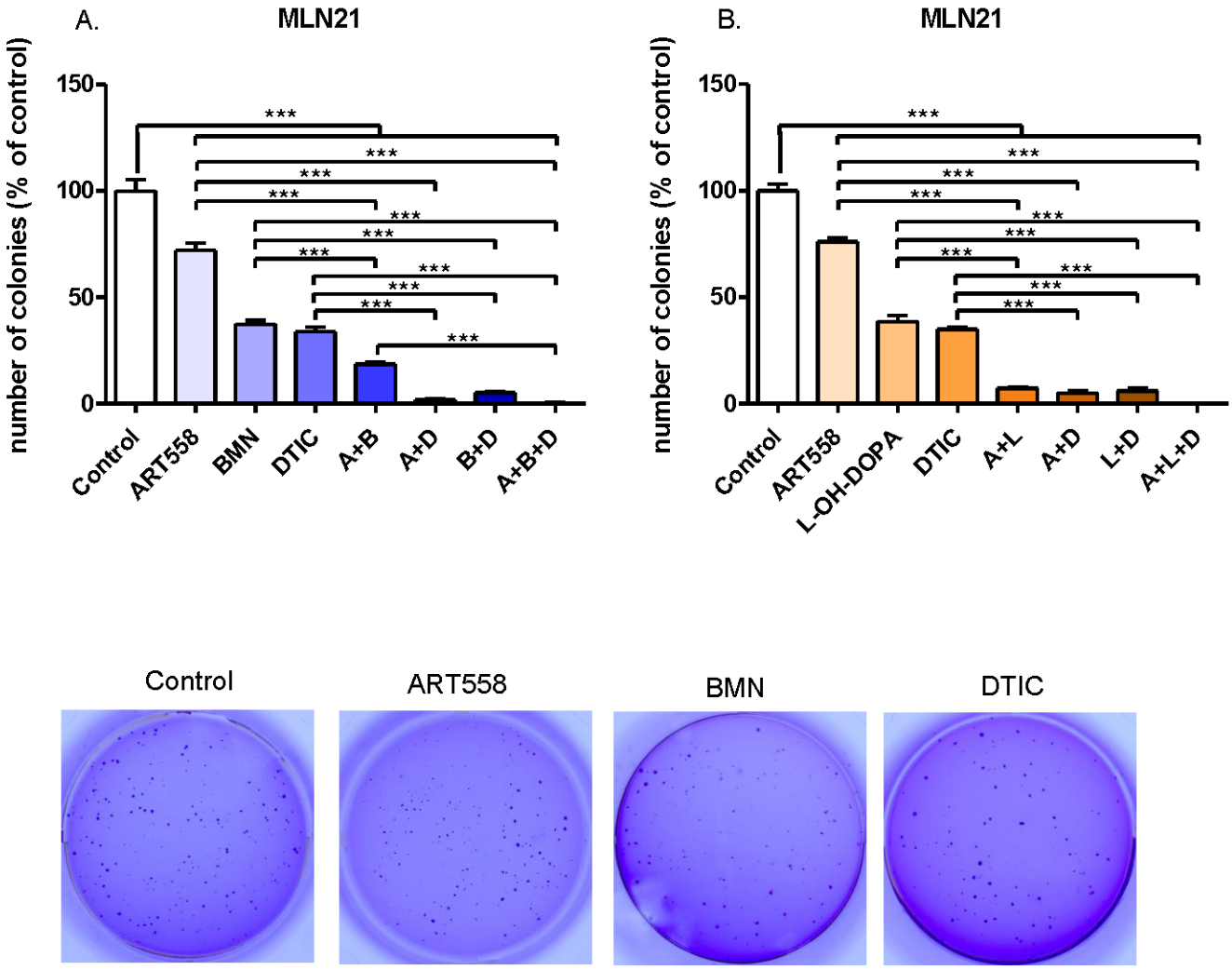


Fig. 2 Proapoptotic effect of DNA repair proteins inhibition and alkiliting agent and their combinations on cancer MLN21 and normal NHEM cells. Three independent experiments were performed, and the results are shown as the mean ± standard error of the mean (SEM). * $p \leq 0.05$, ** $p \leq 0.01$, *** p-value ≤ 0.001; C. late apoptosis, in comparison to the control all results are statisticaly significant with p-value ≤ 0.001; A – ART558, B – BMN673, L – L-OH-DOPA, D – DTIC.

3.2. Inhibition of Polθ and its combination with PARP1 or Rad52 inhibition decreases invasive characteristic of the melanoma cells

We observed decreased cell proliferation shown in reduced colony formation after all variants of treatment in comparison to the control. The combination of inhibitors, Polθi with PARP1i or RAD52i decreased the number of colonies significantly more, compared to separate inhibitions. Moreover, the addition of DTIC to dual inhibitions reduced colony formation almost completely, what is visible both in graphs and pictures (Fig. 3). Nevertheless, the PARP1i, RAD52i or DTIC used separately have around 50% stronger effect than Polθi alone. Overall, these results show that treatment with inhibitors, alone or in combination reduce spreading activity of melanoma cells.



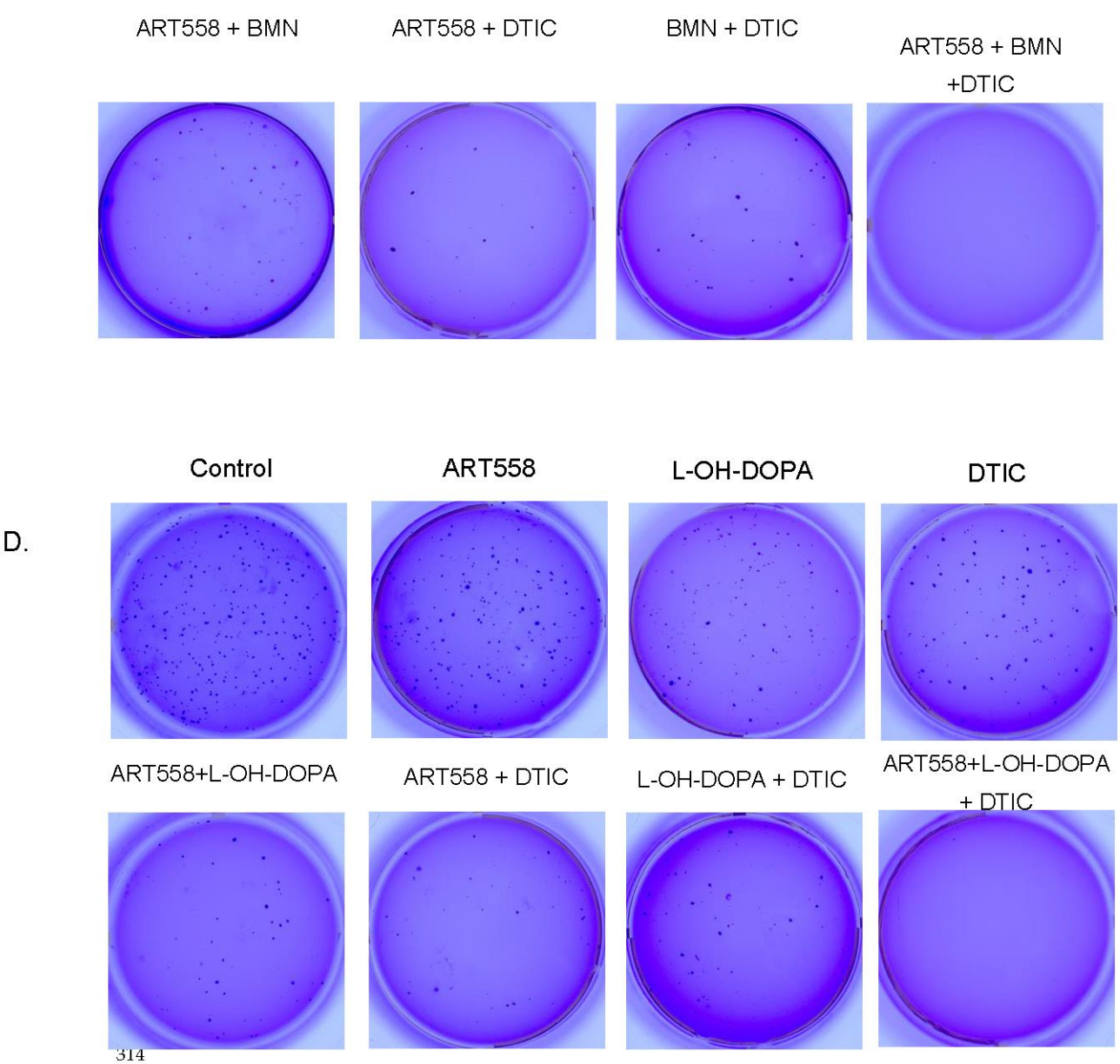


Fig. 3 Reduction of colony formation induced by DNA repair proteins inhibition and alkiliting agent and their combinations in MLN21 cells. Three independent experiments were performed, and the results are shown as the mean ± standard error of the mean (SEM). * $p \leq 0.05$, ** $p \leq 0.01$, *** p-value ≤ 0.001; A – ART558, B – BMN673, L – L-OH-DOPA, D – DTIC.

3.3 Combined inhibition of Polθ with either PARP1 or Rad52 and DTIC leads to decrease of G0/G1 and arrest of the melanoma cell population in S phase

The significant decrease of MLN21 cell population in phase G0/G1 of cell cycle was observed after treatment with Polθi, PARPi and DTIC, in comparison to the control, single inhibition of Polθ and dual inhibition of Polθ and PARP. Simultaneous cumulation of cells in S phase was observed, after the same combination of treatments, also versus Polθi and the combination of Polθi with PARPi (Fig. 4 A). The dual inhibition of Polθ and RAD52 caused significant increase of the MLN21 cells in phase subG0/G1 and decrease of G0/G1

phase verus the control and Polθ inhibition. The suplementation of this treatment with DTIC gave similar results and, additionally, significant elevation of S phase, in comparison to separate usage o Polθi. Moreover, the combination of Polθi with DTIC induced significant elevation of subG0/G1, while decrease of G0/G1 and G2/M phases, in comparison to the control and Polθi, Polθi alone and the control, respectively (Fig. 4 C).

The changes in cell cycle distributuions of NHEM were not statisticly significant, except elevation of subG0/G1 and S phase after combined treatment with PARPi and DTIC, compared to the control and PARPi, and PARPi alone, respectively (Fig. 4 B).

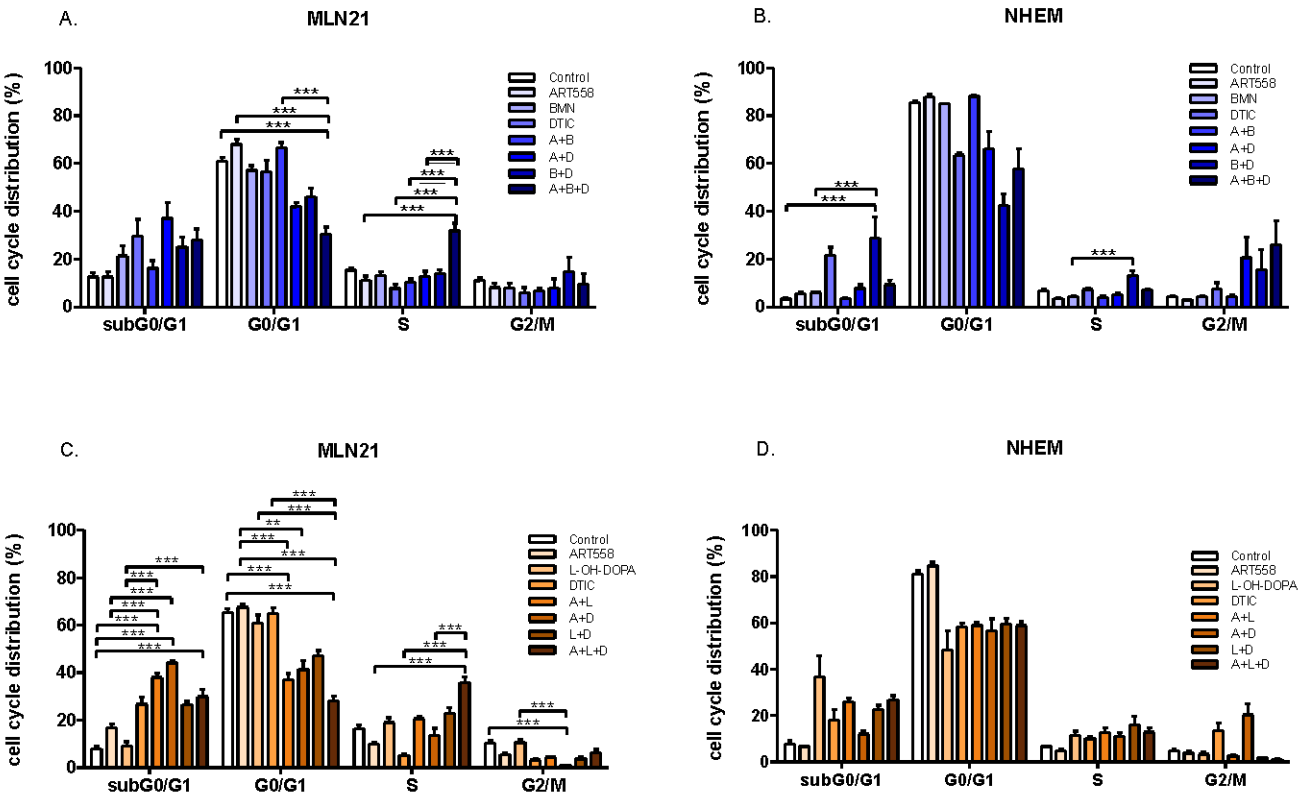


Fig. 4 Distribution of cells through cycle phases after the inhibition of DNA repair proteins and alkiliting agent treatment and their combinations in MLN21 and NHEM cells. At least two independent experiments were performed, and the results are shown as the mean ± standard error of the mean (SEM). * $p \leq 0.05$, ** $p \leq 0.01$, *** p-value ≤ 0.001; A – ART558, B – BMN673, L – L-OH-DOPA, D – DTIC.

3.4. Coinhibition of Polθ with PARP1 increases the number of DSBs in the melanoma cells

The level of H2AX phosporylation in MLN21 cells, serving as marker of DSBs, was significantly elevated after separate Polθ inhibition and the dual inhibition of Polθ and PARP1, compared to the control. The dual inhibition combined with DTIC enhanced notably the γH2AX versus the control and Polθi. Also, the combination of Polθi or PARPi with DTIC induced significant elevation of γH2AX in comapriosn to the separate treatment with these compounds, respectively Polθi or PARPi and DTIC (Fig. 5 A). The changes between treatment variants for NHEM were not statisticly siginificant (Fig. 5

B,D). Also, variant of the experiment with RAD52i did not give significant results (Fig. 5 C).

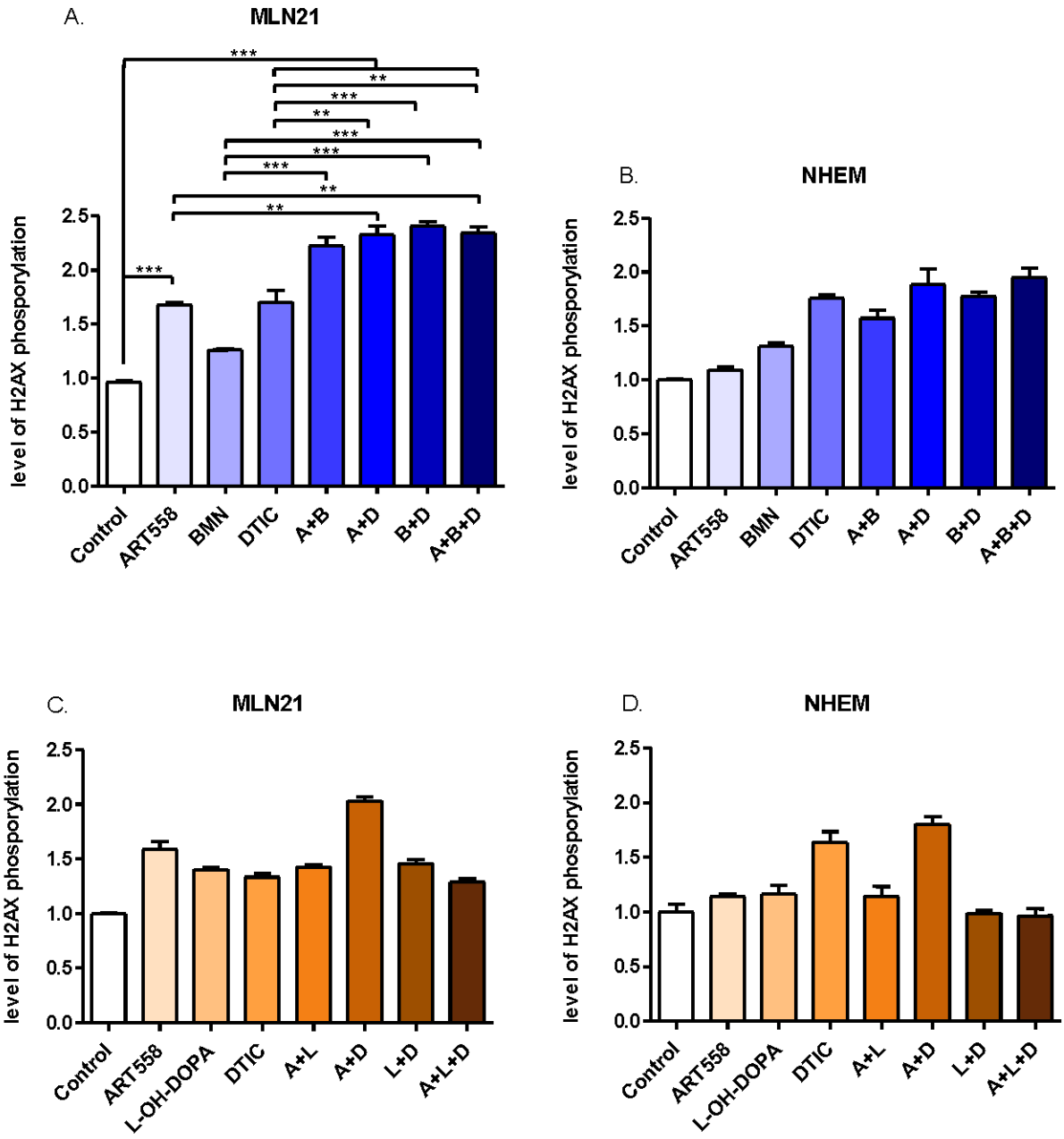


Fig. 5 H2AX phosporylation expressed as level of fluorescence signal relative to the control induced by the inhibition of DNA repair proteins and alkiliting agent treatment and their combinations in MLN21 and NHEM cells. At least two independent experiments were performed, and the results are shown as the mean ± standard error of the mean (SEM). * $p \leq 0.05$, ** $p \leq 0.01$, *** p-value ≤ 0.001; A – ART558, B – BMN673, L – L-OH-DOPA, D – DTIC.

3.5. Coinhibition of Polθ with PARP1 or RAD52 sensitizes melanoma cells to gamma radiation

The amount of DNA in comet tail in neutral version of this assay is a direct indicator of DNA damage, specifically DSBs. The melanoma cells were exposed to gamma radiation in order to simulate radiotherapy and investigate its interaction with the the used compounds. The percentage of DNA in tail in cells without radiation is lower, as could be expected. However, the correlation observed between the variants of treatments are similar in those two cases. Therefore, the amount of DNA in comet tail in MLN21 cells was significantly elevated after combined inhibition of Polθ with PARP or RAD52 compared to the control and Polθi. The addition of DTIC to dual inhibitions further increased level of the DSBs, significantly in comaprison to the control, separate use of the compounds and dual inhibitions, in all variants of the experiment. Also, similarly to other experiments the combiantion of Polθi with DTIC, again in all variants of the experiment, significantly induced DSBs, versus control and Polθi (Fig. 6 A,B,C,D). Moreover, it is worth to notice that Polθi by itself did not increase the DSBs in MLN21 cells in any variant of the experiment, in contrast to RAD52 inhibitor (Fig. 6 C,D).

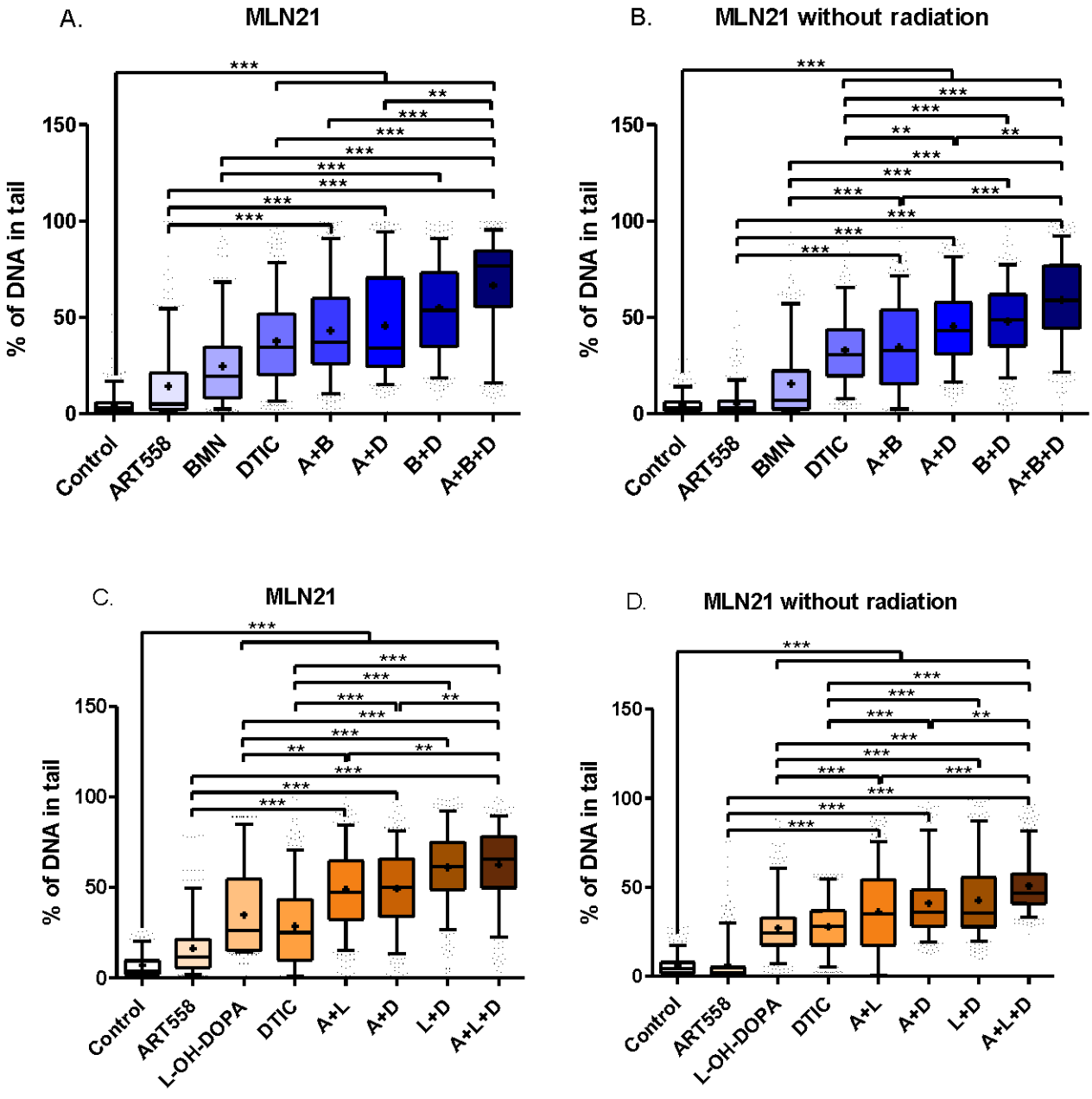


Fig. 6 Double-strand breaks induced by the inhibition of DNA repair proteins and alkiliting agent treatment and their combinations in MLN21 cells, with and without gamma radiaiton, visualized as percentage of DNA in comet tails. 50 randomly selected cells in three independent experiments were analysed and are shown in box plots with whiskers represanting 5-95 percentile. "+" shows mean value; * $p \leq 0.05$, ** $p \leq 0.01$, *** p-value ≤ 0.001; A. In comparison to the control all results are statisticaly significant with p-value ≤ 0.001; A – ART558, B – BMN673, L – L-OH-DOPA,D – DTIC.

3.7. Polθ Inhibitor (RP-6685) in combination with RAD52i (D-IO3) and DTIC reduces melanoma growth in NSG mice

In this part of the study we used different compounds RP-6685 and D-IO3 to inhibit Polθ and RAD52, respectively. We can observe that the highest tumor growth inhibition (TGI) was caused by D-IO3 with RP-6685 solvent. Also, TGI was observed after treatment with RP6685 alone and its combination with D-IO3, giving very similar result. Then, slightly enhanced TGI was observed after addition of DTIC to these two compounds together. Interestingly, tumor growth was also inhibited by separate use of RP6685 solvent. Only, combination of BMN and RP6685 solvent and RP6685 with BMN and DTIC did not lead to reduction of tumor growth, while combined treatment with RP6685 and BMN caused TGI, at the lowest level in comparison to other groups

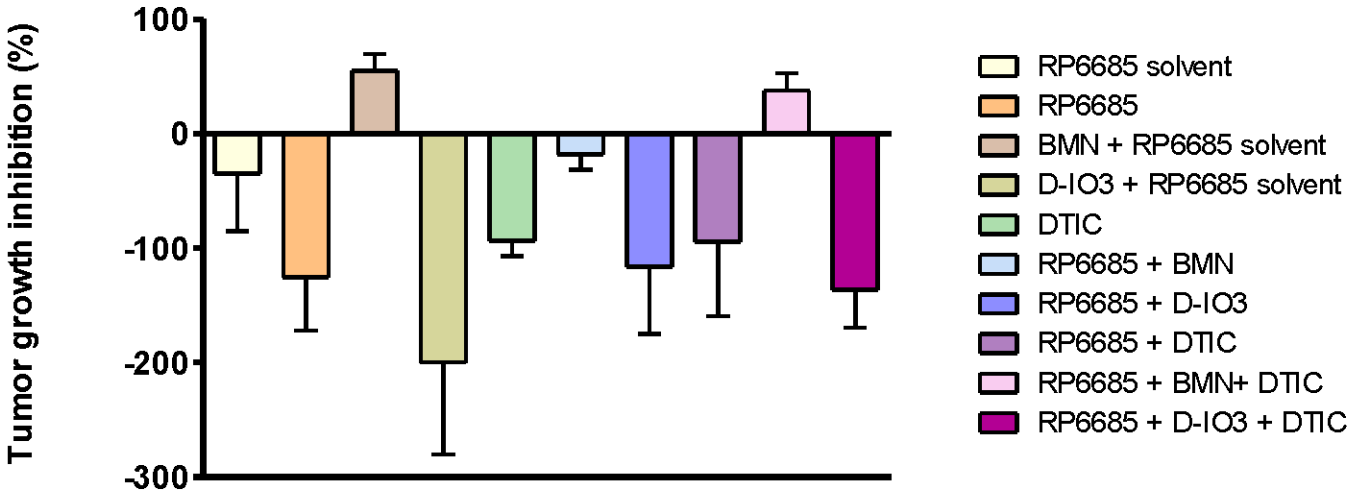


Fig. 7 The differences in tumor growth after Polθ inhibiton with PARPi or RAD52i, and DTIC and their combination, expressed as percentage of tumor growth inhibition (TGI) versus vehicle.

4.Discussion

In our research we proved that inhibition of Polθ alone induce cytotoxic effect, visible in increased cell death via apoptosis, elevated level of DSBs measured by γH2AX, and decreased cell proliferation observed in reduction of colony formation. Moreover, what is more important, addition of PARPi or RAD52i enhances significantly its cytotoxic and genotoxic effect, probably by inducing synthetic lethality. Also, the decrease of MLN21 population in G0/G1 phase of cell cycle corresponds with obtained results. While elevation of S phase could be due to accumulated DNA damages and stalled replication which causes cell's arrest in this phase. Whereas, normal melanocytes were not affected in such extend by the inhibitors, especially Polθi and PARPi. RAD52i - L-OH-DOPA show some cytotoxicity to NHEM, however mostly these results do not exhibit statistical significance.

Other interesting aspect is the addition of alkylating drug dacarbazine to the presented treatment, which further enhanced strongest anticancer effect. The deleterious activity of dual inhibitions to melanoma is supported by DTIC-induced DNA damage. Moreover, other studies report higher sensitivity of cancer cells with inhibited PARP protein to chemotherapeutics such as dacarbazine (22). Similar correlation is possible also in our research, even more while dual inhibition of PARP and Polθ takes place. However, the impact of this combined treatment on normal cells need to be investigated more extensively. As shown in our study, it has the highest effect also on NHEM cells, still not as big as on cancer cells.

Next supplementation to the treatments was gamma radiation. We observed sensitization of melanoma to gamma radiation by prior dual inhibitions with or without DTIC. Although, Rodriguez-Berriguete et al., (2023) present that inhibition of Polθ alone sensitizes cancer cells to radiotherapy, independently on HR-deficiency (23). Similarly, other study shows that Polθ knockout induces radiosensitization of tumor cells with its initial overexpression. Whereas, the normal cells with standard expression of the *POLQ* do not exhibit such sensitivity to radiation (24). This suggests important role of Polθ in observed correlation in our study.

The crucial element of the study, the part of the treatment of human melanoma xenograft, gives promising results that inhibition of Polθ by RP-6685 alone and in combination with RAD52i (D-IO3), and DTIC shows relatively strong tumor growth inhibition. However, the differences between these groups are very small, therefore the mechanism of RP-6685 antitumor activity must be further investigated in context of the future application. Also, the lack of reaction to BMN could suggest that this tumor is resistant to PARPi. Due to described poor activity of ART558 as *in vivo* probe, for this part of the study we chose RP-6685 based on its promising results in animal model (25-27). Also, we excluded L-OH-DOPA due to its potential involvement in Alzheimer's disease in animals, therefore we selected D-IO3 as RAD52 inhibitor (28). The research group, which introduced RP-6685 as a first, demonstrated its beneficial effect as a single agent against BRCA2-deficient HCT116 xenografts, but only for the 8 out of 21 days of the treatment (27). The other study also reported the antileukemia effect of RP-6685 in NRGS mice with HR-deficient primary acute myeloid leukemia xenograft, however in combination with PARPi (olaparib), not separately (29).

Interestingly, even though we did not discover any particular gene deficiency in examined cells we established the cytotoxic effect of inhibitors (Supplementary Fig. S1). This may explain lower effectiveness of the Polθ/PARP1/RAD52 inhibition separately, in comparison to simultaneous inhibition of two repair systems. On the other hand, overexpression of *POLQ* in comparison to normal cells corresponds with the findings of various studies that *POLQ* expression is upregulated in cancer cells and could indicate MLN21 cells dependence on alternative repair pathway such as TMEJ (30-34). The other explanation could be that these cells have specific mutations in any of repair pathways gene and this would have to be further investigated. We have also attempted to perform Polθ knockout

by CRISPR-Cas9 on MLN21 cell line, however the cells did not survive the procedure. This could support the hypothesis that these cells posses mutation in one of the crucial repair pathway, therefore simultaneous knockout of Polθ is lethal for them.

Moreover, in context of the treatment with the compounds applied in this study, we cannot omit the aspect of the drug resistance. The resistance to PARPi, as well as, to dacarbazine, is already studied and described (4,11,35–37). Interestingly, it is Polθ inhibition, which is suspected to be beneficial in overcoming PARPi resistance (13,25). The research suggests that microhomology-mediated DNA repair, which takes place during TMEJ, is involved in forming BRCA-gene reversion mutations (25). Moreover, Zhou et al., (2021) shows that tumor cell line with PARPi resistance, acquired by BRCA2 reversion mutation, additionally, exhibit resistance to Polθ inhibitor (Novobiocin), correlated with low level of Polθ expression (13). In addition, Liddiard et al., (2022) suggest that Polθ inhibition provoking genome instability may lead to variation and clonal evolution and therefore drug resistant tumor (10). It is the important risk, which needs to be taken into consideration in the potential therapy development.

5.Conclusions

In summary, our findings provide support for the idea that combined inhibition of Polθ with PARP or RAD52 could be an effective approach for the therapies against melanoma. While our study show the anticancer effect of analyzed inhibitors on melanoma cells, extension of the study to more cells lines and broader analysis of their genetic profile would be recommended. Also, it could be considered as a limitation of this study that it was performed with only one patient-derived melanoma cell line, therefore it must to be scaled up for further investigation of the topic. However, we believe that our findings may contribute to the development of the future therapy for melanoma based on Polθ inhibition. Moreover, the amount of unrevealed Polθ inhibitors during last 3 years: ART558 an its derivatives, Novobiocin, RP-6685, RTx-161/RTx-152 and 4 ongoing clinical studies involving Polθ inhibitors (NCT05687110, NCT06077877, NCT04991480, NCT05898399) shows the importance of this topic and highlights the direction for future research on cancer treatment (13,23,25-27,38,39).

Supplementary Materials: Figure S1 Melanoma gene expression.

Author Contributions: G.B.-P. original draft preparation, performed the cell culturing and drug treatment, clonogenic assay, gene expression, calcein/propidium iodide double staining, analysis of the data; D.J. and M.D. performed flow cytometric analysis; G.H, D.W. performed experiments with xenografts; I.Z, J.P. provided with surgical specimens; M.T. and K.B. performed comet assay; P.C. reviewed and edited the manuscript, analyzed the data; T. Ś. and T.S. conceived the project, designed the experiments, reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: The APC and the research was funded by the Polish National Science Center UMO-2020/37/B/NZ7/00422 [T Sliwinski]. T. Skorski was supported by the grants from NIH/NCI 1R01 CA244179, CA247707, CA186238, CA244044, and CA2372861.

Institutional Review Board Statement: The study performed on cells derived from surgical specimens was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee the Medical University of Lodz (no RNN/23/22/KE) and informed consent was obtained from all patients.

References

1. Pavri SN, Clune J, Ariyan S, Narayan D. Malignant Melanoma: Beyond the Basics. *Plast Reconstr Surg*. 2016 Aug;138(2):330e-340e. doi: 10.1097/PRS.0000000000002367

2. Hafeez A, Kazmi I. Dacarbazine nanoparticle topical delivery system for the treatment of melanoma. *Sci Rep*. 2017;(September):1–10. http://dx.doi.org/10.1038/s41598-017-16878-1

3. Rastrelli M, Tropea S, Rossi CR, Alaibac M. Melanoma: epidemiology, risk factors, pathogenesis, diagnosis and classification. In Vivo. 2014 Nov-Dec;28(6):1005-11.

4. Koprowska K, Czyż M. Dakarbazyna jako lek przeciwczerniakowy i referencyjny dla nowych programów terapeutycznych * Dacarbazine , a chemotherapeutic against metastatic melanoma and a reference drug for new treatment modalities. *Postepy Hig Med Dosw (online)*, 2011; 65: 734-751

5. Slominski A, Wortsman J, Carlson AJ, Matsuoka LY, Balch CM, Mihm MC. Malignant melanoma. Arch Pathol Lab Med. 2001 Oct;125(10):1295-306. doi: 10.5858/2001-125-1295-MM.

6. Mervic L. Prognostic factors in patients with localized primary cutaneous melanoma. *Acta Derm*. 2012;21:27–31. 2012;21:27-31 doi: 10.2478/v10162-012-0008-1

7. Testori AAE, Blankenstein SA, Akkooi ACJ Van. Primary Melanoma : from History to Actual Debates. *Current Oncology Reports* 2019, 21: 112. https://doi.org/10.1007/s11912-019-0843-x

8. Quintanilla-dieck MJ, Bichakjian CK. Management of Early- Stage Melanoma. *Facial Plast Surg Clin N Am*. 2019;27(1):35–42. https://doi.org/10.1016/j.fsc.2018.08.003

9. Lee S.Y. ScienceDirect Temozolomide resistance in glioblastoma multiforme. *Genes & Diseases* 2016 3(3), 198–210. https://doi.org/10.1016/j.gendis.2016.04.007

10. Liddiard K., Aston-Evans A.N., Cleal K., Baird D.M., Hendrickson E.A.. POLQ suppresses genome instability

and alterations in DNA repeat tract lengths. *NAR Cancer*. 2022;4(3):1–20., https://doi.org/10.1093/narcan/zcac020

11. Baxter J.S., Zatreanu D., Pettitt S.J., Lord C.J. Resistance to DNA repair inhibitors in cancer. *Molecular Oncology* 2022;1–17, doi:10.1002/1878-0261.13224

12. Schrempf A., Slyskova J., Loizou J.I.. Targeting the DNA Repair Enzyme Polymerase θ in Cancer Therapy. *Trends in Cancer* 2021;7(2):98–111, https://doi.org/10.1016/j.trecan.2020.09.007

13. Zhou, J.; Gelot, C.; Pantelidou, C.; Li, A.; Yücel, H.; Davis, R.E.; Färkkilä, A.; Kochupurakkal, B.; Syed, A.; Shapiro, G.I.; et al. A first-in-class polymerase theta inhibitor selectively targets homologous-recombination-deficient tumors. *Nat. Cancer* 2021, 2, 598–610,.

14. Starowicz K., Ronson G., Anthony E., Clarke L., Garvin A.J., Beggs A.D., et al. RAD52 underlies the synthetic-lethal relationship between BRCA1/2 and 53BP1 deficiencies and DNA polymerase theta loss. *bioRxiv* 2022; 485027; doi: https://doi.org/10.1101/2022.03.20.485027

15. Caracciolo D., Riillo C., Teresa M., Martino D. Tagliaferri P. Alternative Non-Homologous End-Joining: Error-Prone DNA Repair as Cancer's Achilles' Heel. *Cancers* 2021; http:// doi.org/10.3390/cancers13061392

16. Chen XS, Pomerantz RT. DNA Polymerase θ : A Cancer Drug Target with Reverse Transcriptase Activity. *Genes* 2021, 12, 1146. https://doi.org/10.3390/ genes12081146

17. Schaub JM, Soniat MM, Finkelstein IJ. Polymerase theta-helicase promotes end joining by stripping single-stranded DNA-binding proteins and bridging DNA ends. *Nucleic Acids Research*, 2022, Vol. 50, No. 7 3911–3921 https://doi.org/10.1093/nar/gkac119

18. Czyż M., Toma M., Gajos-Michniewicz A., Majchrzak K., Hoser G., Szemraj J., et al. PARP1 inhibitor olaparib (Lynparza) exerts synthetic lethal effect against ligase 4-deficient melanomas. *Oncotarget*. 2016 7(46):75551–60.

19. Czyż M., Toma M., Gajos-Michniewicz A., Majchrzak K., Hoser G., Szemraj J., et al. PARP1 inhibitor olaparib (Lynparza) exerts synthetic lethal effect against ligase 4-deficient melanomas. *Oncotarget*. 2016 7(46):75551–60.

20. Drzewiecka M., Gajos-Michniewicz A., Hoser G., Jaśniak D., Barszczewska-Pietraszek G., Sitarek P., et al. Histone Deacetylases (HDAC) Inhibitor—Valproic Acid Sensitizes Human Melanoma Cells to Dacarbazine and PARP Inhibitor. *Genes (Basel)*. 2023;14(6):1295.

21. Drzewiecka M.; Jaśniak D.; Barszczewska-Pietraszek G.; Czarny P.; Kobrzycka A.; Wieczorek M.; Radek,M.; Szemraj J.; Skorski T.; Śliwiński T. Class I HDAC Inhibition Leads to a Downregulation of FANCD2 and RAD51, and the Eradication of Glioblastoma Cells. *J. Pers. Med*. 2023, 13, 1315. https:// doi.org/10.3390/jpm13091315

22. Somnay Y, Lubner S, Gill H, Matsumura JB Chen H. The PARP inhibitor ABT-888 potentiates darbazine-induced cell death in carcinoids. *Cancer Gene Ther*. 2016 October ; 23(10): 348–354. doi:10.1038/cgt.2016.39.

23. Rodriguez-Berriguete G., Ranzani M., Prevo R., Puliyadi R., Machado N., Bolland H.R., et al. Small-molecule Polθ inhibitors provide safe and effective tumor radiosensitization in preclinical models. *Clin Cancer Res*. 2023;(22):1–12.

24. Higgins G.S., Prevo R., Lee Y., Helleday T., Muschel R.J., Taylor S., et al. A siRNA Screen of Genes Involved in DNA Repair Identifies Tumour Specific Radiosensitisation by POLQ Knockdown. *Cancer Res.* 2010 April 1; 70(7): 2984–2993, doi:10.1158/0008-5472.CAN-09-4040

25. Zatreanau, D.; Robinson, H.M.R.; Alkhatib, O.; Boursier, M.; Finch, H.; Geo, L.; Grande, D.; Grinkevich, V.; Heald, R.A.; Langdon, S.; et al. Polθ inhibitors elicit BRCA-gene synthetic lethality and target PARP inhibitor resistance. *Nat. Commun.* 2021, 12, 36, http://dx.doi.org/10.1038/s41467-021-23463-8

26. Stockley ML, Ferdinand A, Benedetti G, Blencowe P, Boyd SM, Calder M, et al. Discovery, Characterization, and Structure-Based Optimization of Small-Molecule in Vitro and in Vivo Probes for Human DNA Polymerase Theta. *J Med Chem.* 2022;65(20):13879–91. https://doi.org/10.1021/acs.jmedchem.2c01142

27. Bubenik, M.; Mader, P.; Mochirian, P.; Vallée, F.; Clark, J.; Truchon, J.F.; Perryman, A.L.; Pau, V.; Kurinov, I.; Zahn, K.E.; et al. Identification of RP-6685, an Orally Bioavailable Compound that Inhibits the DNA Polymerase Activity of Polθ. *J. Med. Chem.* 2022, 65, 13198–13215.

28. Nivsarkar M, Banerjee A. Establishing the probable mechanism of L-DOPA in Alzheimer's disease management. Acta Pol Pharm. 2009 Sep-Oct;66(5):483-6..

29. Sullivan-Reed K., Toma M.M., Drzewiecka M., Nieborowska-Skorska M., Nejati R., Karami A., et al. Simultaneous Targeting of DNA Polymerase Theta and PARP1 or RAD52 Triggers Dual Synthetic Lethality in Homologous Recombination–Deficient Leukemia Cells. *Mol Cancer Res.* 2023;21(10):1017–22.

30. Ceccaldi R, Liu JC, Amunugama R, Hajdu I, Petalcorin MIR, Connor KWO, et al. Homologous recombination-deficient tumors are hyper- dependent on POLQ-mediated repair. *Nature.* 2015 February 12; 518(7538): 258–262. doi:10.1038/nature14184.

31. Pan Q, Wang L, Liu Y, Li M, Zhang Y, Peng W, et al. Knockdown of POLQ interferes the development and progression of hepatocellular carcinoma through regulating cell proliferation, apoptosis and migration. *Cancer Cell Int.* 2021;1–13. https://doi.org/10.1186/s12935-021-02178-2

32. Lemée F., Bergoglio V., Fernandez-vidal A., Machado-silva A, Pillaire M. DNA polymerase θ up-regulation is associated with poor survival in breast cancer , perturbs DNA replication , and promotes genetic instability. *PNAS* 2010;107(30).

33. Higgins GS, Harris AL, Prevo R, Helleday T, Gillies W, Buffa FM. Overexpression of POLQ Confers a Poor Prognosis in Early Breast Cancer Patients. *Oncotarget*, 2010;1(3):175–84.

34. Patterson-Fortin J., Jadhav H., Pantelidou C., Phan T., Grochala C., Mehta A.K., et al. Polymerase θ inhibition activates the cGAS-STING pathway and cooperates with immune checkpoint blockade in models of BRCA-deficient cancer. *Nat Commun.* 2023;14(1):1390, http://www.ncbi.nlm.nih.gov/pubmed/36914658

35. Slade D. PARP and PARG inhibitors in cancer treatment. *Genes Dev.* 2020;34(5):360–94.

36. Chan CY, Tan KV, Cornelissen B. PARP inhibitors in cancer diagnosis and therapy. *Clin Cancer Res.* 2021;27(6):1585–94.

37. Higgins BGS, Boulton SJ. Beyond PARP—POL u as an anticancer target. *Science* 2018;359(6381):1217–9.

38. Fried W., Tyagi M., Minakhin L., Chandramouly G., Tredinnick T., Ramanjulu M., et al. Discovery of a small-molecule inhibitor that traps Polθ on DNA and synergizes with PARP inhibitors. *Nat Commun.* 2024;15(1):1–15, https://doi.org/10.1038/s41467-024-46593-1

39. Wang Y., Wang C., Liu J., Sun D., Meng F., Zhang M., et al. Discovery of 3-hydroxymethyl-azetidine derivatives as potent polymerase theta inhibitors. *Bioorg Med Chem.* 2024 Apr 1;103:117662.

Figure S1 Melanoma MLN21 gene expression

Methodology : RNA isolation and gene expression

Analysis of mRNA expression of 28 selected genes, which products are involved in following DSB repair mechanisms: HR, NHEJ, TMEJ and a-NHEJ, began with the isolation of total RNA using RNeasy Mini Kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions. After the detachment from culture bottles, cells were centrifuged and then resuspended in PBS to determine cell density. Approximately $5x10^6$ cells were used for isolation, with a survival rate of 97-99%.

Subsequently, the purity and quantity of RNA were evaluated by spectrophotometric analysis in Picodrop, then 10 ng/µL RNA was converted into complementary DNA (cDNA), using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Real-time PCR was performed with TaqMan® probes dedicated to detect 4 reference and the selected genes. The RT-PCR reactions were conducted on CFX96 ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California, USA) with parameters consisted of an initial step of 95 ◦C for 10 min, followed by 30 cycles of denaturation at 95 ◦C for 15 s and annealing/extension at 60 ◦C for 60 s., using TaqMan™ Universal Master Mix II probes, no UNG (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Each reaction was performed in triplicates. *ACTB* was used as a reference gene after analysis in Geneinvestigator where it was established with the most stable expression between 4 selected reference genes. The results were calculated as fold change of genes expression in cancer versus normal cells ($2^{-\Delta\Delta Ct}$).

Description of the reuslts*:* To assess genetic profile of cancer cell line and potential deficinency in any of repair pathway we determine expression level of 28 genes, selected due to their activity in DSBs repair pathways. We did not observed a downregulation of any recalled gene versus NHEM cells. However, relatively high expression of HR-related genes and *POLQ* of TMEJ, while all genes involved in NHEJ have realtively low expression. Therefore, this may explain higher effectivness of dual inhibition with Polθi and PARPi or RAD52i.

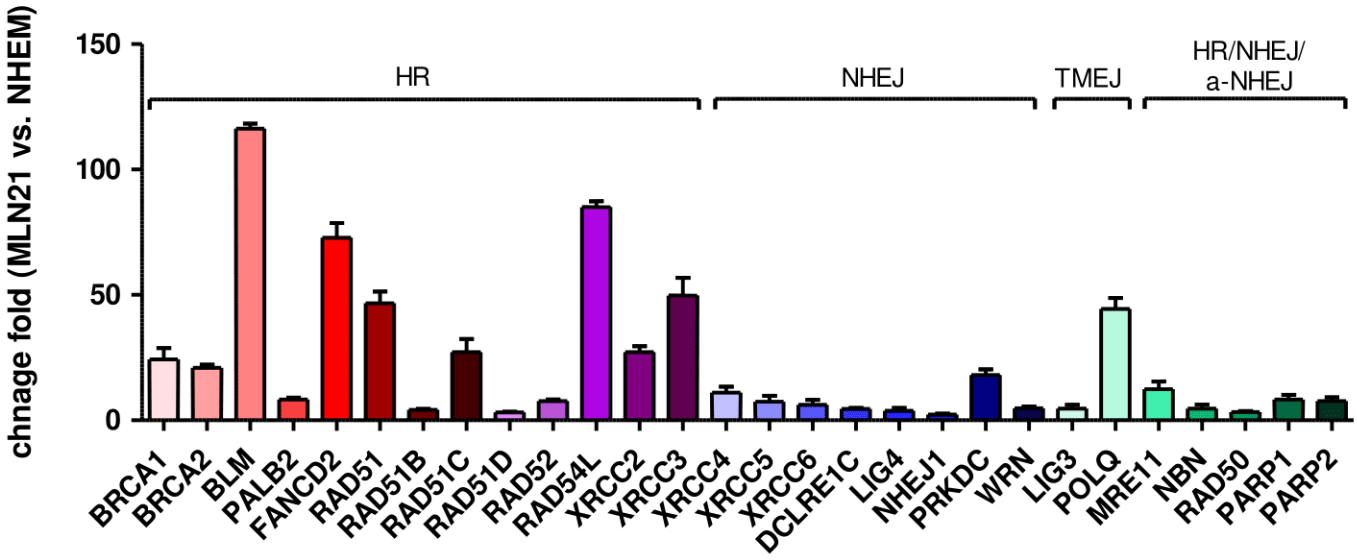


Fig. S1 Expression profile of 28 genes, categorized due to their activity in following DNA double strand breaks repair mechanisms: homologous recombination (HR), non-homologous end joining (NHEJ), theta-mediated end joining (TMEJ), alternative non-homologous end joining (a-NHEJ) in MLN21 melanoma cells, presented as a fold change in reference to normal cells NHEM. Results represent mean value ± SEM from the experiment performed in triplicate.

Fwd: Submission received: MCT-24-0486 at Molecular Cancer Therapeutics

Tomasz Śliwiński <tomasz.sliwinski@biol.uni.lodz.pl>
Wed 19/06/2024 21:53
To:Gabriela Barszczewska-Pietraszek <gabriela.barszczewska.pietraszek@edu.uni.lodz.pl>

Wysłane z iPhone'a

Początek przekazywanej wiadomości:

Od: Molecular Cancer Therapeutics <mct@msubmit.net>
Data: 19 czerwca 2024 o 21:24:28 CEST
Do: Tomasz Śliwiński <tomasz.sliwinski@biol.uni.lodz.pl>
Temat: Submission received: MCT-24-0486 at Molecular Cancer Therapeutics
Odpowiedz-do: brittany.rubio@aacr.org

Dear Dr. Śliwiński,

Thank you for submitting your manuscript to *Molecular Cancer Therapeutics*. Your manuscript has successfully completed our initial quality control process and will now be assessed by an editor.

Please note that our office will primarily confer with you, the corresponding author, with regard to this submission. Pertinent details are below. Please use the listed manuscript number in all future correspondence with our office relating to this submission.

Manuscript Number: MCT-24-0486
Manuscript Type: Research Article
Corresponding Author: Dr. Śliwiński

Polθ inhibition with simultaneous treatment with PARP or RAD52 inhibitors induces cyto- and genotoxic effect in mela-noma cells and reduces tumor growth of human melanoma xenograft
Gabriela Barszczewska-Pietraszek, Piotr Czarny, Grazyna Hoser, Dominika Jaśniak, Malgorzata Drzewiecka, Izabela Zaleśna, Janusz Piekarski, Monika Toma, Katarzyna Białek, Danuta Wasilewska, Tomasz Skorski, and Tomasz Śliwiński
Polθ inhibitors (Polθi) hold great potential as anticancer treatment due to its ability to tar-get cancer cells specifically. The particular Polθi targets are tumors with DNA repair defi-ciencies, which often become reliant on theta-mediated end-joining, a DNA repair mecha-nism facilitated by Polθ. The studies performed on HR-deficient cancer cells show that simultaneous inhibition of Polθ with other DNA repair protein such as PARP or RAD52 leads to synthetic lethal correlation killing cancer cells selectively. In this study, we show that combining Polθi (ART558) with either PARP1 or RAD52 inhibitors enhances anti-cancer effect on melanoma in comparison to separate treatment with

Polθi, especially when paired with dacarbazine (DTIC). In addition, we observed reduced tumor size in human melanoma xenografts after the combined treatment with Polθ inhibitor (RP-6685), RAD52 inhibitor (D-IO3) and DTIC. The treatment's impact on normal cells appears min-imal compared to cancer cells, although further research is needed to ensure safety and ef-ficacy. The potential drug resistance poses the greatest challenge, but the promising results of combined Polθ inhibition suggest a new direction for melanoma therapy.

You may view the manuscript files, send manuscript correspondence and check on the status of this manuscript by clicking on the link below or by logging into the AACR journals user account associated with this submission from any AACR journal submission site.

https://mct.msubmit.net/cgi-bin/main.plex?el=A5Cv4CSSL6A7HyNd5F7A9ftdcvX7wxy7BHC5lSxMViDAhQZ

Thank you again for submitting your work to *Molecular Cancer Therapeutics*. Please feel free to contact me or our office with any questions throughout the course of consideration.

Sincerely,

Molecular Cancer Therapeutics Editorial Office
mct@aacr.org

Oświadczenia współautorów

19.06.2024

mgr inż. Gabriela Barszczewska-Pietraszek

Katedra Genetyki Molekularnej

Wydział Biologii i Ochrony Środowiska

Uniwersytet Łódzki

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy

Barszczewska-Pietraszek G, Drzewiecka M, Czarny P, Skorski T, Śliwiński T. Polθ Inhibition: An Anticancer Therapy for HR-Deficient Tumours. Int J Mol Sci. 2022 Dec 24;24(1):319. doi:10.3390/ijms24010319.

mój udział polegał na koncepcji, tworzeniu manuskryptu oraz wizualizacji. Swój udział w artykule oceniam na 70%.

Gabriela Barszczewska-Pietraszek

Oświadczam, że w pracy:

Barszczewska-Pietraszek G., Czarny P., Drzewiecka M., Błaszczyk M., Radek M., Synowiec E., Wigner-Jeziorska P., Sitarek P., Szemraj J., Skorski T., Śliwiński T. Polθ inhibitor (ART558) demonstrates synthetic lethal effect with PARP and RAD52 inhibitors in glioblastoma cells.

mój udział polegał na współtworzeniu koncepcji i treści manuskryptu oraz przeprowadzeniu praca eksperymentalnych w zakresie hodowli komórkowej, analiz cytometrycznych, testu klonogennego, ekspresji genów oraz analizy i wizualizacji danych. Swój udział w artykule oceniam na 68%.

Gabriela Barszczewska-Pietraszek

Oświadczam, że w pracy:

Barszczewska-Pietraszek G., Czarny P., Hoser G., Jaśniak D., Drzewiecka M., Zaleśna I., Piekarski J., Toma M., Białek K., Wasilewska D., Skorski T., Śliwiński T., Polθ inhibition with simultaneous treatment with PARP or RAD52 inhibitors induces cyto- and genotoxic effect in melanoma cells and reduces tumor growth of human melanoma xenograft.

mój udział polegał na współtworzeniu koncepcji i treści manuskryptu oraz przeprowadzeniu praca eksperymentalnych w zakresie hodowli komórkowej, barwienia kalceiną, testu klonogennego, ekspresji genów oraz analizy i wizualizacji danych. Swój udział w artykule oceniam na 66%.

Gabriela Barszczewska-Pietraszek

prof. dr hab. Tomasz Śliwiński

Katedra Genetyki Molekularnej

Wydział Biologii i Ochrony Środowiska

Uniwersytet Łódzki

19.06.2024

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy:

Barszczewska-Pietraszek G, Drzewiecka M, Czarny P, Skorski T, Śliwiński T. Polθ Inhibition: An Anticancer Therapy for HR-Deficient Tumours. Int J Mol Sci. 2022 Dec 24;24(1):319. doi:10.3390/ijms24010319.

mój udział polegał na pozyskiwaniu funduszy, współtworzeniu koncepcji, edycji i nadzorze powstawania manuskryptu. Swój udział w artykule oceniam na 10%.

Digitally signed by Tomasz Śliwiński
Date: 2024.06.19 15:22:28 +02'00'

Oświadczam, że w pracy:

Barszczewska-Pietraszek G., Czarny P., Drzewiecka M., Błaszczyk M., Radek M., Synowiec E., Wigner-Jeziorska P., Sitarek P., Szemraj J., Skorski T., Śliwiński T. Polθ inhibitor (ART558) demonstrates synthetic lethal effect with PARP and RAD52 inhibitors in glioblastoma cells.

mój udział polegał na pozyskiwaniu funduszy, współtworzeniu koncepcji, edycji i nadzorze powstawania manuskryptu. Swój udział w artykule oceniam na 7%.

Digitally signed by Tomasz Śliwiński
Date: 2024.06.19 15:22:57 +02'00'

Oświadczam, że w pracy:

Barszczewska-Pietraszek G., Czarny P., Hoser G., Jaśniak D., Drzewiecka M., Zaleśna I., Piekarski J., Toma M., Białek K., Wasilewska D., Skorski T., Śliwiński T., Polθ inhibition with simultaneous treatment with PARP or RAD52 inhibitors induces cyto- and genotoxic effect in melanoma cells and reduces tumor growth of human melanoma xenograft.

mój udział polegał na pozyskiwaniu funduszy, współtworzeniu koncepcji, edycji i nadzorze powstawania manuskryptu. Swój udział w artykule oceniam na 5%.

Digitally signed by Tomasz Śliwiński
Date: 2024.06.19 15:23:16 +02'00'

19.06.2024

dr n.biol. Piotr Czarny

Zakład Biochemii Medycznej

Uniwersytet Medyczny w Łodzi

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy:

Barszczewska-Pietraszek G, Drzewiecka M, Czarny P, Skorski T, Śliwiński T. Polθ Inhibition: An Anticancer Therapy for HR-Deficient Tumours. Int J Mol Sci. 2022 Dec 24;24(1):319. doi:10.3390/ijms24010319.

mój udział polegał na edycji i nadzorze powstawania manuskryptu. Swój udział w artykule oceniam na 10%.

ADIUNKT
Zakładu Biochemii Medycznej
Katedry Biochemii Medycznej
Uniwersytetu Medycznego w Łodzi
dr n. biol. Piotr Czarny

Oświadczam, że w pracy:

Barszczewska-Pietraszek G., Czarny P., Drzewiecka M., Błaszczyk M., Radek M., Synowiec E., Wigner-Jeziorska P., Sitarek P., Szemraj J., Skorski T., Śliwiński T. Polθ inhibitor (ART558) demonstrates synthetic lethal effect with PARP and RAD52 inhibitors in glioblastoma cells.

mój udział polegał na edycji i nadzorze powstawania manuskryptu. Swój udział w artykule oceniam na 5%.

ADIUNKT
Zakładu Biochemii Medycznej
Katedry Biochemii Medycznej
Uniwersytetu Medycznego w Łodzi
dr n. biol. Piotr Czarny

Oświadczam, że w pracy:

Barszczewska-Pietraszek G., Czarny P., Hoser G., Jaśniak D., Drzewiecka M., Zaleśna I., Piekarski J., Toma M., Białek K., Wasilewska D., Skorski T., Śliwiński T., Polθ inhibition with simultaneous treatment with PARP or RAD52 inhibitors induces cyto- and genotoxic effect in melanoma cells and reduces tumor growth of human melanoma xenograft.

mój udział polegał na edycji i nadzorze powstawania manuskryptu. Swój udział w artykule oceniam na 5%.

ADIUNKT
Zakładu Biochemii Medycznej
Katedry Biochemii Medycznej
Uniwersytetu Medycznego w Łodzi
dr n. biol. Piotr Czarny

dr Małgorzata Drzewiecka

Katedra Genetyki Molekularnej

Wydział Biologii i Ochrony Środowiska

Uniwersytet Łódzki

19.06.2024

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy

Barszczewska-Pietraszek G, Drzewiecka M, Czarny P, Skorski T, Śliwiński T. Polθ Inhibition: An Anticancer Therapy for HR-Deficient Tumours. Int J Mol Sci. 2022 Dec 24;24(1):319. doi:10.3390/ijms24010319.

mój udział polegał na współtworzeniu powstawania manuskryptu. Swój udział w artykule oceniam na 5%.

Małgorzata Drzewiecka

Oświadczam, że w pracy

Barszczewska-Pietraszek G., Czarny P., Drzewiecka M., Błaszczyk M., Radek M., Synowiec E., Wigner-Jeziorska P., Sitarek P., Szemraj J., Skorski T., Śliwiński T. Polθ inhibitor (ART558) demonstrates synthetic lethal effect with PARP and RAD52 inhibitors in glioblastoma cells.

mój udział polegał na współudziale w wykonaniu analiz cytometrycznych. Swój udział w artykule oceniam na 3%.

Małgorzata Drzewiecka

Oświadczam, że w pracy

Barszczewska-Pietraszek G., Czarny P., Hoser G., Jaśniak D., Drzewiecka M., Zaleśna I., Piekarski J., Toma M., Białek K., Wasilewska D., Skorski T., Śliwiński T., Polθ inhibition with simultaneous treatment with PARP or RAD52 inhibitors induces cyto- and genotoxic effect in melanoma cells and reduces tumor growth of human melanoma xenograft.

mój udział polegał na współudziale w wykonaniu analiz cytometrycznych. Swój udział w artykule oceniam na 3%.

Małgorzata Drzewiecka

dr Ewelina Synowiec

Katedra Genetyki Molekularnej

Wydział Biologii i Ochrony Środowiska

Uniwersytet Łódzki

19.06.2024

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy:

Barszczewska-Pietraszek G., Czarny P., Drzewiecka M., Błaszczyk M., Radek M., Synowiec E., Wigner-Jeziorska P., Sitarek P., Szemraj J., Skorski T., Śliwiński T. Polθ inhibitor (ART558) demonstrates synthetic lethal effect with PARP and RAD52 inhibitors in glioblastoma cells.

mój udział polegał na współudziale w wykonaniu testu kometowego. Swój udział w artykule oceniam na 2%.

E. Synowiec

dr Paulina Wigner-Jeziorska

Katedra Genetyki Molekularnej

Wydział Biologii i Ochrony Środowiska

Uniwersytet Łódzki

19.06.2024

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy:

Barszczewska-Pietraszek G., Czarny P., Drzewiecka M., Błaszczyk M., Radek M., Synowiec E., Wigner-Jeziorska P., Sitarek P., Szemraj J., Skorski T., Śliwiński T. Polθ inhibitor (ART558) demonstrates synthetic lethal effect with PARP and RAD52 inhibitors in glioblastoma cells.

mój udział polegał na współudziale w wykonaniu testu kometowego. Swój udział w artykule oceniam na 2%.

paulina Wigner-Jeziorska

dr Katarzyna Białek

Zakład Biochemii Medycznej

Uniwersytet Medyczny w Łodzi

19.06.2024

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy:

Barszczewska-Pietraszek G., Czarny P., Hoser G., Jaśniak D., Drzewiecka M., Zaleśna I., Piekarski J., Toma M., Białek K., Wasilewska D., Skorski T., Śliwiński T., Polθ inhibition with simultaneous treatment with PARP or RAD52 inhibitors induces cyto- and genotoxic effect in melanoma cells and reduces tumor growth of human melanoma xenograft.

Mój udział polegał na współudziale w wykonaniu testu kometowego. Swój udział w artykule oceniam na 2%.

Katarzyna Białek

17.06.2024

Tomasz Skorski, MD, PhD, DSc
Director, Fels Cancer Institute for Personalized Medicine
Professor, Department of Cancer and Cellular Biology
Temple University, Lewis Katz School of Medicine
3307 N. Broad Street, Room 154 PAHB
Philadelphia, PA 19140, USA

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy

Barszczewska-Pietraszek G, Drzewiecka M, Czarny P, Skorski T, Śliwiński T. Polθ Inhibition: An Anticancer Therapy for HR-Deficient Tumours. Int J Mol Sci. 2022 Dec 24;24(1):319. doi:10.3390/ijms24010319.

mój udział polegał na edycji i nadzorze powstawania manuskryptu. Swój udział w artykule oceniam na 5%.