Kierunek:
Interdyscyplinarne Studia Doktoranckie
„InterDOC-STARt" realizowane na
Wydziale Biologii i Ochrony Środowiska
Uniwersytetu Łódzkiego

Małgorzata Maria Drzewiecka

Inhibitory białek naprawy DNA jako potencjalne leki w spersonalizowanej terapii przeciwnowotworowej, opartej o syntetyczną letalność

Inhibitors of proteins participating in DNA repair as potential drugs in personalized cancer therapy based on synthetic lethality

Praca doktorska

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

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3. Spis publikacji wchodzących w skład rozprawy doktorskiej

Praca przeglądowa

- **Drzewiecka, M**.; Barszczewska-Pietraszek, G.; Czarny, P.; Skorski, T.; Śliwiński, T. Synthetic Lethality Targeting Polθ. Genes 2022, 13, 1101, doi:10.3390/genes13061101
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4. Pozostały dorobek naukowy

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5. Komunikaty zjazdowe

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- **Małgorzata Drzewiecka**, Tomasz Skorski, Tomasz Sliwiński „Odpowiedź komórek glejaka U87MG na inhibicję białek naprawy pęknięć dwuniciowych DNA" Ogólnopolska Konferencja Naukowa „Nauka Okiem Młodego Naukowca" VII edycja, 03.06.2023 online
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- **Małgorzata Drzewiecka**, Tomasz Skorski, Tomasz Sliwiński „Zjawisko syntetycznej letalności w terapii przeciwnowotworowej" V Ogólnopolska Konferencja Genetyczna „Genomica" 5-7.04.2019, Kraków

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01.01.2020 - 01.04.2020 Department of Microbiology and Immunology and Fels Institute for Cancer Research and Molecular Biology Lewis Katz School of Medicine, Temple University, Philadelphia PA, USA. Staż wsparty z finansowania z projektu "InterDOC-STARt – Interdyscyplinarne Studia Doktoranckie” realizowanego na Wydziale Biologii Ochrony Środowiska Uniwersytetu Łódzkiego – Program Operacyjny Wiedza Edukacja Rozwój 2014–2020, Oś priorytetowa III. Szkolnictwo wyższe dla gospodarki i rozwoju, Działanie 3.2 Studia doktoranckie. Nr projektu: POWR.03.02.00–IP.08–00–DOK/16. Realizowany w latach 2018–2023. Kierownik — prof. dr hab. Agnieszka Marczak

7. Wprowadzenie

Choroby nowotworowe stanowią szeroką kategorię chorób cywilizacyjnych, które powodowane są przez gromadzenie się mutacji nadających niosącym je komórkom nieograniczony potencjał replikacyjny [1]. W rezultacie, istnieje wzmożone ryzyko mutacji i utraty funkcji genów, które są istotne dla przeżycia komórki, takich jak geny odpowiedzialne za naprawę DNA. W takim przypadku komórki nowotworowe muszą znaleźć substytuty utraconych mechanizmów i aktywować szlaki alternatywne [2]. Jeśli mutacje prowadzące do śmierci komórek występują tylko w określonych parach genów, podczas gdy inaktywacja każdego z tych genów osobno nie ma wpływu na przeżycie komórki, mówimy o interakcjach syntetycznej letalności [3]. W ostatnich latach coraz większą uwagę przywiązuje się do spersonalizowanej terapii przeciwnowotworowej opartej na zjawisku syntetycznej letalności, wykorzystującej inhibitory białek, mające na celu zablokowanie szlaków alternatywnych. To podejście nie tylko może być selektywne i skuteczne w zwalczaniu nowotworów, ale także przyczynia się do rozwoju naszej wiedzy na temat genetycznych interakcji zachodzących wewnątrz komórek [4,5,6].

Dwuniciowe pęknięcia w DNA (DSB – ang. *double-strand breaks*) stanowią najpoważniejsze, formy uszkodzeń DNA. Jeśli nie zostaną odpowiednio naprawione, mogą prowadzić do powstawania rearanżacji genetycznych i progresji nowotworu [7]. DSB mogą być indukowane przez różne czynniki egzogenne (np. promieniowanie jonizujące) i endogenne (np. wolne rodniki tlenowe – ROS). Dwuniciowe pęknięcia w DNA mogą wynikać między innymi z dwóch niezależnych pęknięć pojedynczej nici DNA (SSB – ang. *single strand break*) zlokalizowanych na dwóch niciach DNA, zaburzonej replikacji, transkrypcji i rekombinacji DNA [8].

W komórkach eukariotycznych naprawa podwójnych pęknięć DNA przebiega poprzez dwa podstawowego mechanizmy - homologiczną rekombinację (HR – ang. *homologous recombination*) w późnej fazie S i G2 (po replikacji) lub mechanizm łączenia końców niehomologicznych (NHEJ – ang. *non-homologous end joining*) zachodzący w fazie G1/S cyklu komórkowego (przed replikacją). Innym szlakiem naprawy DSB jest opierający się na recesji 5' końców DNA szlak SSA (ang. *single-strand annealing*), ale ma on mniejsze znaczenie w porównaniu z NHEJ i HR.

HR może przebiegać zgodnie z klasycznym modelem rekombinacji homologicznej opartym na naprawie DSB i rozdzieleniu podwójnych połączeń Hollidaya lub przybierać postać SDSA (ang. *synthesis-dependent strand annealing*) lub BIR (ang. *break-induced replication*). W przypadku szlaku NHEJ można wyodrębnić dwa warianty - c-NHEJ (kanoniczny ang. *canonical/classical non-homologous end joing*) polegający na łączeniu chronionych końców DNA oraz zależne od PARP1 alternatywne NHEJ (alt-NHEJ, alternative non-homologous end joing) wymagające recesji 5’ końców i bazujące na mikrohomologii (5-25 bp) sekwencji, stąd również zwane MMEJ (ang. microhomology-mediated end joining). Łączenie końców za pośrednictwem polimerazy DNA theta (Polθ) (TMEJ) jest obok rekombinacji homologicznej i niehomologicznego łączenia końców, jednym z najważniejszych mechanizmów naprawy potencjalnie śmiertelnych pęknięć podwójnej nici DNA [9].

TMEJ jest inicjowany przez rekrutację PARP1 do wyciętych końców DNA [10–12]. Po aktywacji przez fosforylowany CtIP, helikazy, takie jak kompleks MRE11 – RAD50 – NBS1 (MRN), generują wystające fragmenty 3'. POLQ następnie wiąże się z długimi nawisami jednoniciowego DNA (ssDNA ang. *signle strand DNA*) generowanymi przez resekcję 5’–3’ DSB i łączy sekwencje z 2–6 parami zasad mikrohomologii, aby wykorzystać je jako startery do syntezy DNA [13–15]. Stabilizowane końce DNA są następnie ligowane przez LIG3 – XRCC1 lub LIG1 [16–19].

Deacetylazy histonowe (HDAC – ang. *histone deacetylase*) to grupa enzymów, które odgrywają istotną rolę w regulacji ekspresji genów poprzez usuwanie grup acetylowych z białek histonowych co powoduje zwartą strukturę chromatyny. Inhibitory HDAC (HDACi – ang. *histone deacetylase inhibitor*) można podzielić na cztery klasy. Klasa I (HDAC1, HDAC2, HDAC3 i HDAC8), klasa II (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 i HDAC10), klasa IV (HDAC11) [21,22]. HDAC klasy III, znane również jako sirtuiny (SIRT1-7), są zależne od NAD(+) i niezależne od cynku w swojej aktywności enzymatycznej. HDAC tworzą złożone kompleksy z innymi białkami, które są niezbędne do wiązania się z DNA lub łączenia się pośrednio lub bezpośrednio z czynnikami transkrypcyjnymi. Należą do nich takie białka jak p53, Rb, NFkB, BRCA1 i YY1. Klasa I HDAC jest stosunkowo najlepiej scharakteryzowana grupą występującą głównie w jądrze komórkowym [23]. Wykazano, że leki HDACi, zwłaszcza te o zdolności hamowania wszystkich klas HDAC, znane również jako "pan HDACi", zmniejszają ekspresję kilku czynników odpowiedzialnych za naprawę uszkodzeń DNA. Zmniejszony poziom ekspresji

może wynikać z mniej wydajnej transkrypcji cząsteczek, takich jak BRCA-1, kinaza kontrolna punktu 1 (CHK1) i RAD51, lub z zwiększonej acetylacji białka HSP90. Wzrost acetylacji wpływa negatywnie na funkcję opiekuńczą HSP90, co prowadzi do obniżonej stabilności białek odpowiedzialnych za naprawę uszkodzeń DNA (DDR ang. *DNA damage response)* [24]. HDAC klasy I często przejawiają nadekspresje w nowotworach i mogą przyczyniać się do oporności komórek na terapię [25].

Ze względu na różnorodność występujących nowotworów, a także odmienną odpowiedź na leczenie, nawet wśród podobnych morfologicznie nowotworów, wciąż istnieje zapotrzebowanie na poprawę skuteczności istniejących terapii oraz opracowywanie nowych metod leczenia. A żeby zminimalizować ryzyko wystąpienia lekooporności coraz częściej jest rekomendowane zastosowanie terapii skojarzonych z użyciem dwóch lub więcej leków o odmiennym mechanizmie działania, które wykazują addytywny lub najlepiej synergistyczny efekt umożliwiający nie tylko skuteczną terapię, ale także obniżenie dawek poszczególnych chemioterapeutyków. Na przestrzeni ostatnich lat liczne badania wykazały, że inhibitory HDAC i inhibitory PARP działają synergistyczne w leczeniu nowotworów, dlatego w niniejszej pracy podjęto się poszukiwania kolejnych interakcji opartych na zjawisku syntetycznej letalności i inhibicji deacetylazy histonowej w połączniu z inhibitorami PARP1 i związkiem alkilującym w komórkach guzów litych o złym rokowaniu – glejaka i czerniaka.

7.1. Hipoteza

Zastosowanie inhibitora deacetylazy histonowej – kwasu walproinowego (VPA) uwrażliwia komórki czerniaka i glejaka na działanie inhibitora PARP1 – talazoparibu (BMN-673) i związku alkilującego temozolomidu (TMZ) i dakarbazyny (DTIC).

7.2. Cel pracy

Celem niniejszej rozprawy doktorskiej była ocena skuteczności zastosowanego inhibitora HDAC (VPA) w skojarzeniu z inhibitorem PARP1 (BMN-673), HDAC i związku alkilującego na wyizolowanych z guzów litych (mózgu i skóry) liniach komórkowych.

7.3. Cele szczegółowe

- Pozyskanie komórek glejaka do hodowli in vitro z materiału pobranego od pacjentów
- Analiza efektów komórkowych indukowanych przez inhibitor HDAC, PARP1 i związek alkilujący stosowanych samodzielnie i w kombinacji
- Określenie zależności pomiędzy inhibicją deacetylaz histonów a odpowiedzią komórkową po ekspozycji na inhibitor PARP1 i związek alkilujący.

8. Materiały i metody badawcze

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- -Wycinki glejaka pobrano od pacjentów:
- Kliniki Neurochirurgi i Chirurgii Nerwow Obwodowych, Uniwersyteckiego Szpitala Klinicznego im. Wojskowej Akademii Medycznej
- Oddziału Neurochirurgii i Nowotworow Układu Nerwowego, Wojewódzkiego Szpitala Specjalistycznego im. M. Kopernika w Łodzi Badania uzyskały zgodę Komisji Bioetycznej UMED - Nr zgody RNN/194/12/KE

Metody in vitro

- Wyprowadzenie linii pierwotnych czerniaka i *glejaka
- Identyfikacja linii pry uzyciu markerow powierzchniowych* (m.in. CD133)
- Analiza ekspresji genów* - RealTime PCR
- Analiza Western Blot*
- Badanie przeżywalności* - barwienie blekitem trypanu.
- Analiza zdolności proliferacyjnej komórek* - test klonogennosci;
- Badanie akumulacji DSB* - analiza poziomu ufosforylowanego histonu HA.X - analiza cytometryczna; Neutralny test kometowy;
- Badanie cytometryczne cyklu komórkowego*;

- Badanie cytometryczne apoptozy/nekrozy* po utrwaleniu komorek i barwieniu Aneksyna V i PI;

Metody in vitro

- Myszy NSG podskórne ksenografty linii czerniaka**.** Badania prowadzone w Pracowni Cytometrii Przepływowej, Centrum Medyczne Kształcenia Podyplomowego (dr Grażyna Hoser)

* badania wykonane samodzielnie

Materiał badawczy wykorzystany w eksperymentach do niniejszej rozprawy doktorskiej stanowiły linie pierwotne glejaka wielopostaciowego oraz czerniaka.
Linię komórkowa DMBC11 (ang. ***D**epartament of **M**olecular **B**iology of **C**ancer*) wyprowadzono z fragmentu guza od pacjenta (ze zdiagnozowanym czerniakiem złośliwym skóry) Kliniki Chirurgii Onkologicznej ,Katedry Onkologii Uniwersytetu Medycznego w Łodzi w Zakładzie Biologii Molekularnej Nowotworów, Uniwersytetu Medycznego w Łodzi. Poprzez zastosowanie charakterystycznych dla komórek czerniaka markerów powierzchniowych i analizy cytometrycznej potwierdzono tożsamość nowotworową komórek w uzyskanych hodowlach. Linia DMBC11 hodowana była w pożywce SCM (*ang – stem cell medium).*

Linię komórkową GBM113 i GBM114 wyprowadzono z guzów od pacjentów (ze zdiagnozowanym glejakiem wielopostaciowym w stadium III i IV) hospitalizowanych w Klinice Neurochirurgii i Chirurgii Nerwów Obwodowych, Uniwersyteckiego Szpitala Klinicznego im. Wojskowej Akademii Medycznej oraz Oddziału Neurochirurgii i Nowotworów Układu Nerwowego Wojewódzkiego Szpitala Specjalistycznego im. Mikołaja Kopernika w Łodzi. Prowadzone prace zostały zaaprobowane przez Komisję Bioetyczną Uniwersytetu Medycznego w Łodzi (nr zgody RNN/194/12/KE). Komórki glejaka GBM113 i GBM114 zostały wyizolowane z wycinków guza w Zakładzie Biochemii Medycznej Uniwersytetu Medycznego w Łodzi. W celu potwierdzenia obecności komórek glejaka zastosowano marker komórek nowotworowych macierzystych - antygen powierzchniowy CD133. Komórki zostały posortowane z wykorzystaniem kulek magnetycznych MACS (ang. *magnetic-activated cell sorting*). Linie GMB113 i GBM114 hodowane były w pożywce DMEM z dodatkiem 10%

surowicy bydlęcej – FBS oraz antybiotyków penicylina-streptomycyna (kolejno 50 U/mL oraz 50 µg/mL) w 5% CO_2.

Jako kontrole do linii nowotworowych zastosowano komercyjnie dostępną linię komórek prawidłowych melanocytów (NHEM – ang. *Normal Human Epidermal Melanocytes*) dla komórek czerniaka oraz astrocytów (NHA – ang. *Normal Human Astrocytes*) dla komórek glejaka.

Izolację całkowitego RNA z badanych linii przeprowadzono z użyciem komercyjnie dostępnego zestawu Total RNA Mini (A&A Biotechnology, Gdynia, Poland) a następnie w reakcji odwrotnej transkrypcji przepisano na cDNA. W celu analizy poziomu ekspresji badanych genów na poziomie mRNA przeprowadzono reakcję łańcuchowej reakcji polimerazy w czasie rzeczywistym z użyciem sond TaqMan wykrywającymi transkrypty genów *FANCD2, BRCA1, BRCA2, LIG3, LIG4, RAD51B, RAD51D, PARP1.*

W celu potwierdzenia poziomu ekspresji badanych genów na poziomie białka przeprowadzono badania Western Blot.

Na podstawie poziomu ekspresji transkryptów i białek będących produktami wybranych genów, ale także ze względu na tempo wzrostu komórek do dalszych eksperymentów zostały wybrane trzy linie komórkowe (jedna linia DMBC11 -czerniak i dwie linie glejaka wielopostaciowego GMB113, GBM114). Pierwszą serię eksperymentów poświęcono odpowiedzi komórek na zastosowane związki – inihbitor deacetylazy histonowej klasy I (HDAC1) – kwas walproinowy stosowany samodzielnie bądź w kombinacji z inhibitorem PARP1 talazoparibem (BMN-673) oraz związkiem alkilującym temozolomidem - TMZ (w liniach glejaka), dakarbazyną - DTIC (w linii czerniaka). Komórki były wstępnie inkubowane z kwasem walproinowym przez 168 godzin (z wymianą medium co 48 godzin) a następnie przez 72 godziny inkubowane z inhibitorem PARP1 i związkiem alkilującym. Zgodnie z postawioną hipotezą wstępna inkubacja komórek z inhibitorem HDAC1 miała uwrażliwić badane komórki na stosowane związki (DTIC/TMZ, BMN-673).

W celu określenia stopnia przeżywalności komórek po ekspozycji na testowane związki wykorzystano barwienie przyżyciowe błękitem trypanu. Z uwagi na negatywny ładunek, związek ten nie jest zdolny do przenikania do żywych komórek, których błona komórkowa jest

nienaruszona. Jednak, gdy dochodzi do trwałej utraty integralności błony komórkowej co prowadzi do zaniku potencjału między wewnętrzną a zewnętrzną stroną błony, błękit trypanu jest w stanie przeniknąć do wnętrza komórki. Powoduje to zabarwienie cytoplazmy i/lub jądra na kolor niebieski.

Do oceny potencjału proliferacyjnego i tworzenia kolonii badanych linii nowotworowych wykorzystano test klonogenny, który polega na zliczeniu kolonii, które są w stanie utworzyć pojedyncze, zdolne do proliferacji komórki. W celu przeprowadzenia eksperymentu, komórki umieszczono na 12 dołkowych płytkach w agarze i hodowano w inkubatorze (5% CO2, 37°C) iprzez okres dwóch tygodni. Po zakończonej inkubacji kolonie komórkowe barwiono fioletem krystalicznym a następnie liczbę utworzonych kolonii zliczono pod mikroskopem. Zdolność do proliferacji komórek wyrażono jako procent liczby kolonii uzyskanej z płytki kontrolnej (komórki, które nie były poddane ekspozycji na badane związki).

W celu oceny powstającej ilości dwuniciowych pęknięć w DNA w liniach komórkowych czerniaka i glejaka wielopostaciowego po ekspozycji na badane związki wykonano analizę cytometryczną histonu γH2AX. W celu potwierdzenia wyników uzyskanych z cytometru przepływowego dodatkowo wykonano neutralny test kometowy.

Aby określić, w jaki sposób inhibicja HDAC klasy I powoduje uwrażliwienie na badane związki, przeprowadzono reakcje PCR w czasie rzeczywistym z sondami TaqMan wykrywającymi trankrypty genów *RAD51, RAD51D, FANCD2, BRCA1, BRCA2, PALB2, PARP1*. HDAC klasy I były hamowane w komórkach czerniaka i glejaka poprzez zastosowanie kwasu walproinowego. Wartość *„Fold change”* została obliczona przez porównanie poziomu ekspresji genów w komórkach traktowanych VPA do komórek nietraktowanych. W celu potwierdzenia poziomu ekspresji badanych genów na poziomie białka przeprowadzono analizę Western Blot.

Wyniki zostały uzyskane w trzech niezależnych powtórzeniach i przedstawione jako wartość średnia ± SD. Wyniki zostały porównane wykorzystując niesparowany test-t Studenta. Wartości $p<0.05$ zostały uznane za istotne statystycznie.

Eksperymenty *in vivo* zostały wykonane na linii czerniaka DMBC11 z wykorzystaniem myszy NSG (NOD scid gamma).

Przydzielono po trzy zwierzęta do ośmiu różnych grup (podział na grupy ze względu na podawane związki: grupa kontrolna, VPA; BMN-673; BMN-673+VPA, DTIC; DTIC+VPA; BMN-673+DTIC i BMN-673+DTIC+VPA). Sześć dni przed wstrzyknięciem BMN-673 i DTIC myszom z odpowiedniej grupy wstrzykiwano dootrzewnowo raz dziennie 500 mg/kg VPA (rozcieńczonego w PBS) a następnie podawano BMN-673 (35 mg/kg masy ciała, rozcieńczone w PBS), DTIC (8 mg/kg masy ciała co drugi dzień, rozcieńczone w PBS) lub BMN-673 z DTIC (dawkowanie takie samo jak w monoterapii) przez okres 24 dni. Po zakończeniu eksperymentu guzy zebrano i zważono. Myszy NSG (samice i samice w wieku od 10 do 12 tygodni) hodowano w sterylnym środowisku i zapewniano swobodny dostęp do pożywienia i wody.

9. Wyniki

W liniach komórkowych glejaka wykazano obniżony poziom *LIG4* w porównaniu do komórek prawidłowych astrocytów NHA. Ligaza IV działa w kompleksie z innym białkiem, XRCC4, przyczyniając się do skutecznej ligacji końców DSB w szlaku cNHEJ. Kompleks LIG4-XRCC4 pełni również rolę w regulacji i stabilizacji procesu NHEJ, zatem obniżony poziom LIG4 może prowadzić do nieefektywnej naprawy DSB skutkując aktywacją szlaku alternatywnego altNHEJ.

W celu blokowania alternatywnego mechanizmu naprawy dwuniciowych pęknięć w DNA zastosowano inhibitor PARP1- BMN-673. Wstępna inkubacja komórek z kwasem walproinowym miała na celu uwrażliwić komórki na działanie związków wykorzystanych w dalszej części eksperymentów – BMN-673 i TMZ. Wynik barwienia komórek błękitem trypanu po zakończonej inkubacji wykazał, specyficzną eliminację komórek po zastosowaniu kombinacji kwasu walproinowego, BMN-673 i TMZ. Po wstępnej 168 godzinnej inkubacji z VPA i następnie 72 godzinnej inkubacji z kombinacją TMZ i BMN-673 zaobserwowano, że testowana potrójna kombinacja związków działa synergistycznie co prowadziło do nasilonej śmierci komórek glejaka, na drodze syntetycznej letalności. Ten sam schemat traktowania nie spowodował efektu toksycznego w przypadku komórek prawidłowych – astrocytów.

Eksperyment oceniający zdolność komórek glejaka wielopostaciowego do proliferacji w teście klonogennym wykazał niemal całkowite zahamowanie podziału komórek po

ekspozycji na kombinacje inhibitorów PARP1, HDAC1 i TMZ. Związki stosowane samodzielnie bądź skojarzeniu podwójnym prowadziły mniejszej wydajności do obniżenia proliferacji

Poziom fosforylowanego histonu γH2A.X, który jest wskaźnikiem uszkodzeń dwuniciowych pęknięć, po podaniu inhibitora PARP1, HDAC1 i związku alkilującego samodzielnie lub w kombinacji potwierdził genotoksyczne działanie stosowanych związków. Jednakże, wykazano, że zarówno w komórkach glejaka linii GBM113 i GBM114, kombinacja tych związków spowodowała zwiększenie liczby dwuniciowych pęknięć. Akumulacja liczby dwuniciowych pęknięć w komórkach glejaka wielopostaciowego została dodatkowo potwierdzona poprzez test kometowy w warunkach neutralnych.

Drugą część eksperymentów poświęcono na zidentyfikowanie mechanizmu, który odpowiada za zwiększoną wrażliwości komórek na temozolomid i talazoparib po inhibicji HDAC1. W tym celu przeprowadzono badania z użyciem reakcji łańcuchowej polimerazy w czasie rzeczywistym (PCR). W komórkach glejaka zastosowano inhibitor HDAC klasy I - kwas walproinowy, a następnie monitorowano zmiany w poziomach mRNA za pomocą PCR w czasie rzeczywistym. Geny związane z naprawą DNA, takie jak RAD51, FANCD2, wykazywały obniżony poziom ekspresji zarównocw linii GBM113 jak i GBM114 Aby potwierdzić te wyniki, zbadano zmiany na poziomach białek, aby sprawdzić, czy korelują z wynikami dotyczącymi ekspresji genów. Analizowano wpływ VPA na poziomy białek RAD51, FANCD2. W wyniku inhibicji HDAC1 potwierdzono zmniejszenie poziomów białek RAD51 i FANCD2, co sugeruje, że zmniejszona zdolność do naprawy DNA na drodze HR, jest przyczyną zwiększonej wrażliwości komórek glejaka na temozolomid i talazoparib po zastosowaniu inhibitora HDAC1.

Analiza cytometryczne, w której zastosowano barwienie aneksyną V i jodkiem propidyny, wykazała, że komórki nowotworowe, które wykazują obniżoną ekspresję LIG4, eliminowane są na drodze apoptozy najskuteczniej po inkubacji z VPA, BMN-673 i TMZ. Dodatkowo, analiza rozmieszczenia komórek linii GBM114 i GBM114 w fazach cyklu komórkowego wykazała, że po zastosowaniu badanych z związków, komórki glejaka występowały najliczniej się w fazie subG1.

W eksperymentach mających zbadać odpowiedź komórek czerniaka na inhibicję HDAC1 w połączniu z inhibitorem PARP1 i związkiem alkilującym wykorzystano inhibitor BMN-673 oraz dakarbazynę (DTIC). Stosując barwienie przyżyciowe błękitem trypanu wykazano, że kwas walproinowy wpływał na uwrażliwienie komórek na działanie BMN-673 oraz DTIC.

Natomiast najbardziej skuteczna okazała się stosowana w skojarzeniu kombinacja badanych związków - kwasu walproinowego, talazoparibu i dakarbazyny obniżyła przeżywalność linii czerniaka DMBC11 do 10%.

Podobną zależność zaobserwowano przy określeniu stopnia proliferacji komórek. Wstępna inkubacja komórek z kwasem walproinowym skutkowała zwiększoną skutecznością hamującego proliferacje działania BMN-673 i DTIC, ale zastosowanie kombinacji potrójnej było w stanie obniżyć potencjał proliferacyjny poniżej 5%.

Analiza cytometryczna po zastosowaniu barwników aneksyny V i jodku propidyny wykazała, że śmierć komórek nowotworowych, następowała na drodze apoptozy po inkubacji z inhibitorem HDAC1, inhibitorem PARP1 i związkiem alkilującym.

Ocena liczby powstających dwuniciowych pęknięć w DNA po ekspozycji na badane związki wykazała, że wcześniejsza inkubacja komórek z kwasem walproinowym uwrażliwia komórki na działanie BMN-673 oraz DTIC (co przekłada się na wzrost liczby powstających DSBs w komórce). Wynik ten potwierdzono testem kometowym w wersji neutralnej, wykrywającym bezpośrednio dwuniciowe pęknięcia w DNA.

Podobnie jak w przypadku eksperymentów wykonanych na liniach komórkowych glejaka, aby zbadać mechanizm, za pomocą którego hamowanie HDAC klasy I zwiększa wrażliwość na dakarbazynę i talazoparyb, przeprowadzono PCR w czasie rzeczywistym. Linia DMBC11 po inkubacji z VPA wykazała obniżony profil genów RAD51D, RAD51 i FANCD2. W celu potwierdzenia otrzymanego wyniku przeprowadzono analizę ekspresji na poziomie białek, która wykazała obniżenie ekspresji RAD51 i FANCD2.

Przeprowadzone eksperymenty *in vivo* na ksenograftach pierwotnej linii czerniaka DMBC11 w myszach NSG wykazały niemal całkowitą, redukcję masy guza po zastosowaniu kombinacji badanych związków.

10.Wnioski

Badania, które przeprowadziłam w ramach niniejszej pracy doktorskiej pozwoliły mi pozytywnie zweryfikować postawioną hipotezę badawczą zakładającą, że zastosowanie inhibitora deacetylazy histonowej – kwasu walproinowego (VPA) uwrażliwia komórki czerniaka i glejaka na działanie inhibitora PARP1- BMN-673 i związku alkilującego.

Na podstawie zgromadzonych danych i przeprowadzonych analiz w ramach niniejszej pracy doktorskiej można wyciągnąć następujące wnioski:

1. Inhibicja HDAC klasy I spowodowała zahamowanie naprawy dwuniciowych pęknięć w DNA przez rekombinację homologiczną w wyniku obniżenia ekspresji RAD51 i FANCD2 co uwrażliwiło komórki na związki alkilujące wprowadzające cytotoksyczne uszkodzenia i w konsekwencji powodując syntetycznie letalną interakcję z inhibitorem PARP1 - BMN-673

2. Akumulacja dwuniciowych pęknięć w DNA skutkowała istotnym zmniejszeniem zdolności proliferacyjnych komórek oraz indukcję apoptozy.

3. Terapia skojarzona inhibitorów PARP1, HDAC i związku alkilującego dała znacznie silniejszy efekt eradykacji badanych komórek czerniaka i glejaka, w porównaniu do pojedynczego zastosowania tych związków

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12.Streszczenie w języku polskim

Wraz z rosnącą wiedzą na temat zmian genetycznych i epigenetycznych obserwowanych w komórkach nowotworowych, coraz większe zainteresowanie wzbudza spersonalizowane podejście do terapii przeciwnowotworowej oparte na zjawisku syntetycznej letalności. Obecnie, jedynie inhibitory PARP1, które są w stanie eliminować komórki posiadające mutacje w genach BRCA1/2, znajdują praktyczne zastosowanie w leczeniu nowotworów. Jednak, inhibitory PARP1 mają potencjał do rozszerzenia swojego zastosowania na inne typy nowotworów, które wykazują defekty w szlakach naprawy DNA.

Aby zbadać terapeutyczne działanie strategii przeciwnowotworowej opartej na zjawisku syntetycznej letalności w komórkach guzów litych o złym rokowaniu i efektu inhibicji deazetylaz histonów, przeprowadzono szereg eksperymentów na pierwotnych liniach komórkowych czerniaka i glejaka pochodzących od pacjentów.

Przeprowadzone badania w ramach niniejszej rozprawy doktorskiej wykazały, że inhibicja deacetylazy histonowej klasy I uwrażliwia komórki czerniaka i glejaka wielopostaciowego na działanie inhibitora PARP1 i związku alkilującego. Inkubacja komórek z badanymi związkami po wcześniejszej ekspozycji na kwas walproinowy, prowadziła do gromadzenia się większej liczby podwójnych pęknięć DNA (powyżej poziomu naprawy), zahamowania proliferacji komórek nowotworowych a także do indukcji apoptozy.

Ponadto inhibicja HDAC klasy I spowodowała obniżenie poziomu białek FANCD2, RAD51 we wszystkich badanych liniach komórkowych.

Zastosowanie inhibitora PARP1 w połączeniu z związkiem alkilującym nie wywoływało toksycznego efektu w prawidłowych melanocytach i astrocytach.

Otrzymane wyniki sugerują, że zastosowanie kwasu walproinowego w połączniu z inhibitorem PARP1 i związkiem alkilującym prowadzi do synergistycznego działania, co skutkuje m.in. zmniejszoną przeżywalnością, proliferacją a także indukcją apoptozy w komórkach czerniaka i glejaka. Ze względu na kluczową rolę białek RAD51 i FANCD2 w mechanizmie naprawy przez rekombinację homologiczną (HR), zmniejszenie ich poziomów może być przyczyną zaobserwowanej zwiększonej wrażliwości na PAPRi i związek alkilujący.

13.Streszczenie w języku angielskim | Summary

With the advancing knowledge of genetic and epigenetic changes observed in cancer cells, personalized approaches to anticancer therapy based on the concept of synthetic lethality are attracting increasing interest. Currently, only PARP1 inhibitors, capable of eliminating cells carrying mutations in the BRCA1/2 genes, have practical application.

To investigate the therapeutic effects of the anticancer strategy based on the concept of synthetic lethality and the inhibition of histone deacetylases in solid tumors with poor prognosis, a series of experiments were conducted on primary melanoma and glioma cell lines derived from patients.

The conducted studies in this doctoral thesis have demonstrated that the inhibition of class I histone deacetylase (HDAC) sensitizes melanoma and glioblastoma multiforme cells to the action of PARP1 inhibitors and alkylating agents. Incubating cells with the tested compounds after prior exposure to valproic acid resulted in the accumulation of a greater number of DNA double-strand breaks (beyond the level of repair), inhibition of tumor cell proliferation, and induction of apoptosis.

Furthermore, the inhibition of class I HDAC led to a decrease in the levels of FANCD2 and RAD51 proteins in all tested cell lines. The application of the PARP1 inhibitor in combination with an alkylating agent did not induce a toxic effect in normal melanocytes and astrocytes.

The obtained results suggest that the combination of valproic acid with a PARP1 inhibitor and an alkylating agent leads to a synergistic effect, resulting in decreased cell viability, proliferation, and induction of apoptosis in melanoma and glioblastoma cells. Due to the crucial role of RAD51 and FANCD2 proteins in the homologous recombination (HR) DNA repair mechanism, the reduction in their levels may be responsible for the observed increased sensitivity to PARP inhibitors and alkylating agents.

14.Kopie publikacji wchodzących w zakres rozprawy doktorskiej

Article

Class I HDAC Inhibition Leads to a Downregulation of FANCD2, RAD51 and Eradication of Glioblastoma Cells

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Abstract: HDAC inhibitors (HDACi) hold great potential as anti-cancer therapies due to their ability to regulate the acetylation of both histone and non-histone proteins, which is frequently disrupted in cancer and contributes to the development and advancement of the disease. Additionally, HDACi have been shown to enhance the cytotoxic effects of DNA-damaging agents like radiation or cisplatin. In this study, we found that histone deacetylase inhibitor, valproic acid (VPA), synergized with PARP1 inhibitor (PARPi), talazoparib (BMN-673), and, alkylating agent, temozolomide (TMZ) , to induce DNA damage and reduce glioblastoma multiforme. At molecular level, VPA leads to a downregulation of FANCD2, RAD51 and eradication of glioblastoma cells. The results of this study indicate that combining HDACi with PARPi could potentially enhance the treatment of glioblastoma, the most aggressive type of cancer that originates in the brain.

Keywords: glioblastoma multiforme; valproic acid; HDAC; DNA damage response; double strand break; synthetic lethality

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1. Introduction

Glioblastoma multiforme (GBM), which accounts for 54% of all gliomas, is recognized as the most aggressive primary brain tumor. Without treatment, the average life expectancy for patients with glioblastoma multiforme is approximately 14-15 months. Currently, a multimodal approach is the standard treatment path for glioblastoma multiforme [1]. It typically begins with surgical removal of the tumor, followed by radiation therapy. Additionally, patients receive chemotherapy with temozolomide (TMZ) for a duration of 6.5 months [2]. Flollowing completion of the initial chemotherapy phase, a maintenance therapy period of another 6 months follows, during which patients continue to receive temozolomide chemotherapy. In this maintenance phase, the chemotherapy is administered for 5 consecutive days each month[1].

Synthetic lethality is a concept that has gained significant attention in cancer research and targeted therapy approaches. It refers to the phenomenon where the simultaneous disruption of two genes, neither of which is individually lethal, results in cell death. This concept has been particularly explored in the context of DNA repair pathways and the development of novel treatment strategies. One notable example of synthetic lethality involves the inhibition of poly (ADP-ribose) polymerase (PARP) enzymes The PARP protein family, also known as poly(ADP-ribose) polymerases, plays a crucial role in DNA

repair and genomic stability. PARP enzymes are involved in detecting and signaling DNA damage, recruiting other repair proteins to the site of damage, and facilitating the repair process. PARP inhibitors (PARPi), are a class of drugs designed to block the activity of PARP enzymes[1]. PARPi work by binding to the catalytic domain of PARP enzymes, preventing them from adding poly(ADP-ribose) chains to target proteins involved in DNA repair[3]. By inhibiting PARP activity, these drugs impair the DNA repair process, leading to the accumulation of DNA damage and ultimately causing cell death, particularly in cancer cells. PARPi have shown significant clinical success in the treatment of certain types of cancer, such as ovarian and breast cancer, especially in patients with specific mutations in DNA repair genes like BRCA1 and BRCA2. PARPi have also been investigated for their potential use in combination with other cancer therapies, such as chemotherapy and radiation therapy in relation to their ability to, to enhance the effectiveness of these therapies. Ongoing research aims to further understand the mechanisms of PARPi and identify new therapeutic strategies to maximize their benefits in cancer treatment while minimizing potential side effects [4,5].

Histone deacetylases (HDACs) are a family of enzymes that have an essential role in controlling gene expression and modifying chromatin[6]. The main function of HDACs is to eliminate acetyl groups from histone proteins, leading to the condensation of chromatin structures and the suppression of gene transcription [7]. However, aberrant HDAC activity has been implicated in various diseases, including cancer and neurodegenerative disorders. Inhibition of HDACs has emerged as a promising therapeutic strategy for the restoration of normal gene expression patterns and provides a potential treatmen for these disease[8,9]. Based on extensive clinical experience in treating epilepsy, valproic acid (VPA) has been found to be a safe drug with excellent bioavailability[10]. Recent clinical trials conducted for different types of cancers have demonstrated that the serum concentration of VPA, achieved during epilepsy treatment with a daily dose, acts as a potent inhibitor of HDACs that are crucial for histone acetylation. The inhibitory effect of VPA on HDACs leads to an increase in histone acetylation levels, resulting in chromatin relaxation and the activation of silenced genes [10,11]. By modulating gene expression, VPA has shown potential in diverse therapeutic applications. Apart from its antiepileptic properties, VPA has been investigated for its anti-cancer effects. It has demonstrated the ability to induce differentiation, inhibit cell proliferation, and promote apoptosis in various cancer cell types[6]. Furthermore, VPA has been explored for its neuroprotective properties in neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease. Studies have shown that VPA treatment can enhance neuronal survival, reduce neuroinflammation, and improve cognitive function in animal models of these diseases [7].

In our previous studies, we demonstrated that HDAC inhibitors effectively reduce the viability of melanoma cells and may render this type of cancer more susceptible to the cytotoxic effects of DNA-damaging agents, such as dacarbazine [12]. However, whether HDAC inhibitors could sensitize LIG4 deficient glioblastoma to PARPi and TMZ remains to be investigated. In this article, to explore this aspect, we use a class I HDAC inhibitor, VPA, in combination with BMN-673, a PARP1 inhibitor, and the alkylating agent TMZ. At the molecular level, we assess the impact of these HDACi treatments on the expression of FANCD2 and RAD51, molecules highly involved in HR repair.

2. Materials and Methods

2.1. Cell Lines and Culture Conditions

Glioblastoma specimens were were obtained from patients of the Department of Neurosurgery, Surgery of Spine and Peripheral Nerves at the University Hospital WAM-CSW Łódź. The cell cultures derived from these glioblastoma specimens were established in the Laboratory of Molecular Genetics at the University of Lodz. These cell cultures were given the names GMB113 and GBM114. for identification purposes. To initiate the cell cultures, the glioblastoma tissue fragments were washed multiple times and then minced

using a scalpel. The resulting cells were filtered through a cell strainer with a pore size of 70 μM. The glioblastoma cells were then cultured in DMEM medium (Lonza, Basel, Switzerland) supplemented with 10% FBS (Lonza), 100 IU/ml penicillin, 100 μg/ml streptomycin (Lonza), and 50 μg/ml gentamicin (Lonza). The cultures were maintained in a humidified atmosphere with 5% CO2 at a temperature of 37°C. In addition to the glioblastoma cell cultures, normal human astrocytes (NHA) obtained from Lonza were also used in the study. The NHA cells were grown in ABM Basal Medium supplemented FBS 15ml/l,L-Glutamine 5ml/l, GA-1000 500μl /l; Ascorbic Acid 500μl/l; HEGF 500μl/l; Insulin 1,25ml/l

2.2. Proliferation Determination

Cells proliferation was tested by a clonogenic assay. Cells were seeded ($3x10^4$ cells/well) into 12 well plate and pre-treatment with VPA(10mM) (for 168h) and then treated with BMN-673(50nM) used alone or in combination with TMZ(6.25μM) (for 72h). After treatment cell were resuspend in 700μl of soft agar (DMEM, 0,4% w/v) and reseeded over 700μl of solidified agar underlay (DMEM, 0,5% agar) into 6-well plate ($2x10^3$ cells/well). After solidifying cell layer was covered with medium (changed weekly). After two weeks cells were washed gently with PBS (Phosphate-Buffered Saline). After washing step, the cells were fixed and stained using a DNA intercalating dye, specifically crystal violet (0.5% w/v), for a minimum of 30 minutes. To remove the excess stain, a technique involving the gentle dunking of the plates in beakers filled with water was employed. This process continued until all excess stain was eliminated, leaving only bright-purple colonies on the plates. Cells were counted under the microscope and clonogenic efficiency was expressed as percent of untreated control (no. of colonies after treatment vs no. of colonies in control sample × 100%).

2.3. Cell Viability

Glioblastoma cells were seeded a density of 7×10^3 viable cells per well in 12 well-plate. Then cells were pretreated with 1mM valproic acid for a duration of 168 h with a refreshment of VPA-containing medium every 48 h. After a 168 h pretreatment period cells were cultured with 50nM BMN-673 (MedChemExpress; Cat#HY-16106), 6,25μM TMZ (Sigma Aldrich, Burlington, MA, USA) used alone or in combination. To determine cell viability after treatments with VPA, TMZ and BMN673, a trypan blue exlusion assay was performed. Cells were counted (within 3 to 5 min of mixing with 0,4 % trypan blue) by light microscopy using a Neubauer hemocytometer. The experiments were carried out three times, in triplicate.

2.4. RNA Isolation and Real-Time Polymerase Chain Reaction

In this study, RNA was isolated from cultured GBM113 and GBM114 cell pellets containing approximately 2.5 x 106 cells. The extraction was performed using a total RNA isolation kit (A&A Biotechnology; Cat#031-100). Subsequently, the RNA samples were converted into complementary DNA (cDNA) using SuperScript II Reverse Transcriptase from Invitrogen Life Technologies. For quantitative reverse transcription PCR (qRT-PCR), TaqMan Re-al-Time PCR Master Mix from Life Technologies was utilized. The qPCR reactions were conducted on an Agilent Technologies Stratagene Mx3000P system with MxPro software. The expression levels of seven genes, which are involved in DNA double-strand break repair pathways, were examined using TaqMan probes from Life Technologies. To ensure accurate normalization of the expression data, 18S RNA was employed as the reference gene. TaqMan probes from Life Technologies were also utilized for this purpose.The qPCR cycling parameters consisted of an initial step of 95°C for 10 minutes, followed by 30 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 60 seconds.

2.5. Comet Assay

The Comet assay was conducted following the methodology outlined in a previous study [13]. Cells were cultured for a period of 72 hours in the presence of either drugs or a vehicle. Prior to the 72h culture, the cells were pre-treated with VPA (Valproic acid) for a duration of 168 h. Fifty comet images were randomly selected for each treatment variant and the percentage of DNA in the tail (% tail DNA) was measured. The mean value for this parameter was taken as an index of DSBs in the given sample.

2.6. Apoptosis and necrosis

To assess apoptosis and necrosis in the cell population, FITC Annexin V staining was performed along with a vital dye such as propidium iodide (PI) or 7-Amino-Actinomycin (7-AAD). This staining method allows for the identification of early apoptotic cells (PI negative, FITC Annexin V positive) before the loss of membrane integrity that occurs in the later stages of apoptotic or necrotic cell death. In this study, a FITC Annexin V Apoptosis Detection Kit from BD Biosciences (Catalog #556547) was used to quantitatively determine the percentage of cells actively undergoing apoptosis, following the manufac'urer's instructions. The experimental procedure involved plating the cells at a density of 1 x 105 cells per well in a 24-well plate. The cells were then pretreated with 1 mM valproic acid (VPA) for 168 hours, with a refreshment of VPA-containing medium every 48 hours. Subsequently, the VPA-containing medium was removed before further treatment. After the 168-hour pretreatment period, the cells were cultured with 50 nM talazoparib from MedChemExpress (Catalog #HY-16106) and 2 m TMZ from Sigma Aldrich, Burlington, MA, USA. These treatments were administered individually or in combination. Following a 24-hour incubation with VPA, BMN-673, and TMZ, the cells were washed twice with cold PBS and resuspended in 1X Binding Buffer at a concentration of 1×10^5 cells/ml. Then, 100 µl of the solution (1×10^4 cells) was transferred to a 5 ml culture tube. FITC Annexin V (5 µl) and PI (5 µl) were added to the tube. The cells were gently vortexed and incubated in the dark at room temperature (25°C) for 15 minutes. Finally, 400 µl of 1X Binding Buffer was added to each tube, and the samples were analyzed by flow cytometry within 1 hour.

2.7. Histone H2AX

The GBM113 and GBM114 cell line was cultured at a density of 5×10^3 cells per well. To detect the levels of phosphorylated Histone H2AX, the H2AX Phosphorylation Assay Kit (Flow cytometry) from Millipore (Catalog #17-344) was utilized. The assay was performed on cultured cells that were treated with agents capable of inducing DNA damage or apoptosis. Prior to treatment, the cells were pre-treated with 1 mM valproic acid (VPA) for 168 hours, with a refreshment of VPA-containing medium every 48 hours. Subsequently, the VPA-containing medium was removed before further treatment. After the 168-hour pre-treatment period, the cells were cultured with 50 nM BMN-673 and 6.25µM TMZ, which promoted H2AX phosphorylation. These mentioned agents were used either individually or in combination. Following 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. Cell cycle

For the analysis of cell cycle distribution, GBM113 and GBM114 cells, either untreated or treated, were subjected to propidium iodide (PI). After a 48-hour treatment period, the cells were washed with cold 1× PBS and fixed in 70% ethanol on ice for at least 1 hour. Following centrifugation, the cell pellet was washed with cold 1× PBS and stained with a solution containing 50 µg/mL PI and RNase, incubated for 15 minutes at 37°C, and subsequently analyzed using FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA).

2.9. Statistical Analysis

All graphs show the mean ± standard deviation (SD), and statistically significant differences were analyzed using Student's t-test with GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Expression of genes involved in DSB repair in normal human astrocytes and glioblastoma cells

To implement a personalized synthetic lethality approach, we conducted an analysis of gene expression profiles in two patient-derived glioblastoma primary cell lines and compared them with the profile of normal human astrocytes (NHA). Our focus was on 12 genes involved in DNA double-strand break (DSB) repair pathways. These genes included BRCA1, BRCA2, PALB2, RAD51B, RAD51C, RAD51D, XRCC2, XRCC3, RAD52 (part of HR – homologous recombination pathway), LIG4, XRCC5, (part of D-NHEJ DNA-PK dependent non-homologous end-joining pathway) and PARP1 (part of B-NHEJ the backup non-homologous end-joining pathway). Significant changes in the mRNA expression profile of LIG4 was observed between the glioblastoma cell lines and NHA, as shown in Figure 1.

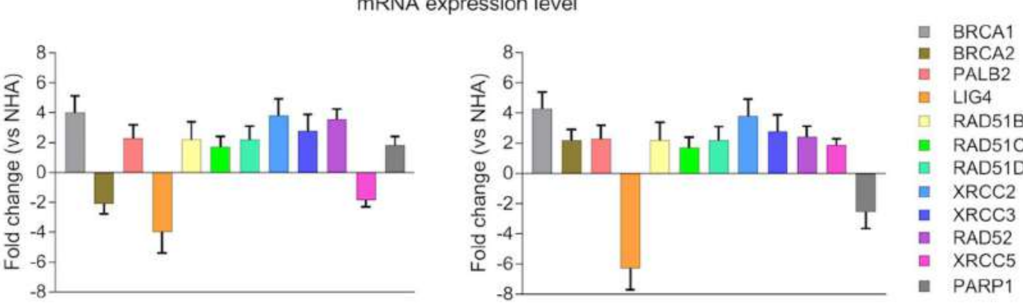

Figure 1. Expression profiles of 12 specified genes involved in HR (homologous recombination), D-NHEJ (DNA-dependent non-homologous end joining), and B-NHEJ (backup non-homologous end joining) repair systems were compared between glioblastoma cells and normal human astrocytes. The expression levels of these genes in primary human glioblastoma cell lines (GBM113 and GBM114) were normalized to the expression of the reference gene, 18S rRNA. The data are presented as fold changes relative to normal human astrocytes (NHA). The results represent the mean value ± standard deviation (SD) obtained from three independent experiments, each performed in triplicates.

3.2. Response of patient-derived glioblastoma cells and normal astrocytes to VPA inhibitor used alone or in combination with alkylating agent and PARP1 inhibitor.

Next, we investigated whether the pretreatment with HDAC inhibitor alters the cytotoxicity induced by TMZ and BMN-673. GBM113 and GBM114 cell lines were treated with appropriate IC50 concentrations of each drug alone or in various combinations for 240 hours: 168 h of pre-treatment with VPA (10mM), followed by 72 h of 6.25 μM TMZ and 50 nM BMN-673[13]. When compared to individual agents, the combination of VPA, BMN 673, TMZ exerted significantly stronger anti GBM113 and GBM114 glioblastomas effect with only minimal toxicity to normal astrocytes . Cell viability was determined using trypan blue staining. Clonogenic assay was used to examine the influence of drugs on the colony-forming ability of cancer cells. VPA in combination with BMN-673 or TMZ had an influence on clonogenic efficacy, while the triple combination of VPA+BMN673+TMZ was able to almost completely abolish the clonogenic growth of LIG4-deficient glioma cells.

A.

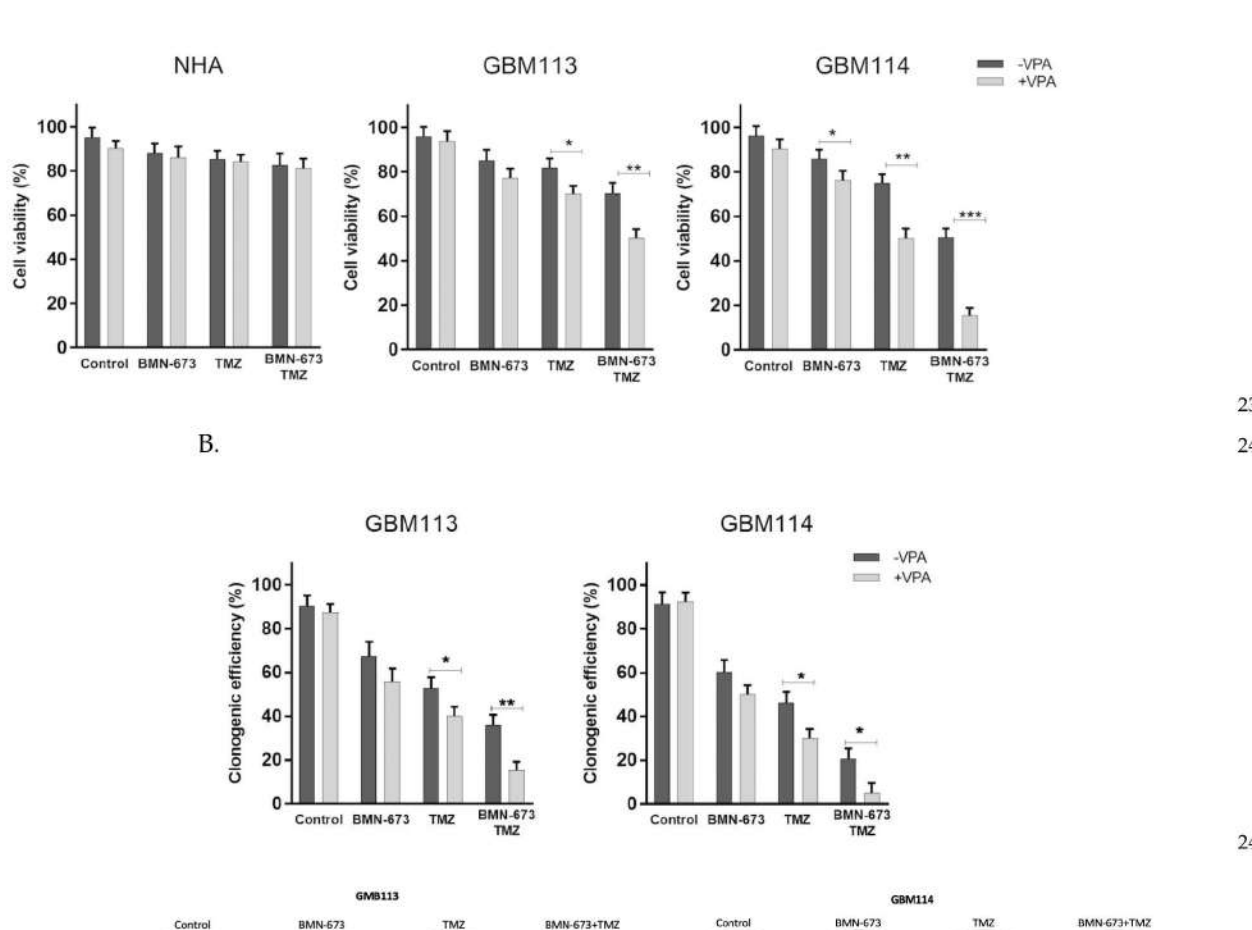

Figure 2. The effect of VPA, BMN-673, and TMZ treatment on cell viability(A) and proliferation (B) after 240h incubation (168h pretreatment with VPA and 72h incubation with BMN-673 and TMZ). At least three independent experiments were performed, and the results are shown as the mean ± standard deviation (SD). * $p \leq 0.05$, ** $p < 0.001$, compared with the control group.

3.2. *Valproic acid Down-Regulate RAD51 and FANCD2*

To examine how inhibiting class I HDACs with VPA enhances sensitivity to temozolomide and talazoparib, a real-time PCR array was conducted in GMB113 and GBM114 glioblastoma cells. The aim was to monitor changes in mRNA expression of six genes involved in the double strand break repair pathway (RAD51, RAD51D, FANCD2, BRCA1, BRCA2, PALB2). In Figure 3, the results of the PCR array showed alterations in the mRNA expression profile of RAD51, FANCD2. The findings revealed that RAD51 and FANCD2 protein levels decreased following inhibition of HDACs.

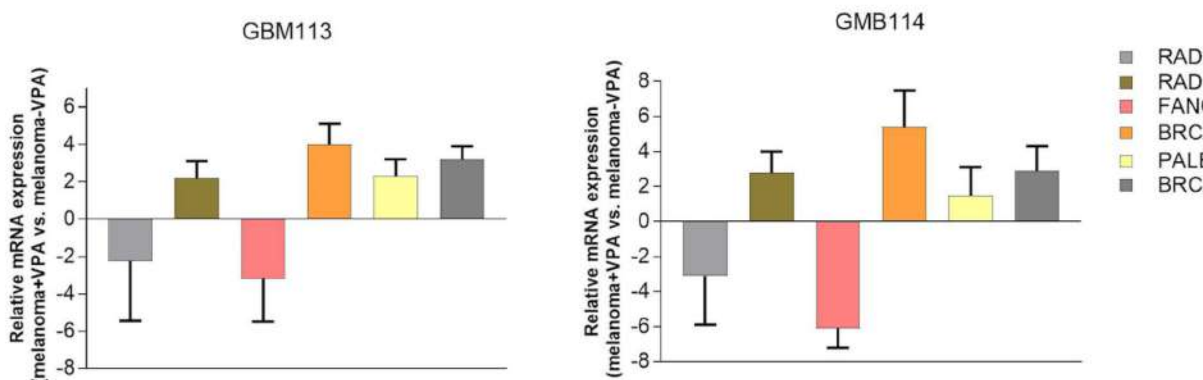

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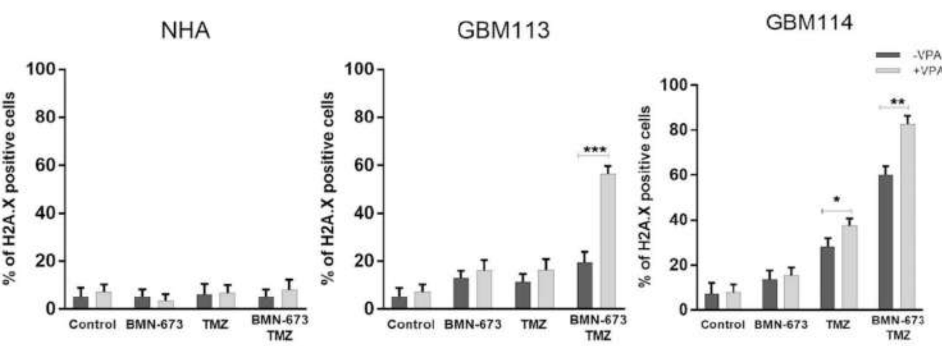
Figure 3. VPA downregulate RAD51 and FANCD2 in glioblastoma cells. GMB113 and GBM114 cells were treated with VPA (10 mM) for 168 h. (**A**) Real-time PCR results showing the down and upregulation of the listed genes. 256 257 258

3.3. Inhibition of class I HDACs increase the number of DSBs in glioblastoma cells 259

GBM113 and GB114 cells showed elevated levels of γ-H2AX after treatment with the tested compounds. Figure 2A. Furthermore, when GMB113 cells were treated with a combination of VPA, TMZ and BMN-673 there was approximately a twofold increase in phosphorylation levels of H2AX compared to treatment with either drug alone. In NHA cells the level of γH2A.X positive cells stayed at relatively low level regardless from the treatment used. The neutral comet assay was utilized to identify DNA double-strand breaks (DSBs) following the administration of VPA, BMN673, and/or TMZ. The combination treatment involving VPA, BMN673, and TMZ resulted in a substantial and notable elevation in DSBs in both glioblastoma cell lines. 260 261 262 263 264 265 266 267 268



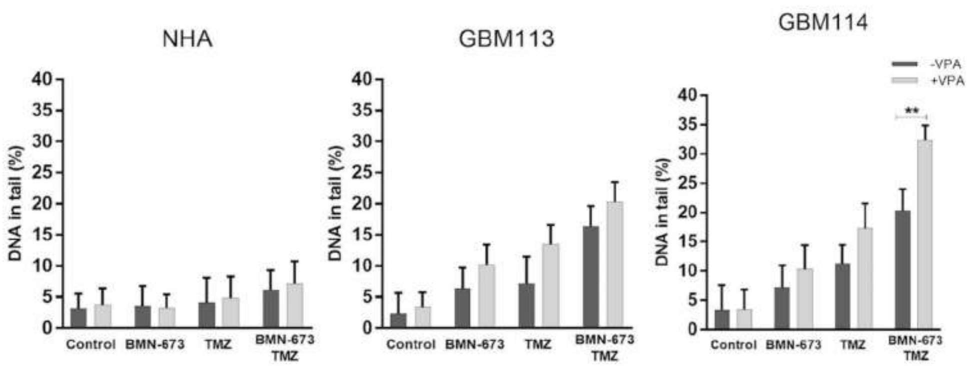


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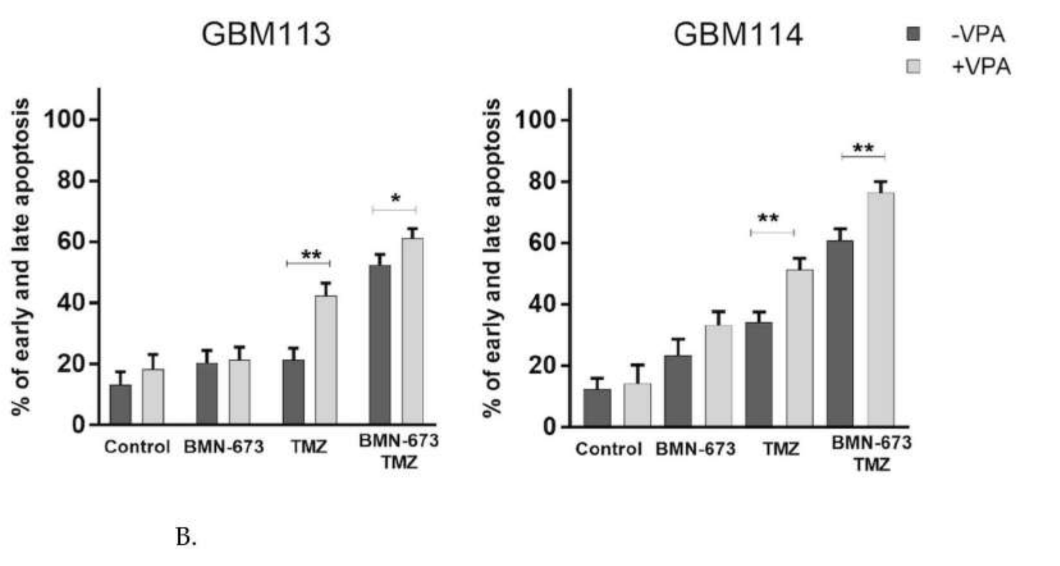
Figure 4. Accumulation of DSB in VPA+BMN673+TMZ-treated GMB113 and GBM114 glioblastoma cells. (A) DSBs were detected by γH2A.X immunofluorescence. Bars show mean percentage of γH2A.X –positive cells ± SD from 3 independent experiments. (B) DSBs were detected by neutral comet assay. Bars show mean percentage of DNA in tail ± SD from 50 randomly selected cells in 3 independent experiments. *$p < 0.05$ and **$p < 0.001$ *** p-value ≤ 0.001 when compared to control.

3.4. *Effects of valproic acid on the cell cycle and apoptosis*

In order to analyze the potential anti-glioblastoma effect of the HDAC inhibitor, either used alone or in combination with a PARP1 inhibitor and an alkylating agent a double staining method utilizing propidium iodide (PI) and Annexin V was employed. This staining technique allows for differentiation between live and dead cells, as well as early apoptotic vs late apoptotic/necrotic cells. The combination of VPA+BMN673+TMZ exhibited a significantly stronger anti-glioma effect on GBM113 and GBM114 cells. Flow cytometry analysis indicated that cell death primarily occurred through the process of apoptosis. Such an effect was accompanied by an increase in subG1 events in both cell lines, providing confirmation of a more potent pro-apoptotic effect.

A. 285



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B. 287

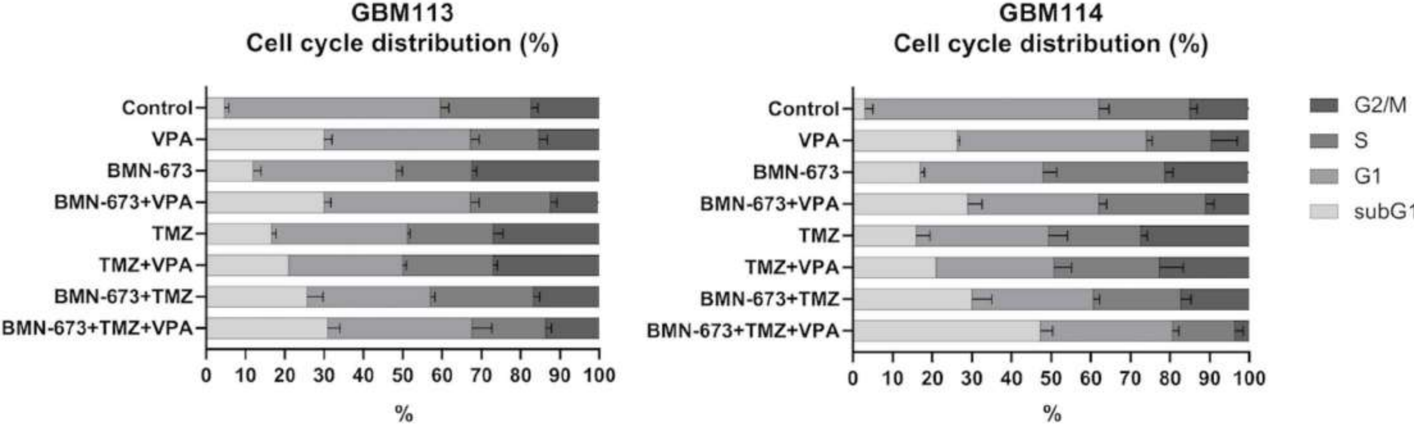

Figure 5. Effects of valproic acid on the cell cycle and apoptosis A. The percentage of GBM113 and GBM114 glioblastoma cells in early and late apoptosis **B**. Cell cycle analysis was evaluated by FACS analysis after 48 h of treatments, following staining with PI. The bars represent the mean of the percentage of cells in each phase of cell cycle (subG1, G1, S and G2) plus S.D. of three experiments. At least three independent experiments were performed, and the results are shown as the mean ± standard deviation (SD). * $p \leq 0.05$, **$p < 0.001$, compared with the control group. 289 290 291 292 293 294

4. Discussion 295

Recent advancements in understanding genetic and epigenetic changes in tumors have highlighted the concept of synthetic lethality as a promising area for identifying new therapeutic candidates. Synthetic lethality occurs when the simultaneous loss of two genes leads to cell death, while the loss of each gene individually is not lethal. For example, tumors with BRCA1/2 deficiencies, impairing homologous recombination repair, have been found to be sensitive to PARP inhibition. PARP inhibitors (PARPi) can be used in combination with agents that induce DNA damage, such as doxorubicin, radiation, or alkylating drugs . 296 297 298 299 300 301 302 303

The Involvement ofIICs in cancer has raised hope that these enzymes may represent valuable targets in drug discovery programs. Recent clinical trials demonstrated that, at 304 305

least for hematological cancers small molecules that inhibit HDACs can be effective pharmacological agents, either when administered alone or in combination with other drugs. HDACi exhibit a number of anti-proliferative effects, such as cell cycle arrest, differentiation, angiogenesis inhibition and apoptosis. A significant number of HDACi, such as suberoylanilide hydroxamic acid (SAHA, also known as vorinostat or Zolinza), entinostat (MS-275), romidepsin (Istodax), and belinostat (PXD-101) are at various stages of drug development.The use of HDAC inhibitors (HDACi) has proven to be an effective strategy in anticancer treatment, both as single agents and in combination therapies targeting various types of cancer, including pancreatic, solid tumors, and hematologic malignancies. HDAC inhibitors have demonstrated their ability to interfere with DNA repair processes [23], leading to their incorporation into combination therapies with DNA-damaging agents like cisplatin, doxorubicin, and ionizing radiation. Furthermore, HDAC inhibitors have been shown to enhance the sensitivity of cancers, such as triple-negative breast cancer and prostate cancer , to PARP inhibitors, suggesting their potential in combination therapies.

In our study, we discovered that the combination of PARP inhibitors (PARPi) and HDAC inhibitors (HDACi) holds promise as a potential therapeutic approach for glioblastoma. VPA, which selectively inhibits class I HDACs, demonstrated increased cytotoxicity when combined with BMN-673, a PARP1 inhibitor and alkylating agent. This enhanced cytotoxic effect was correlated with the induction of more pronounced DNA damage in cells treated with the HDACi/PARP1 combination, attributed to the down-regulation of FANCD2, RAD51 by VPA.

In the first part of our study, we investigated the response of patient-derived glioblastoma cells and normal astrocytes to the VPA inhibitor, both as a single agent and in combination with an alkylating agent and PARP1 inhibitor. Our findings demonstrated that while VPA sensitized the treated cells, the triple combination exhibited the most significant efficacy.

Apoptosis is a crucial process characterized by biochemical and morphological changes that ultimately lead to cell death . The TP53 DNA damage checkpoint tumor suppressor genes, regardless of the specific tumor, are among the most frequently mutated genes. Apoptosis initiation relies on the delicate balance between two apoptosis regulators, Bcl-2 and Bax, wherein maintaining the appropriate expression levels of these proteins is essential for cell survival. Higher expression of Bcl-2 inhibits apoptosis [37, 38]. Common anticancer therapies, including chemotherapy and radiotherapy, induce apoptosis in various cancer cells. Literature suggests that DNA damage or damage to other critical molecules is often the initial trigger in the apoptotic process. It was observed that each of the three compounds tested in this study induces apoptosis and increases the levels of phosphorylated H2A.X, thereby influencing the expression of genes involved in HR repair in glioblastoma cell lines. Other studies have shown that the tested inhibitors PARP1 and HDAC induced apoptosis in DMBC11, H6, and H7 cell lines by causing DNA damage[12–14]. Similarly, Romeo et al reported that VPA and TSA sensitizing pancreatic cancer cell to AZD2461 PARP inhibitor. They propose that the mechanism of action I associated with downregulation of CHK1 and RAD51 [15] The heightened cytotoxicity achieved by the combination of HDACi and PARPi and alkylating agent may be attributed to the impairment of the HR DNA repair pathway, a mechanism often associated with resistance to PARP inhibitor treatment [9].

5. Conclusions

The combination of VPA (valproic acid), a histone deacetylase inhibitor (HDACi), with a PARP inhibitor and an alkylating agent shows promising results in reducing the survival rate of melanoma cells. This combination treatment induces stronger DNA damage specifically in glioblastoma cells without affecting astrocytes. What is particularly interesting is that the interconnected effects induced by HDACi have not been previously highlighted in cancer cells. The findings of this study suggest that HDACi, especially

those targeting class I HDACs, could be used in combination with PARP inhibitors and alkylating for the treatment of glioblastoma. This combination therapy may offer new therapeutic possibilities for melanoma patients. 359 360 361

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Review

Synthetic Lethality Targeting Polθ

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Abstract: Research studies regarding synthetic lethality (SL) in human cells are primarily motivated by the potential of this phenomenon to be an effective, but at the same time, safe to the patient's anti-cancer chemotherapy. Among the factors that are targets for the induction of the synthetic lethality effect, those involved in DNA repair seem to be the most relevant. Specifically, when mutation in one of the canonical DNA double-strand break (DSB) repair pathways occurs, which is a frequent event in cancer cells, the alternative pathways may be a promising target for the elimination of abnormal cells. Currently, inhibiting RAD52 and/or PARP1 in the tumor cells that are deficient in the canonical repair pathways has been the potential target for inducing the effect of synthetic lethality. Unfortunately, the development of resistance to commonly used PARP1 inhibitors (PARPi) represents the greatest obstacle to working out a successful treatment protocol. DNA polymerase theta (Polθ), encoded by the POLQ gene, plays a key role in an alternative DSB repair pathway—theta-mediated end joining (TMEJ). Thus, it is a promising target in the treatment of tumors harboring deficiencies in homologous recombination repair (HRR), where its inhibition can induce SL. In this review, the authors discuss the current state of knowledge on Polθ as a potential target for synthetic lethality-based anticancer therapies.

Keywords: DNA damage; DNA repair; personalized medicine; polymerase theta; synthetic lethality

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1. Introduction

The phenomenon of synthetic lethality (SL) was discovered and described for the first time in *Drosophila melanogaster* almost one hundred years ago [1]. One can define synthetic lethality as follows: when pathway A is defective, a redundant pathway B enables cell viability. If pathway B is inactivated or inhibited in cells deficient for pathway A, then both A and B are not functional, which leads to cell death. After 85 years, it was first applied in targeted cancer therapies [2,3]. However, it took time to change it into an efficient treatment protocol. Nowadays, there are several commercialized drugs that utilize this mechanism, which have been approved by the Food and Drug Administration (FDA), for example, olaparib, rucaparib, niraparib, and talazoparib [4]. All of these are poly (ADP-ribose) polymerase 1 inhibitors (PARPi) and their application has successfully been translated into therapy, mainly in combination with homologous recombination deficient (HRD) tumors, not only including BRCA mutations, but also the ones imitating BRCA-mutated cancers, called BRCA-associated or BRCAness. Interestingly, there have also been several cases found of patients with ovarian cancer who did not present these mutations but relapsed platinum-sensitivity disease after the administration of PARPi. However, there are some drawbacks of this solution, namely, cancer cells can develop resistance to PARPi

due to homologous recombination repair pathway restoration [4]. Therefore, scientists are continuing their research to explore new synthetic lethal relationships, which can be used to treat drug resistant cancer.

Recent studies demonstrate that DNA polymerase theta (Polθ), encoded by the *POLQ* gene, might play a significant role in alternative DNA double-strand breaks (DSBs) repair pathways [5]. Thus, Polθ is suggested to maintain genome stability, however, its activity is correlated with cancer progression [6]. Accordingly, cancer cells have elevated expression of Polθ, which promotes their survival. Normal cells, however, have expressed a low or a non-existent level of Polθ. Furthermore, silencing Pol in HR-deficient cells reveals a synthetically lethal correlation between Polθ and HR genes. In addition, Polθ depletion causes tumor cells to become more sensitive to other treatments such as radiation or chemotherapy. Polθ is fated to become a new target in customized cancer treatment due to this Polθ feature and its probable engagement in PARPi resistance mechanisms in tumors [7].

In this review, the authors described Polθ and its role in the DSB repair mechanisms as well as focus on the aspect of synthetic lethality in the context of anticancer therapies. Finally, the authors emphasize the promising role of Polθ as a target of this kind of treatment.

Google Scholar and PubMed were used to review the most relevant papers (published until March 2022) that focused on the role of polymerase theta in the context of synthetic lethality and potential anticancer therapy. The authors considered studies performed on animals as well as human subjects (in vivo, in vitro) along with the clinical trials. Keywords applied were as follows: synthetic lethality, dual synthetic lethality, microhomology-mediated end joining, DNA damage response, helicase, polymerase, DNA repair, polymerase theta, cancer, targeted cancer therapy, polymerase theta-mediated end joining, TMEJ, double strand break repair, MMEJ, homologous recombination repair, HR, and anticancer therapy.

2. Polθ: Structure and Functions

Mammalian cells are known to contain at least 16 different DNA polymerases that function in semiconservative DNA replication (pols α, δ, ε), base excision repair (pol β), mitochondrial DNA replication, repair and degradation (pol γ), DSB repair and immunological diversity (pols λ, μ, pol θ, and terminal deoxynucleotidyl transferase), and DNA damage tolerance by translesion synthesis [8]. Among them, DNA polymerase theta has the most unique structure. This protein with a mass of 290 kDa is comprised of an N-terminal helicase-like domain (superfamily 2 helicase domain, Polθ-Hel) and a C-terminal DNA polymerase domain (family-A polymerase domain, Polθ-Pol) separated by a long central domain [9]. As such, it is the only eukaryotic DNA polymerase containing a helicase domain [10].

Polθ-Hel shares a close homology to HELQ (helicase Q, POLQ-like helicase, HEL308) belonging to superfamily 2 helicases conserved in eukaryotes and archaea. It is thought to function in the early stages of recombination following replication fork arrest and has a specificity for the removal of the lagging strand in model replication forks [11]. The finding that Polθ has a role in the regulation of DNA replication timing in human cells is noteworthy because this program's regulation is exceedingly complex. Up to this point, only the loss of Rap-interacting-factor-1 (Rif1) has been found to impact the replication timing of a subset of domains in vertebrates. Rif1 was first identified as a telomere-binding protein in yeast and a double-strand break response factor in mammals, and it has also been proven to regulate replication timing in yeast [12].

HELQ can displace streptavidin from a biotinylated DNA molecule, suggesting that one function of the enzyme may be in the removal of bound proteins at stalled replication forks and recombination intermediates. Similarly, Polθ-Hel has the ability to promote annealing of complementary ssDNA in an ATP-independent manner and RPA-coated ssDNA in an ATP-dependent manner. The latter counteracts RPA activity, which results in the promotion of polymerase theta-mediated end joining (TMEJ). Another similarity

between Polθ-Hel and HELQ is the ability to unwind lagging threads on the replication fork in an ATP-dependent manner in $3' \rightarrow 5'$ direction. Furthermore, its activity allows short DNA fragments to be disentangled in an ATP-dependent manner in $3' \rightarrow 5'$ direction, not unlike DNA HELQ. In TMEJ, the activity of the Polθ-Hel domain drastically increases the yield of the long ssDNA fragments conducted by Polθ-Pol through the inhibition of non-productive snap-back replication [1,13,14].

Polθ-Pol is arranged structurally like most other A-family polymerases, meaning that it contains palm, thumb, and fingers subdomains, which structurally resemble a closed right hand. The domain facilitates DNA synapse formation between the 3′ ssDNA overhangs, then, through the use of the opposing overhang as a template in trans, the protein promotes microhomology-mediated annealing and subsequent extension of the 3′ ssDNA terminus. At the ssDNA/dsDNA junction, the presence of a 5′-terminal phosphate increases the rate of the ssDNA extension step, which indicates that Polθ-polymerase exhibits an affinity to this kind of structure, similar to NHEJ polymerases (i.e., Polμ, Polλ). Finally, another Polθ can perform two gap-filling steps by extending the second overhang [15].

Because most of the secondary structural motifs in the central domain of human Polθ are mostly absent, it is generally considered disordered. However there have been suggestions that it contains two binding sites for RAD51, which is an essential recombinase in HR. According to the same studies, Polθ-Hel also contains a putative RAD51 binding site and may act as an anti-recombinase and suppress HR in favor of TMEJ [11].

3. The Role of Polθ in Normal Cells

3.1. Mechanisms of DNA Double Strand Break Repair: Where Is Polθ?

DSBs might arise directly due to an exposition to ionizing radiation or indirectly due to ultraviolet radiation, reactive oxygen species, or genotoxic stress (e.g., chemotherapy). This kind of lesion is most cytotoxic, because if left unrepaired, it may lead to transcription and replication blockage. As a result, it can trigger apoptosis or necrosis, genetic rearrangements (mutations, deletions, insertions, translocations), and cause disruption in the course of meiosis, weakened functioning of the immune system, abnormal development of the nervous system, or the development of genetic diseases and cancer [16]. It has been suggested that the loss of the function mutations of genes involved in DSB repair and the activation of a back-up pathway is a source of the therapy-refractory character of some cancer cells.

The choice of the method of DSB repair depends on many factors (i.e., the expression, activity, and availability of repair complex components (regulated, among others, by post-translation modifications such as phosphorylation or poly-ubiquitination)) as well as template availability. In canonical pathways, HR (or simply homologous recombination—HR) can only occur in the G2 and late S phases when a sister chromatid is available, whereas non-homologous end joining—NHEJ (also referred as canonical or classical NHEJ—cNHEJ, or DNA-PK-depended NHEJ—D-NHEJ) is used to repair DSBs during the G1 and early S cell cycle phases [17]. It is estimated that more than 90% of DNA double-strand breaks in mammals are repaired by NHEJ, while most of the damage in yeast and bacteria is repaired by HR [18]. A DNA double-strand break (DSB) repair (DSBR) pathway that employs homologous repeats flanking a DSB is known as single-strand annealing (SSA).

Another DSB repair pathway has been named theta-mediated end joining (TMEJ) because it requires DNA polymerase theta [19]. Apart from this, alternative pathways can be distinguished for both NHEJ and HR: alt-NHEJ, also referred to as microhomology-mediated end joining (MMEJ) and transcription-associated homologous recombination (TA-HR), respectively (Figure 1) [16].

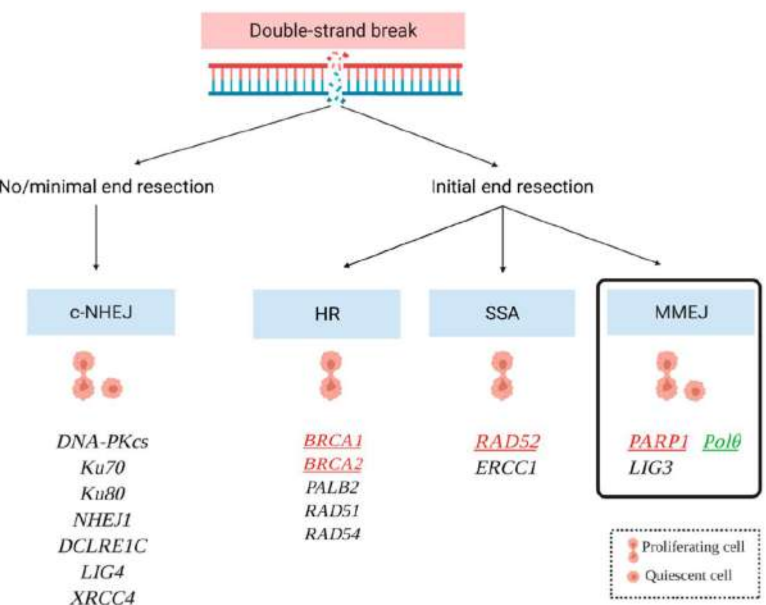

Figure 1. Double-strand break (DSB) repair mechanisms in the quiescent and proliferating cells and major proteins participating in them. Homologous recombination (HR), single-strand annealing (SSA), and microhomology-mediated end joining (MMEJ), in contrast to canonical non-homologous end joining (c-NHEJ), require DNA end resection to expose 3′ single stranded DNA fragments. Polθ as a potential target for synthetic lethality-based therapy has been marked in green. The PARP1, Rad52, BRCA1, BRCA2 partners for dual synthetic lethality have been marked in red.

3.1.1. Homologous Recombination Repair (HRR)

Homologous recombination is deemed as the only extremely accurate DSB repair process and is critical during DNA replication. HR has high accuracy because of its use of a homologous DNA template (i.e., sister chromatid). This allows the lost sequence information to be copied with high-fidelity during DNA repair synthesis [20]. Accordingly, HR is specifically active in the S and G2 cell cycle phases when the sister chromatid is available, preserving genome integrity by preventing mutations that can occur if an error-prone pathway is used. Apart from this, homologous recombination is activated during meiosis, in an event called crossing-over, when it switches the sequence information between homologous chromosomes to promote genetic variation in gametes. Thus, major defects in this pathway cause embryonic lethality [21].

The initial events in DSB repair are crucial in deciding which cellular pathway to choose. Ku proteins, for example, have a high affinity for DSBs in non-homologous end-joining (NHEJ), but the MRN complex (MRE11-RAD50-NBS1), in particular, MRE11, competes with the Ku proteins. MRN is a key DNA damage response (DDR) factor that binds to DSB ends, activates the ATM (ataxia telangiectasia mutated) signaling kinase responsible for cell cycle checkpoint regulation and phosphorylation of H2AX histone, and is directly involved in the two primary DSB repair process, HR and NHEJ. Nevertheless, P53 binding protein 1 (51BP1) is one of the first proteins that binds to the ends of DSBs. However, during S/G2 cell cycles, a critical HR factor, BRCA1, suppresses 53BP1 by excluding it from the vicinity of DSBs, thus promoting DSB resection. 53BP1 has the ability to inhibit end resection via effector proteins. Shieldin, a 53BP1 effector complex containing C20orf196 (otherwise known as SHLD1), FAM35A (SHLD2), CTC-534A2.2 (SHLD3), and REV7, has been identified [22]. Shieldin is dependent on 53BP1 and RIF1 to localize to double-strand break sites, and its SHLD2 subunit binds to single-stranded DNA via OB-fold domains that are comparable to those of RPA1 and POT1 [23–25]. MRN works in conjunction with CtIP to initiate DNA resection of DSBs by promoting nicks near DSB ends. MRN-CtIP can then excise one of the strands in a 3′-5′ direction toward the break. Then, nucleases such as Dna2 and Exonuclease I, which are more processive, are recruited

along with DNA helicases (i.e., Bloom's helicase) to execute an extended 5′-3′ resection step that ends in long 3′ ssDNA overhangs. These structures are bonded and stabilized by replication protein A (RPA), which results in the activation of ATR (ATM- and Rad3-related) kinase. The activation is mediated by ATRIP (ATR-interacting protein) and plays a crucial role in DDR. BRCA1 is hypothesized to help recruit BRCA2 by forming a complex with PALB2 (i.e., BRCA1-PALB2-BRCA2), which, along with other recombination mediator proteins, mediates the replacement of RPA with RAD51 on ssDNA. RAD51 is a Walker A/B containing recombinase that promotes homology search and strand invasion into the sister chromatid, which serves as a template for DNA repair synthesis catalyzed by polymerases [26]. Another essential cofactor RAD54 regulates RAD51 activity by stabilizing the RAD51 nucleofilament and remodeling of nucleosomes. It also stimulates the homology search and strand invasion activity of RAD51 [27].

3.1.2. Single Strand Annealing (SSA)

Single-strand annealing (SSA) occurs during the S and G2 cell cycle phases and uses DNA homology regions of 8–20 base pairs (bp) in length to connect the ends of widely resected DNA. Overall, SSA is considered to be highly error-prone because it eliminates the DNA fragments between repeats because it eliminates DNA fragments between the repeats as well as one repeat [16,28]. Moreover, it typically occurs between repetitive DNA sequences, and as a result, induces massive deletions and DNA rearrangements that can trigger tumorigenesis. The first steps of SSA and HR are a shared MRN complex with CtIP initiating $5' \rightarrow 3'$ DNA resection. Other factors such as Exonuclease I and BLM helicase as well as Dna2 helicase/nuclease promote further resection. RPA binds to single-stranded DNA (ssDNA), prevents secondary ssDNA structures, and interacts with a variety of replication and repair factors. RAD52 anneals the homologous sequences flanking the break sites, generating a synapsed intermediate that is subsequently subjected to endonucleolytic cleavage of the 3′ ssDNA tails by ERCC1, forming a complex with XPF [29].

After this, the mechanisms of HR and SSA start to diverge. Particularly, SSA is a RAD51 independent pathway, where the ssDNA overhang homology search and annealing is mediated by RAD52 (RAD52 Double Strand Break Repair Protein). Following annealing, the ERCC1 (Excision Repair Cross Complementation Group 1) interacts with XPF (Xeroderma Pigmentosum, Complementation Group F), creating a complex that removes the flanking unannealed non-complementary 3′ssDNA. Finally, DNA Ligase I (LIG1) ligates the paired DNA ends [27,30].

3.1.3. Nonhomologous End Joining (NHEJ)

In mammalian cells, NHEJ is a significant DSB repair pathway, particularly during the G0/G1 phases of the cell cycle. As previously mentioned, BRCA1 suppresses NHEJ in S and G2 despite the fact that this pathway is functional throughout all of the cell cycle phases except mitosis. Although this pathway is considered to be error-prone because it can cause minor insertions and/or deletions, along with chromosomal translocations, it is overall thought to be important for preventing genome instability [31,32].

NHEJ is initiated by the Ku70/Ku80 heterodimer. Particularly, Ku70/Ku80 binds to the ends of DSBs with high affinity, protecting them from resection, which causes HR. Only after this will the recruitment of all downstream NHEJ factors such as DNA-PKcs (DNA dependent Protein Kinase catalytic subunit), XRCC4 (X-ray repair cross-complementing protein 4)-LIG4(DNA ligase IV)-XLF (XRCC4-Like Factor) complex, and X-family DNA polymerases (Pols) lambda (Pol λ) and mu (Pol μ), be possible [33]. Ku70/80 specifically activates DNA-PKcs, causing it to autophosphorylate and phosphorylate other proteins. During NHEJ, Polλ and Polμ enable DNA extension and gap filling in NHEJ-mediated DNA synapses [34]. XRCC4 is an essential auxiliary factor for LIG4 that boosts its activity responsible for ligation of the ends of DSBs, it is involved in the latter steps of NHEJ. This step is also stimulated by XLF [35].

3.1.4. Polymerase Theta-Mediated End Joining (TMEJ)

Polymerase theta is mainly found to be present in TMEJ, which in itself is an alternative pathway of NHEJ, sometimes treated as a separate, third pathway. The role of Polθ ortholog, Mus308, in the repair of DSB was first discovered in studies of Drosophila. Mus308 was found to play a role in a synthesis-dependent and Ku70/LIG4-independent end joining pathway in vivo break joining assays of DSBs produced by I-SceI homing endonuclease. There are similarities between TMEJ and SSA and HR as they engage a common intermediate, the 3′ssDNA tails generated after the resection of chromosome breaks [36].

The common steps include the activation of helicases such as the MRN complex as phosphorylated CtIP generates 3′ DNA overhangs. Polθ binds this long single strand DNA overhang and anneals to the microhomologous sequences, at least 2 base pairs in length, in order to utilize them as primers for DNA synthesis [6,29]. Polymerization of DNA stabilizes the ends that undergo ligation by either the LIG3-XRCC1 complex or LIG1 alone [37–39].

Polθ uses the ssDNA overhang on the opposite side of the break from its binding site as a template for DNA extension, which ultimately results in the stabilization of the intermediate DNA structure formed during the initial phases of the repair process. Subsequently, polymerase is thought to lengthen the second overhang, thereby filling the gap caused by the occurrence of DSB. LIG3 is required for the final fusion of the gap after the processing of the transition structure by other enzymes such as endonuclease [38,39]. Furthermore, due to the lack of proper stabilization, the overhangs elongated by Polθ may detach from the structure. If detached overhangs are then annealed and used as templates once more, the templated insert fragments (additional inserts) are created between the deletion junctions [21].

Polθ has been shown to be able to promote DNA synapse formation from DSB-adjacent 3′ ssDNA containing microhomologous sequences of at least 2 bp in length. In this situation, Pol synthesis is compatible with the extremely efficient capacity of the polymerase to elongate DNA strands from mismatched termini as well as a tendency for primer-template slippage [15].

A different recently published study discovered that Pol-pol DNA endonuclease activity, which has been associated with end-trimming during TMEJ. Furthermore, the polymerase domain has been found to exhibit 5′-deoxyribose phosphate lyase activity, which suggests that it might take part in base excision repair (BER) [40–42].

However, under certain circumstances, neither NHEJ nor HR are sufficient, and only TMEJ is able to mend the lesion. For example, the selection of the repair mechanism could be based on DNA end resection. TMEJ is favored in situations when the overhangs of DNA are 30–70 nucleotides long. Additionally, as could be expected, TMEJ is engaged in DNA repair in different HR- or NHEJ-deficient backgrounds such as BRCA1/2 or Ku70 deficiency. This is reflected in various studies showing synthetic lethality between Polθ, the main protein of TMEJ repair, and the canonical DSB repair pathway genes. Despite frequently being described as an alternative NHEJ, TMEJ differs from it substantially as it does not require the presence of Ku heterodimers and is able to act only on resected DNA ends. Polθ utilizes homologous fragments from both sides of the lesion during repair initiation, which invariably results in the loss of one of these fragments. Consequently, TMEJ is an intrinsically mutagenic repair pathway [1,43].

4. The Role of Polθ in Malignant Cells

4.1. Expression of Polθ in Cancer Cells

The Polθ expression in human malignancies was investigated since it has been linked to chromosomal instability in *D. melanogaster*. Many human neoplasms including those of the lung, stomach, small intestine, rectum, and colon overexpress this protein, which correlates with poor survival [44–48]. It is estimated that approximately 70% of breast cancers are characterized by the overexpression of Polθ [45]. In addition, the expression level of the polymerase is particularly high in lung, breast, and ovarian HR-deficient

cancer cells. Due to the fact that Polθ has low or no expression in normal tissue, one can speculate that it constitutes an ideal tumor-specific radiosensitization target. Accordingly, its depletion causes the sensitization of cells deficient in homologous repair to radiation and their decreased viability [7,46,49,50]. Moreover, the knockdown of Polθ with short interfering RNA (siRNA) has been associated with increased DSB formation, destabilization of replication forks, and enhanced sensitivity to some genotoxic agents, suggesting its role as a guardian of the genome [51]. It has been proposed that while keeping a correct level of Polθ expression is crucial for maintaining genome stability, it is possible that the elevated level may favor the occurrence of chromosomal rearrangements, which may ultimately lead to the generation of more aggressive neoplastic phenotypes [52].

Accordingly, the Japanese patients showed not only a significant increase in Polθ expression in colorectal and lung tumors compared to the adjacent tissues, but also an elevated level of Polθ significantly lowered the survival rate within a timeframe of 24 months. Another study of patients with colorectal cancer, but conducted in France, explored the expression levels of 47 genes involved in replication: 18 were downregulated while 17 were upregulated. Not only was *POLQ* among the upregulated genes, but its mRNA level also correlated with a substantial drop in the patients' survival [6,49]. Based on an analysis of early-stage non-small cell lung tumors, increased Polθ expression within a five-gene prognostic panel was associated with a worse prognosis for patients. Moreover, data from The Cancer Genome Atlas (TCGA) showed that the level of Polθ expression in ovarian cancer was correlated with the degree of tumor development [49,53,54]. Overall, the above data suggest that the presence of the overexpression of Polθ promotes the survival and/or growth of cancer cells.

Still, it is possible that Polθ takes part in yet unknown mechanistic interactions within cancer development, other than DNA repair or replication [45]. An excess of Polθ may deplete cofactors needed for translesion replication or DNA repair pathways such as TMEJ or BER, in which the polymerase is also involved. Decreased resistance to alkylating agents and more frequent short-fragment DNA replication in Polθ overexpressing cells may—over time—lead to DNA damage accumulation and, eventually, to a severe reduction in the efficiency of replication fork progression. Polθ overexpression has also been shown to lead to other neoplastic cell traits such as chromosome end fusions and dicentric chromosomes. This may point toward defects in telomere formation and suggests that Polθ overexpression is a factor for a more aggressive tumor phenotype and a higher likelihood of disease recurrence [23,45,53]. Such traits make Polθ a highly attractive target of clinical interventions [6,42].

4.2. *HR-Deficient Tumors*

It has been discovered by previous research that HR-deficient tumors have a high sensitivity to Polθ, implying that TMEJ is required for HR-deficient tumor survival. To test whether there is a synthetic lethality between the HR genes and POLQ, Ceccaldi et al. created a HR-deficient ovarian cancer cell line. To determine the cell survival, this cell line was depleted of Polθ and exposed to cytotoxic agents. Following exposure to a variety of inhibitors, Polθ depletion reduced the lifespan of these HR-deficient cells, further supporting that HR-deficient cells are dependent on Polθ for survival. Furthermore, in mice, the deletion of HR and Polθ resulted in embryonic lethality [51]. HR-deficiency and high levels of replication stress characterize BRCA1/2-deficient breast and ovarian cancers, leading to genomic instability [55]. BRCA1/2 and Fanconi Anemia (FA) proteins work together in healthy cells to keep replication forks stable and maintain genomic integrity [56]. BRCA1/2-deficient tumors have previously been demonstrated to upregulate FANCD2, a protein necessary for the protection of replication forks, thus protecting DNA strands from excessive nucleolytic degradation [53,54].

In a study conducted by Kais et al., tumors with BRCA1/2 deficiencies were found to exhibit a compensatory increase in the expression of FANC [57]. The function of FANCD2 is to stabilize stalled replication forks and promote alt-EJ repair in tumors deficient in

BRCA1/2. FANCD2 knockout in those tumor cells resulted in acute DNA repair defects and an upsurge in cell death. Unstable replication forks cause copy number variation mutations and chromosomal translocations in cells deficient in BRCA1/2 [51]. Genomic instability, while crucial to tumor progression, can also limit cancer cell survival if it is excessive. Thus, mechanisms have evolved in BRCA1/2-deficient cells, which allow them to tolerate genomic instability and replicative stress, ultimately allowing the cells to survive and replicate DNA. For example, in BRCA1/2-deficient cells, an upregulation of the error-prone Polθ/PARP1-mediated alt-EJ DNA repair pathway was found as a mechanism compensation for HR defects [1,2]. According to one study, FANCD2 promotes Polθ recruitment at the sites of damage and TMEJ repair. The loss of FANCD2 in BRCA1/2-deficient tumors increased cell death. This finding shows that FANCD2 and BRCA1/2 have synthetic lethality [58].

4.3. Synthetic Lethality Targeting Polθ

Tumor cells that are reliant on alternative backup pathways of DNA repair become novel targets for anticancer treatment based on the phenomenon of synthetic lethality, which is one of the innovative approaches in eliminating cancer cells [45]. In the case of synthetic lethality, losing a gene in a cell involved in a metabolic process that is important for cell survival is compensated for by the action of another gene engaged in a pathway alternative to this process (Figure 2) [59].

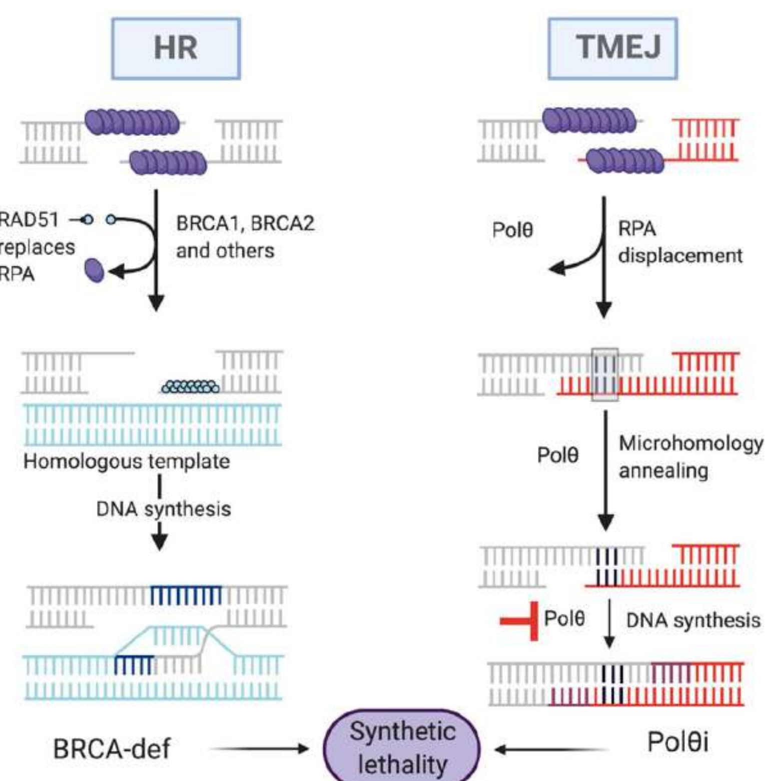

Figure 2. The synthetic lethality (SL) strategy in homologous recombination (HR) deficient cells.

The results of studies on primary glioblastoma, melanoma, and other cancer cells revealed that the inhibition of certain DNA repair mechanisms can lead to synthetically lethal effects in cancer cells with simultaneous sparing of normal cells [60,61]. The initial success associated with the use of PARPi in the treatment of ovarian and breast cancer with BRCA1/2 mutations has established a foundation for targeting DSB repair pathways by inducing synthetic lethality [62].

The TMEJ pathway is yet to be defined exactly, and numerous research groups are in the process of seeking therapeutics that may serve to limit Polθ activity, and to utilize this pathway in cancer therapy. In recent research, inhibiting Pol was found to sensitize cells to replication stress produced by drugs such as topoisomerase poisons or ATR inhibitors, leading to new cancer treatment candidates. DSB repair-proficient cancer cells expressing high levels of Polθ may be sensitive to the inhibition of Polθ in combination with standard cytotoxic drugs [8].

Since the DNA synthesis activity of Polθ is essential for TMEJ and the proliferation of DSB repair-deficient cells, it is anticipated that pharmacological inactivation of the Polθ polymerase domain will eliminate HR/NHEJ-deficient cancer cells [63]. Additionally, DSB repair-proficient cancer cells expressing high levels of Polθ may be sensitive to the inhibition of Polθ in combination with standard cytotoxic drugs. The combination of Polθ inactivation, together with PARPi or RAD52i, will exert a synergistic "dual synthetic lethality" in NHEJ- or HR-deficient solid tumors.

It has been proven that Polθ inhibition results in HR repair enhancement and an increase in RAD51 clusters. This can be explained by the fact that Polθ can bind directly to RAD51 causing HR inhibition [51,63]. These findings suggest that Polθ prevents the formation of HR repair complexes, which are toxic in BRCA1/2 defective cells. Both the depletion of Polθ in ovarian cancer cells with inactive FANCD2, a gene crucial for the DNA repair pathway through HR, and the inactivation of these genes in the cells of embryos from mice produced a synthetically lethal effect [64,65]. Moreover, the *POLQ* gene knockout in xenografts prepared from the cells of the tumor with dysfunctional HR genes increased the degree of its sensitivity to PARP inhibitors and enhanced survival in the knockout mice compared to the control group maintaining Polθ expression [66].

5. Polθ—A Therapeutic Target for Cancer Therapy

5.1. Why Target Polθ to Trigger Synthetic Lethality?

DSB-repair-deficiency depends on Polθ mediated-TMEJ. It is anticipated that the pharmacological inhibition of Polθ will selectively kill cancer cells, which depend on Polθ mediated-TMEJ. Moreover, recent studies have suggested that secondary mutations restoring BRCA1/2 function are caused by the activity of Polθ mediated-TMEJ and that Polθ inhibition can prevent the development of PARPi resistance. PARP1 and RAD52 inhibitors are involved in the already known synthetic lethal mechanism in cancer treatment. Initially, their success was undoubted; however, with the passing of time, research brought about evidence of cells becoming resistant to PARPi [67–69]. This has motivated scientists to search for further potential inhibitor targets, and they focused on Polθ. Despite being an important player in DNA DSBR, it has been found that Polθ may be one of the factors responsible for its resistance to radiation and chemotherapeutics including PARP inhibitors (PARPi) [42,51,70–72]. Nevertheless, the exact mechanism has not yet been discovered; there are certain assumptions that the engagement of Polθ inhibitors may improve treatment effectiveness.

In the authors' opinion, the main interest should be focused on the type of tumor with alterations to the HR genes since the studies showed that cells of this type are 'hyper-dependent' on Polθ mediated repair (like with PARP1). Moreover, they express higher levels of Polθ, which is attributed to their high survival rate [68,73]. Therefore, the use of Polθi would be a great opportunity to selectively kill these cancer cells. Furthermore, referring to other studies that have shown a broad range of synthetic lethal genes with Polθ, it is also possible to work on other therapeutic strategies than those only known with PARPi and RAD52i [67].

It may look like Polθ acts similarly to PARP1, however, there is still evidence that Polθ inhibitors lead to SL in a different way from PARPi [72]. Additionally, as above-mentioned, HR-deficient cells with a reduced level of Polθ expression are more sensitive to radiation and probably other antitumor agents such as chemotherapy, cisplatin, and mitomycin C. Furthermore, it is possible that several DNA lesions are repaired only via a Polθ mediated

mechanism, hence we can hypothesize that cancer cells with such lesions may be even more sensitive during treatment with Polθi [74].

Considering its complex characterization, Polθ appears to be a promising therapeutic target, especially in the case of PARPi resistance. However, this does not mean that Polθ has to replace the PARP and RAD52 inhibitors; rather, it could work simultaneously to enhance their action or serve as the inhibitor's target itself [75].

5.2. Polθ Inhibition as a Potential Target for Synthetic Lethality-Based Anticancer Therapy

Based on the knowledge gained during the research, it has been proposed that the inhibition of Polθ in HR-deficient cells might induce SL and cause the elimination of cancer cell [76]. Moreover, "dual pathway synthetic lethality" expands the synthetic lethal approach to simultaneous targeting of two repair mechanisms. The inhibition of Polθ and PARP1 may lead to a dual SL effect because these types of synthetic lethality interactions involve two or more genes and two pathways [77].

A combination of Polθ inactivation together with PARPi or RAD5i will exert synergistic dual synthetic lethality in c-NHEJ or BRCA1/2 HR-deficient solid tumors. However, not only PARP1, but also other DSB repair proteins (e.g., BRCA1/2, RAD52, and ATM) have a potential synthetic lethal connection to Polθ [76,78]. In total, 140 of these genes have been revealed using a CRISPR genetic screen, which provides scientists with a broad field to search for suitable targets in anticancer therapies [64]. Nevertheless, the mechanisms that underlie these relationships still require more profound research and could indicate a good direction for further studies on Polθ inhibitors (Polθi). Moreover, DNA repair proteins interact differently with Polθ, depending on whether they do so with its helicase or polymerase domain, or can interact with both domains; thus it is important to take into account its structure when considering the Polθi design [19,79–81].

The DNA damage repair deficiency has recently been associated with anti-tumor immunity activation, with compelling evidence. BRCA2 inactivation has been shown to induce an innate immune response. Lian Li et al. looked into the link between inactivating POLQ and/or FANCD2, two key DNA damage repair genes, and probable innate immune response activation. In comparison to single POLQ or FANCD2 KOs, double KOs of POLQ and FANCD2, which promote POLQ recruitment at sites of injury, drastically reduced cell proliferation in vitro and in vivo. The POLQ and/or FANCD2 KO esophageal squamous cell carcinoma (ESCC) cells had a considerably higher number of micronuclei. The activation of cGAS (Cycling GMP-AMP Synthase) and the overexpression of interferon-stimulated genes (ISGs) were also seen when POLQ and/or FANCD2 were lost (ISGs). Taken together, these results indicate the potential for the activation of the innate immune response through the cGAS-STING-STAT1 pathway, after the loss of both the Polθ and FANCD2 proteins [6].

Using Polθi may be a promising approach in the treatment of cancer types that have developed resistance to PARP inhibitors due to genetic alterations. This type of inhibition is unlikely to elicit a response in the case of secondary mutations that reactivate BRCA and lead to the recovery of HR activity. However, most relapses of neoplastic diseases result from other changes including decreased expression of DNA repair proteins [82].

In the case of such changes leading to PARPi resistance, the use of Polθ inhibitors could lead to the elimination of tumor cells. Furthermore, the simultaneous inhibition of PARP and Polθ may also provide promising effects. Compared to the use of each of the inhibitors separately, the application of dual inhibition will make it difficult for the tumor to develop resistance. Data from the in vitro studies support this assumption, namely, the use of PARPi in combination with decreased Polθ expression leads to a synergistic reduction in colony formation by cells with inactive BRCA1 [82].

Aside from the synthetically lethal effect, the disruption of Polθ expression also sensitizes cancer cells with dysfunctional HR to the effects of radiotherapy to an extent that may bring significant therapeutic effects. A drastic slowdown in tumor mass growth

observed during studies on the inhibition of Polθ and PARP in xenografts also indicates such a synergistic effect [83].

Novobiocin and ART558

Novobiocin is a coumarin antibiotic that has lately been identified as a Polθ inhibitor in a small-molecule screen. Novobiocin has already been used in cancer studies, however, with poor results. Novobiocin is a coumarin antibiotic that was recently discovered to be a Pol inhibitor in a small-molecule screen. Cancer studies have already utilized novobiocin, albeit with unsatisfactory results [84].

The first paper concerning it was published in 2021 and confirmed the successful inhibition of Polθ in human BRCA1/BRCA2-deficient cells in vitro and in vivo. Novobiocin is reported to inhibit MMA and abolish the recruitment of the Polθ protein to the DNA damage sites [64]. It has been revealed in experiments conducted by Zhou et al. that novobiocin specifically targets Polθ, and has a similar impact on cells as genetic methods of Polθ depletion. Furthermore, novobiocin enhances the activity of PARP inhibitors, allowing it to bypass the PARPi resistance mechanisms. Furthermore, cells that achieve PARPi resistance through BRCA2 gene somatic reversion are also resistant to novobiocin.

Zatreanu et al. reported that another Polθ inhibitor called ART558 could also eliminate cancer cells and tumors that have become resistant to PARP inhibitors. Using this Polθi in combination with a PARPi in the patients with cancer characterized by mutations in BRCA genes might prevent resistance from emerging in the first place. ART558 has the ability to inhibit theta-mediated end joining—a major Polθ-mediated DNA repair process—without affecting non-homologous end joining. Furthermore, BRCA1/BRCA2 mutant tumor cells have been found to exhibit DNA damage and be affected by synthetic lethality upon exposure to ART558. ART558 also enhances the PARP inhibitor effects.

Nevertheless, ART558 has great potential, but so far, it has not been used in vivo due to its poor stability in a rat model. Therefore, another Polθi, ART812 was used in these experiments. It has been revealed through genetic perturbation screening that the 53BP1/Shieldin complex has defects, which result in PARP inhibitor resistance, eliciting both in vitro and in vivo sensitivity to small-molecule Polθ inhibitors. The mechanism of ART558 action involves increasing the ssDNA biomarkers and synthetic lethality in cells with 53BP1 defects. The inhibition of DNA nucleases, and thus the inhibition of end resection, is able to reverse these effects, thus implicating them in the mechanism of synthetic lethality.

Importantly, these two inhibitors have different mechanisms of action: novobiocin targets the ATP-ase domain and ART558 the polymerase domain of Polθ, which could be the advantage in advancing the research on Polθi.

Both novobiocin and ART558 represent powerful tools that prove the relevance of targeting Polθ in the case of cancer. It is still unknown whether these drugs will benefit people with cancer, but the strategy of inhibiting Polθ in tumors with defects in homologous recombination appears promising [63].

5.3. Future Perspectives

Personalized anticancer therapy can result in increased treatment effectiveness and reduced toxic effects. To achieve this goal, it is necessary to create a therapeutic model based not only on the clinical indicators of a specific neoplastic disease, but also on the molecular biology of cancer. Identifying the elements that a personalized therapy will target in diverse types of cancer is still a serious challenge for both researchers and clinicians. The use of carefully selected inhibitors of DNA double-strand break repair proteins with an intention to induce cell death based on the phenomenon of synthetic lethality is a promising approach, in which personalized medicine will be used to treat human solid tumors.

The initial success achieved with PARP inhibitors such as lynparza has indicated a promising direction of treating some patients with tumors having mutations in BRCA1/2. It has also established evidence that supports the concept of DSB repair by inducing

synthetic lethality. However, over time, these cancer types invariably become resistant to drugs. Recent studies have shown that Polθ becomes essential in the cells that are deficient in factors facilitating the canonical DSB repair mechanism (BRCA1, BRCA2, Ku70), which indicates the backup function of Polθ-dependent DNA repair processes. Due to this discovery, more attention is now being paid to Polθ as a new therapeutic target. Currently, new genes involved in the DNA damage repair mechanism, chromatin structure maintenance, and DNA metabolism are recognized as synthetic lethality partners for Polθ [36,81,85]. It is predicted that the pharmacological inhibition of Polθ selectively kills TMEJ-dependent cancer cells mediated by Polθ. Moreover, recent studies suggest that secondary mutations restoring the BRCA1/2 function are caused by the activity of TMEJ with Polθ mediation. In this case, the inhibition of Polθ may prevent the development of resistance to PARPi.

Currently, clinical trials on an anticancer drug from the group of Polθ inhibitors started at the end of 2021.

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Abbreviations

ATM	Ataxia telangiectasia mutated
alt-NHEJ	Alternative non-homologous end joining
B-NHEJ	Backup nonhomologous end-joining
BER	Base excision repair
51BP1	P51 binging protein 1
c-NHEJ	Canonical non-homologous end joining
CtIP	BRCA1 C-terminal Interacting Protein
D-NHEJ	DNA-PK-mediated nonhomologous end-joining
DDR	DNA damage response
DSB	DNA double strand break
ESCC	Esophageal squamous cell carcinoma
FDA	Food and drug administration
HR	Homologous recombination
MMEJ	Microhomology-mediated end-joining
NHEJ	Nonhomologous end-joining
PARPi	Poly (ADP-ribose) polymerase 1 inhibitor
Polθ	Polymerase theta
Polθi	Polymerase theta inhibitor
ROS	Reactive oxygen species
RBBP8	Retinoblastoma binding protein 8
RPA	Replication protein A
RIF1	Replication timing regulatory factor 1
SSA	Single strand annealing
SSB	Single strand break
ssDNA	Single stranded DNA
SL	Synthetic lethality
XPF	Xeroderma pigmentosum complementation group F

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Article

Histone Deacetylases (HDAC) Inhibitor—Valproic Acid Sensitizes Human Melanoma Cells to Dacarbazine and PARP Inhibitor

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Abstract: The inhibition of histone deacetylases (HDACs) holds promise as a potential anti-cancer therapy as histone and non-histone protein acetylation is frequently disrupted in cancer, leading to cancer initiation and progression. Additionally, the use of a histone deacetylase inhibitor (HDACi) such as the class I HDAC inhibitor—valproic acid (VPA) has been shown to enhance the effectiveness of DNA-damaging factors, such as cisplatin or radiation. In this study, we found that the use of VPA in combination with talazoparib (BMN-673—PARP1 inhibitor—PARPi) and/or Dacarbazine (DTIC—alkylating agent) resulted in an increased rate of DNA double strand breaks (DSBs) and reduced survival (while not affecting primary melanocytes) and the proliferation of melanoma cells. Furthermore, the pharmacological inhibition of class I HDACs sensitizes melanoma cells to apoptosis following exposure to DTIC and BMN-673. In addition, the inhibition of HDACs causes the sensitization of melanoma cells to DTIV and BMN-673 in melanoma xenografts in vivo. At the mRNA and protein level, the histone deacetylase inhibitor downregulated RAD51 and FANCD2. This study aims to demonstrate that combining an HDACi, alkylating agent and PARPi could potentially enhance the treatment of melanoma, which is commonly recognized as being among the most aggressive malignant tumors. The findings presented here point to a scenario in which HDACs, via enhancing the HR-dependent repair of DSBs created during the processing of DNA lesions, are essential nodes in the resistance of malignant melanoma cells to methylating agent-based therapies.

Keywords: melanoma; HDACi; PARP1; valproic acid; alkylating agent; DNA damage

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1. Introduction

The use of properly selected inhibitors of DNA double-stranded breaks (DSBs) repair proteins to induce cell death, mediated by the synthetic lethality (SL) phenomenon in tumor cells with a reduced activity of the main components of this type of repair, is a perspective approach to the application of personalized medicine for treating human solid tumors [1].

When looking at the five-year survival rate, melanoma proves to be an aggressive tumor, characterized by a low ten percent survival rate at the advanced stage of disease. Systemic therapy serves as the primary treatment approach for the majority of patients with

metastatic melanoma, although surgery and radiation therapy also play a role. Although single-agent chemotherapy is generally well tolerated, it demonstrates limited efficacy, with response rates ranging from 5% to 20%. Combination chemotherapy and biochemotherapy have the potential to enhance the objective response rates; however, they do not confer a survival benefit and are linked to increased toxicity. Residual disease following combination treatment presents a significant challenge, as it can contribute to disease relapse, metastasis, and reduced overall survival rates. Strategies to address residual disease in this context are actively being explored to improve treatment outcomes and reduce the impact of residual disease in patients with metastatic melanoma [2,3].

The elimination efficacy of tumor cells can be enhanced by the administration of cytotoxic compounds used in standard chemotherapy, i.e., dacarbazine (DTIC) and temozolomide (TMZ) [4,5]. As DNA alkylating agents, they can induce the formation of ssDNA stretches. If these stretches of ssDNA are not bypassed by homologous recombination during DNA synthesis, DSBs will arise and trigger apoptosis in cancer. In cases of cancer cells without any defects in this repair system, it is possible to trigger such defects through the inhibition of histone deacetylases (HDACs), which reduces the activity of DSB repair key proteins, such as RAD51 or FANCD2 [1,6]. Recent studies also suggest that an oral chemotherapeutic agent, TMZ, used in the treatment of glioblastoma, triggers the activation of the DNA damage response (DDR) in resistant glioma cells, leading to the bypass of DNA damage and cell survival. The catalytic activity of a class I HDAC stimulates the expression of the E3 ubiquitin ligase RAD18. Furthermore, data have shown that RAD18 is part of the O6-methylguanine-induced DDR, as TMZ induces the formation of RAD18 foci at DNA damage sites. The downregulation of RAD18 through HDAC inhibition prevents DDR activation in glioma cells following exposure to TMZ [7].

Poly (ADP-ribose) polymerases (PARPs) are a group of enzymes involved in DNA repair pathways, particularly in base excision repair (BER) and single-strand break repair. However, PARPs also play a key role in the repair of DSBs through a pathway known as an alternative non-homologous end joining (al-NHEJ). PARP inhibitors (PARPis) exhibit a synthetic lethal effect in cells with deficiencies in HR repair, such as those with mutations in Breast Cancer gene 1 or 2 (BRAC1, BRAC2) as they rely heavily on the PARP-mediated alt-NHEJ pathway to repair DSBs. Numerous PARP inhibitors (Veliparib, Fluzoparib, Talazoparib, Olaparib, Rucaparib, Niraparib) have obtained approval by the USA Food and Drug Administration (FDA) and European Medicines Agency (EMA) for their application in the treatment of lung, breast, ovarian, pancreatic and prostate cancer. Nevertheless, akin to other targeted therapies, PARPi resistance frequently emerges, facilitated by diverse molecular mechanisms, despite their favorable tolerability and extensive clinical practice.

Histone deacetylases (HDACs) are a class of enzymes that play important roles in regulating gene expression by removing acetyl groups from histone proteins. This leads to a more compact chromatin structure, which can inhibit transcriptional activity. HDACs are divided into four classes based on their sequence homology and catalytic mechanism: Class I (HDAC1, HDAC2, HDAC3, and HDAC8), Class II (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10), and Class IV (HDAC11). The majority of these classes are zinc-dependent and function by using a catalytic zinc ion in their active site to deacetylate histones. However, Class III HDACs, also known as sirtuins (SIRT1-7), are NAD(+)-dependent and do not rely on zinc for their enzymatic activity [8]. Histone deacetylase inhibitors (HDACis) are a diverse group of drugs that modify the epigenome by changing the acetylation of not only histones but also other proteins not associated with histones [9]. HDACi drugs, particularly those that can inhibit all HDACs, also known as "pan HDACis", have been found to reduce the expression of several DNA damage repair molecules [10]. This reduction in expression can occur through transcriptional downregulation of molecules such as BRCA-1, Checkpoint kinase 1 (CHK1), and RAD51, or through increasing the acetylation of HSP90 [11]. This increase in acetylation impairs the chaperoning function of HSP90, which leads to the decreased stability of DNA damage response (DDR) proteins [5]. Since Class I HDACs are often overexpressed in cancers and may contribute to those

cancers' response to therapy, we hypothesize that VPA—a histone deacetylase inhibitor, could sensitize melanomas to genotoxic agents such as dacarbazine (DTIC) administered alone or in combination with a PARP inhibitor.

2. Materials and Methods

2.1. In Vitro Cell Cultures

The patient-derived melanoma cell line DMBC11 was obtained from tumor specimens. The study was approved by the Ethical Commission of the Medical University of Lodz, and informed consent was obtained from all patients. Melanoma cells were cultured in a serum-free Stem Cell Medium (SCM) consisting of DMEM/F12 low osmolality medium (Lonza, Basel, Switzerland) in the presence of B-27 supplement (Gibco, Paisley, UK), growth factors (10 ng/mL bFGF and 20 ng/mL EGF; BD Biosciences, San Jose, CA, USA), insulin (10 μg/mL), heparin (1 ng/mL), and antibiotics (100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 μg/mL fungizone). Cell cultures were maintained in low-adherent flasks (NUNC) at 37 °C in a humidified atmosphere containing 5% CO_2 [12].

Normal Human Epidermal Melanocytes (NHEMs, Lonza) were cultured in Melanocyte Cell Basal Medium (MBM) (CC-3250, Lonza). The culture medium was supplemented with growth supplements containing $CaCl_2$, hFGF-B, PMA, rh-Insulin, hydrocortisone, BPE, FBS (10%), gentamicin/amphotericin-B and endothelin (Lonza). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Drug Treatment

Melanoma cells and NHEMs were plated at a density of 1×10^5 viable cells per well in a 12-well plate one day before drug treatment. Valproic acid sodium salt (VPA, Sigma-Aldrich) was dissolved to 100 mmol/L in PBS and stored at −80 °C after sterile filtration. Cells were pretreated with 1 mM valproic acid (VPA) for 168 h with a refreshment of VPA-containing medium every 48 h, followed by VPA removal before further treatment. After 168 h cells were cultured with 50 nM talazoparib (BMN-673) (MedChemExpress; Cat#HY-16106), 2 mM dacarbazine (DTIC) (Sigma Aldrich, Burlington, MA, USA). After 48 h, 1 mL of fresh medium containing drugs at appropriate concentrations was added to the cell culture for an additional 72 h of culturing.

2.3. Cell Viability

Melanoma cells were plated at a density of 1×10^5 viable cells per well in a 24-well plate and pretreated with 1 mM valproic acid (VPA) for 168 h with a refreshment of VPA-containing medium every 48 h, followed by VPA removal before further treatment. After 168 h, the cells were cultured with 50 nM talazoparib (BMN-673) (MedChemExpress; Cat#HY-16106), and 2 mM dacarbazine (DTIC) (Sigma Aldrich, Burlington, MA, USA) used alone or in combination. A trypan blue exclusion assay was used to determine the viability of the cells after treatments with VPA, BMN-673, and DTIC. Cells were counted within 3 to 5 min of mixing with 0.4% trypan blue using light microscopy with a Neubauer hemocytometer. The experiments were carried out three times, in triplicate.

2.4. Cell Proliferation

Melanoma cells were incubated with compounds at the indicated concentrations for 120 h. Each well of the 12-well culture plates was coated with 350 μL bottom agar mixture (either SCM, 0.5% (*w*/*v*) agar, 5% FBS, or 0.5% (*w*/*v*) agar). After the bottom layer solidified, 350 μL of top agar medium mixture, containing 1×10^3 cells was added (either SCM, 0.35% (*w*/*v*), 5% FBS, or 0.35% (*w*/*v*) agar). The plates were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 10 days of incubation, cell proliferation was determined using a clonogenic assay. For this purpose, the colonies were fixed and stained with 250 μL of 0.005% crystal violet for 1 h, and the spheres were counted under the microscope.

2.5. Apoptosis and Necrosis

FITC Annexin V staining precedes the loss of membrane integrity, which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Therefore, staining with FITC Annexin V is used in conjunction with a vital dye, such as propidium iodide (PI) or 7-Amino-Actinomycin (7-AAD), to allow the identification of early apoptotic cells (PI negative, FITC Annexin V positive). Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. An FITC Annexin V Apoptosis Detection Kit (BD Biosciences, Cat#556547) was used to quantitatively determine the percentage of cells within a population that were actively undergoing apoptosis, according to the manufacturer's instructions. The cells were plated into a 24-well plate (1×10^5 cells/well) and pretreated with 1 mM valproic acid (VPA) for 168 h with a refreshment of VPA-containing medium every 48 h, followed by VPA removal before further treatment. After 168 h, the cells were cultured with 50 nM talazoparib (BMN-673) (MedChemExpress; Cat#HY-16106), and 2 mM dacarbazine (DTIC) (Sigma Aldrich, Burlington, MA, USA). Following 24 h incubation with VPA, BMN-673 and DTIC used alone or in combination, the cells were washed twice with cold PBS and then resuspended in 1X Binding Buffer at a concentration of 1×10^5 cells/mL. Next, 100 μL of the solution (1×10^4 cells) was transferred to a 5 mL culture tube. Subsequently, 5 μL of FITC Annexin V and 5 μL of PI were added. The cells were gently vortexed and incubated for 15 min at room temperature (25 °C) in the dark. Finally, 400 μL of 1X Binding Buffer was added to each tube, and the samples were analyzed using flow cytometry within 1 h.

2.6. Histone γ-H2AX

The DMBC11 cell line was cultured at a density of 1×10^5 cells per well. The H2AX Phosphorylation Assay Kit (Flow cytometry; Millipore, Cat#17-344) was used for the detection of the phosphorylated Histone H2AX levels. The assay was performed on cultured cells that were treated with agents inducing DNA damage or apoptosis (cells were pretreated with 1 mM VPA for 168 h with a refreshment of VPA-containing medium every 48 h, followed by VPA removal before further treatment). After 168 h, the cells were cultured with 50 nM BMN-673, and 2 mM DTIC), thereby promoting H2AX phosphorylation. Following treatment, the cells were fixed and permeabilized to facilitate staining and detection. The presence of Histone H2AX phosphorylated at serine 139 was detected using the FITC-conjugated anti-phospho-Histone H2AX antibody. Flow cytometry was employed to quantify the number of cells exhibiting positive staining for phosphorylated histone H2AX.

2.7. RNA Isolation, cDNA Synthesis and Real-Time PCR

RNA was extracted from the cultured DMBC11 pellet 2.5×10^6 using a total RNA isolation kit (A&A Biotechnology; Cat#031-100). Following that, the RNA was transcribed into complementary DNA using SuperScript II Reverse Transcriptase from Invitrogen Life Technologies. For the quantitative reverse transcription PCR (qRT-PCR), TaqMan Real-Time PCR Master Mix from Life Technologies was utilized, and the qPCR reactions were conducted on an Agilent Technologies Stratagene Mx3000P system with MxPro software. The expression levels of seven genes whose products are involved in the DNA double-strand break repair pathways (*RAD51*, *RAD51D*, *FANCD2*, *BRCA1*, *BRAC2*, *PALB2*, *PARP1*) were analyzed using TaqMan probes (Life Technologies CA, USA). The reference gene used was 18S RNA (Life Technologies, CA, USA). The qPCR cycling parameters were 95 °C for 10 min, 30 cycles of 95 °C for 15 s, and 60 °C for 60 s.

2.8. Preparation of Protein Extracts and Western Blot Analysis

Protein extraction was performed by washing the cell pellet with ice-cold PBS. Next, 1 mL of ice-cold RIPA lysis buffer (Sigma) and a protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA) were added and incubated on ice for 30 min. Then, 30 μg of cell lysates were resolved on 4–20% ExpressPluS PAGE Gel (GenScript, Piscataway, NJ, USA)

following concentration measurements. The eBlot Protein Transfer device (GenScript, Piscataway, NJ, USA) was used to transfer the proteins onto a PDVDF Transfer Membrane (Thermo Scientific, Rockford, IL, USA). The membranes were washed and incubated for 1 h with a secondary anti-mouse antibody conjugated with HRP (Cell Signaling Technology, Danvers, MA, USA). Pierce ECL Western blotting Substrate (Thermo Scientific, Rockford, IL, USA) and BioRad Universal Hood II with a Chemiluminescence System (BioRad, Hercules, CA, USA) were used to visualize the result. Antibodies: Recombinant Anti-FANCD2 antibody [EPR2302] (abcam—ab108928) 1/1000; Recombinant Anti-Rad51D antibody [EPR16205] (abcam—ab202063) 1/1000; Recombinant Anti-β Actin antibody [EPR21241] (abcam—ab213262) 1 μg/mL; Recombinant Anti-Rad51D antibody [EPR16205] (ab202063) 1/1000.

2.9. Neutral Comet Assay

A comet assay was performed according to the protocol used in the previous research [5] on cells cultured for 48 h with either drugs or the vehicle and 168 h of pretreatment with VPA. Fifty comet images were randomly selected for each treatment variant and the percentage of DNA in the tail (% tail DNA) was measured. The mean value for this parameter was taken as an index of DSBs in the given sample.

2.10. In Vivo Experiments

NOD SCID γ (NSG) mice (10- to 12-week-old males and females) were housed in a sterile environment and allowed free access to food and water. The studies with animal experiments were approved by the local Ethical Committee and performed according to Polish federal law and guidelines for the protection of animals. DMBC11 human melanoma xenografts were initiated by injecting (under the right scapula) 2×10^6 cells previously suspended in Matrigel. Three animals were assigned to the eight different groups. The groups that received valproic acid pretreatment were treated as follow. Six days before the BMN-673 and DTIC injection, the mice were injected intraperitoneally once daily with 500 mg/kg VPA (diluted in PBS). Following pretreatment with VPA, the mice were treated with BMN-673 (35 mg/kg bodyweight, diluted in PBS), DTIC (8 mg/kg bodyweight every second day, diluted in PBS) or BMN-673 with DTIC (same dosing as monotherapy) for 24 days. At the end of the experiment, tumors were collected and weighed.

2.11. Statistical Analysis

The data were accessed in three independent experiments and presented as the mean ± SD. The student's *t*-test was performed between the various treatment regimens using Prism 7 (GraphPad Software, La Jolla, CA, USA). Differences were considered statistically significant where $p < 0.05$. The synergistic effect of drugs was studied using the response additivity approach.

3. Results

3.1. VPA Increase the Cytotoxic Effect Induced by BMN-673 and DTIC

To assess how the tested compounds affected the number of viable cells, we used trypan blue to measure plasma membrane integrity (Figure 1A). Combining VPA with DTIC and/or BMN-673 resulted in a synergistic reduction in DMBC11 cell survival, while the normal human epidermal melanocytes (NHEMs) were not affected by the treatments. To evaluate the impact of the compounds on cell proliferation, we used a clonogenic assay to obtain distinct colonies. When used alone, DTIC and BMN-673 reduced the number of colonies (Figure 1B). However, when the drugs were used in combination with VPA, this resulted in increased clonogenic inhibition efficiency. Treatment with a combination of VPA, BMN-367 and DTIC led to more significant changes (Figure 1C) than those observed with single drug treatments. The number of apoptotic cells significantly increased after treatment with the VPA, BMN-673, and DTIC combination compared to what was reported for monotherapy.

3.2. VPA, DTIC and BMN673, Used Alone or in Combination, Increase the Number of DSBs in Melanoma Cells

In normal melanocytes, treatment did not alter the level of γ-H2AX, which marks DSBs. However, the DMBC11 cells exhibited increased levels of γ-H2AX compared to melanocytes (Figure 2A). Additionally, the combined treatment with VPA approximately doubled the level of phosphorylated γ-H2AX in the DMBC11 cells compared to treatment with either drug alone.

The neutral comet assay was used to measure the ability of VPA and/or DTIC and BMN673 to induce DSBs. DMBC11 cells treated with individual drugs exhibited an increased intensity of DNA tails compared to melanocytes, indicating the accumulation of DSBs (Figure 2B). Moreover, the combination of VPA, DTIC, and BMN673 caused more DSBs than individual drugs.

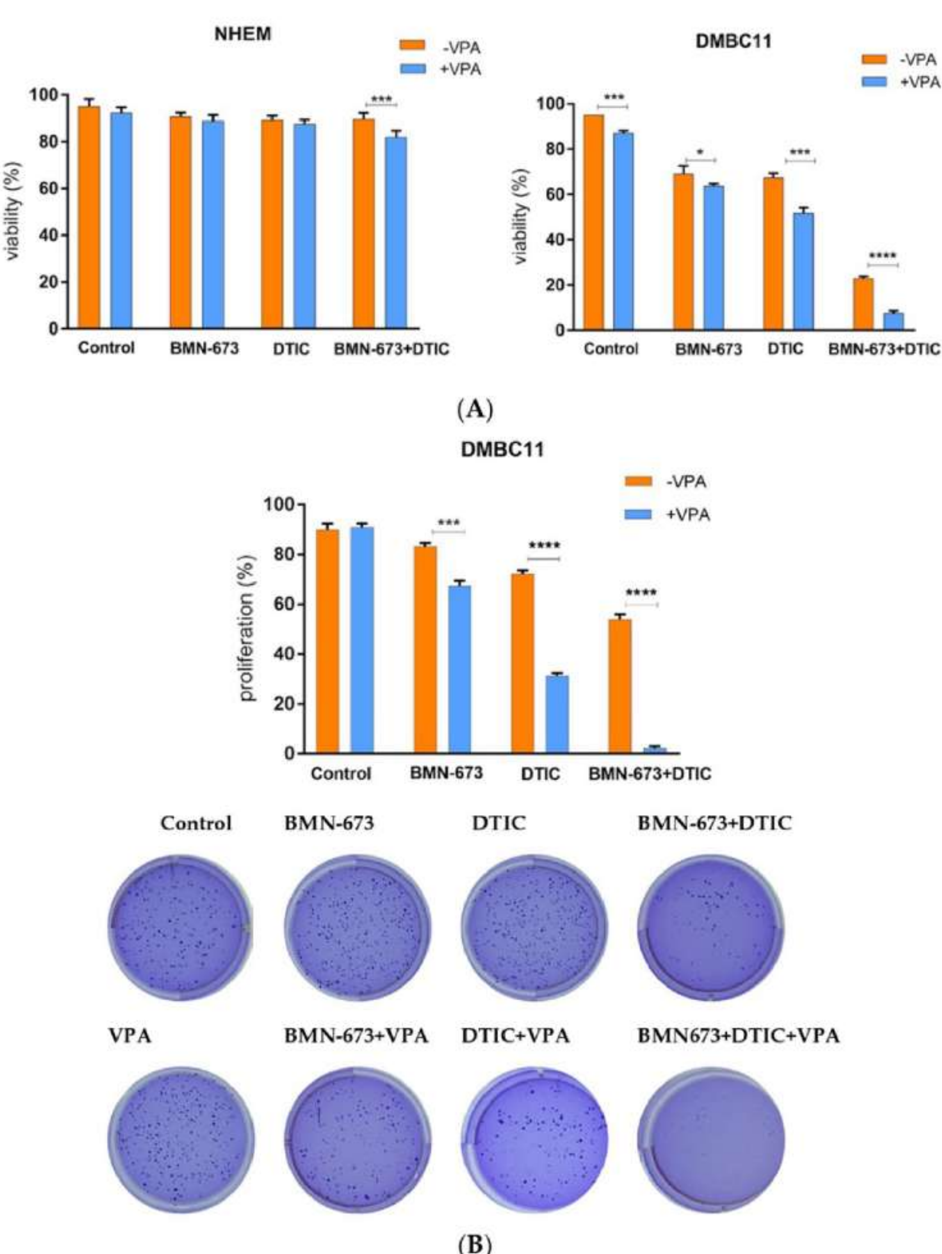

Figure 1. *Cont.*

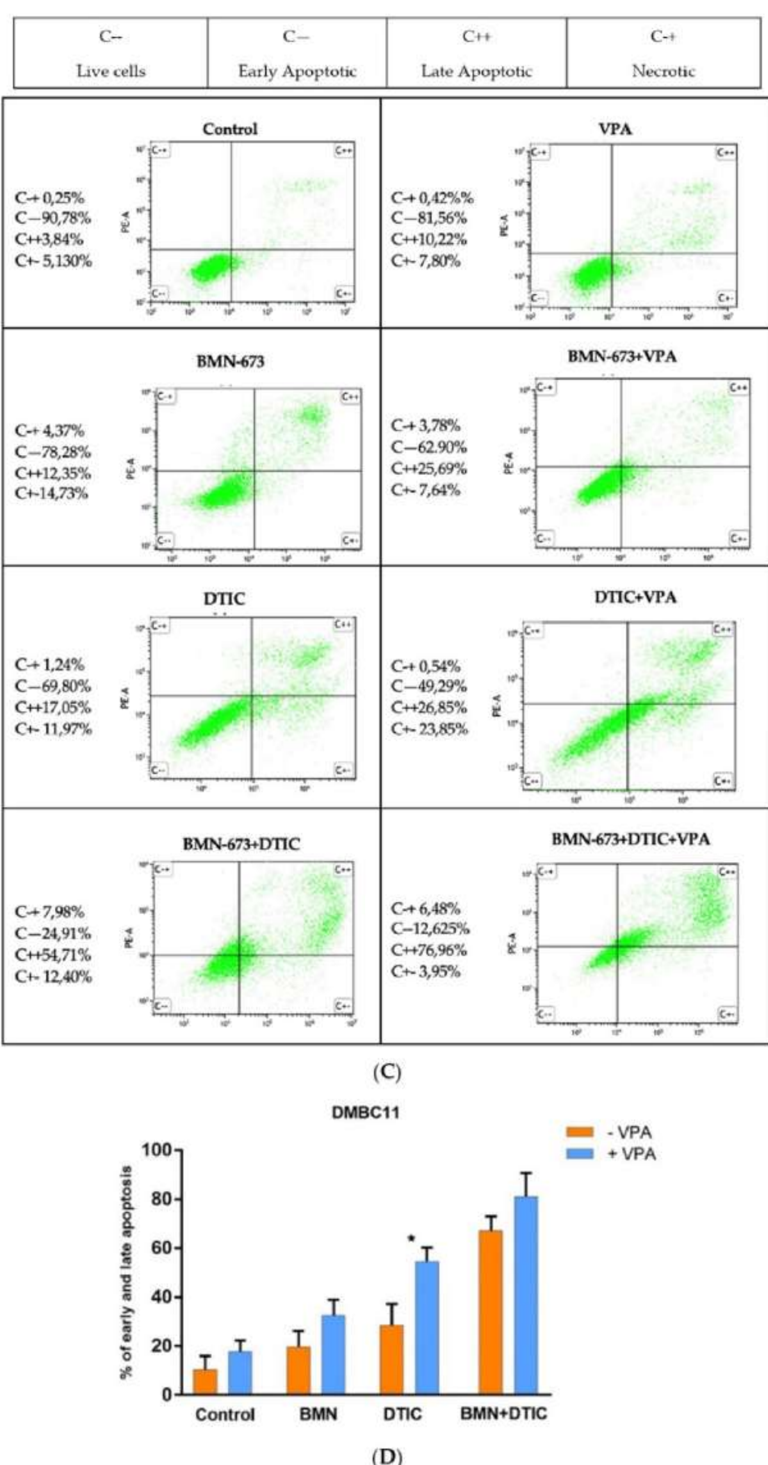

Figure 1. VPA increases the cytotoxic effect of the DTIC alkylating agent and BMN-673 PARP1 inhibitor. DMBC11 cells were pretreated with VPA (1 mmol/L) for 168 h and then treated or not with BMN-673 (10 μM) and DTIC (2 mM). (**A**) Cell viability was evaluated with a trypan blue assay after 72 h of treatment. (**B**) Cell proliferation was evaluated with a clonogenic assay after 72 h of treatment. (**C**) Representative histograms of the apoptosis/necrosis analysis of melanoma cells after 24 h of treatment with DTIC and BMN-673 (and pretreatment with VPA). (**D**) The percentage of DMBC11 melanoma cells in early and late apoptosis. At least three independent experiments were performed, and the results are shown as the mean ± standard deviation (SD). * $p \leq 0.05$, *** $p \leq 0.001$, **** p-value ≤ 0.0001 compared with the control group.

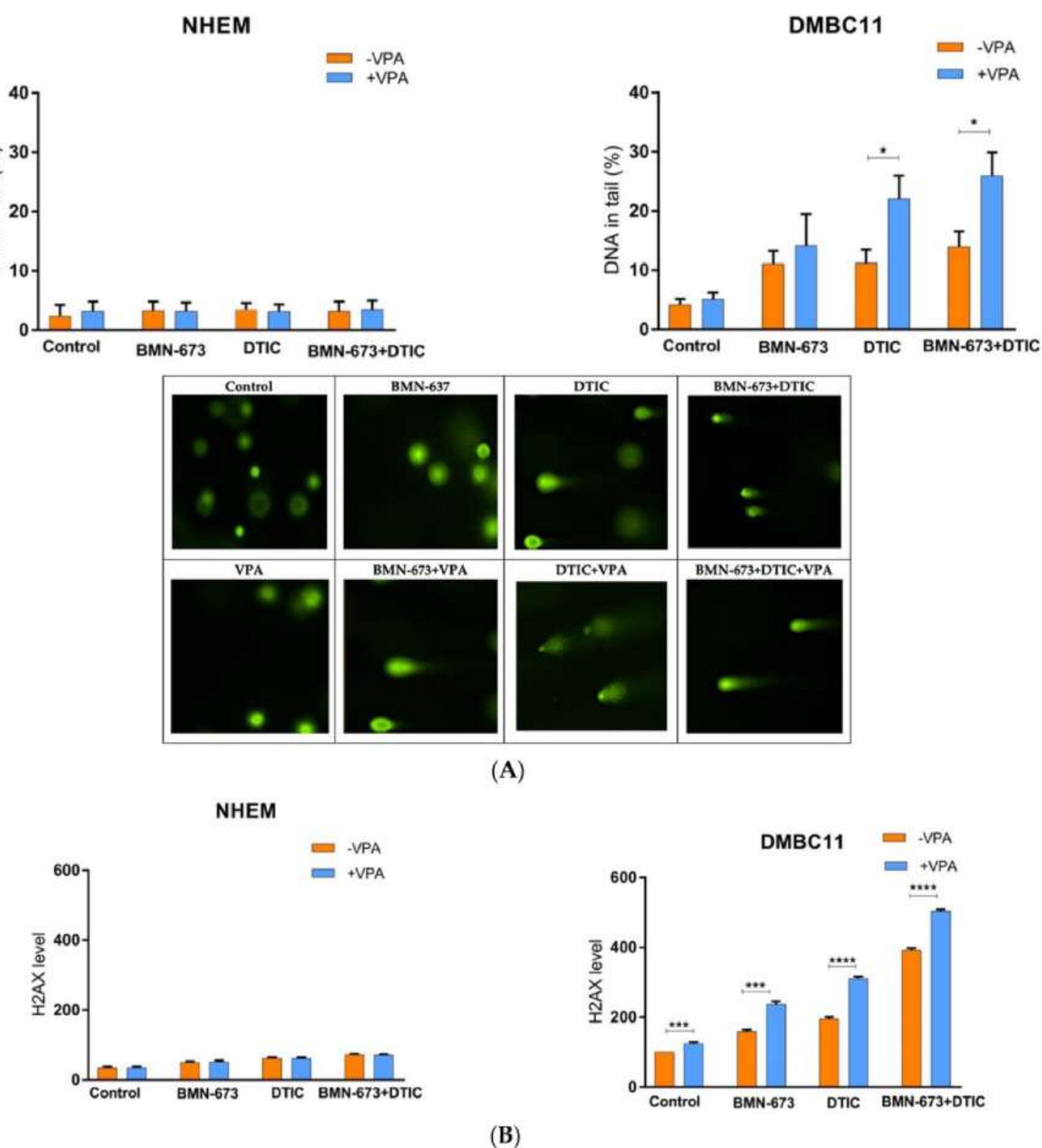

Figure 2. BMN673 and/or DTIC, VPA induced DSBs in melanoma cell lines (DMBC11). Cells were treated with 80 nM BMN-673 and/or 2 mM DTIC and VPA for 48 h (comet assay) and 120 hrs (γ-H2AX). (**A**) The mean percentage $\pm$ SD of DNA in the tails of comets in neutral conditions acquired from the 50 cells/group from 3 experiments. The characteristic tails of comets with damaged DNA are shown under a fluorescence microscope after electrophoresis and 4′,6-diamidino-2-phenylindole (DAPI) gel staining. (**B**) The mean values $\pm$ SD of γ-H2AX were calculated from 3 experiments performed in triplicate. The characteristic tails of comets with damaged DNA are shown under a fluorescence microscope after electrophoresis and 4′,6-diamidino-2-phenylindole (DAPI) gel staining. * p-value ≤ 0.05; *** p-value ≤ 0.001; **** p-value ≤ 0.0001 in comparison with the control.

3.3. Inhibition of HDAC1 Downregulates FANCD2 and RAD51

To investigate the mechanism by which class I HDAC inhibition enhances sensitivity to dacarbazine and talazoparib, we conducted a real-time PCR array to monitor changes in mRNA expression in DMBC11 melanoma cells following the inhibition of class I HDACs using VPA (Figure 3A). The subjects of our interest were seven genes involved in the double

strand break repair pathway (RAD51, RAD51D, FANCD2, BRCA1, BRCA2, PALB2, PARP1). We observed changes in the mRNA expression profile of RAD51, RAD51D, and FANCD2.

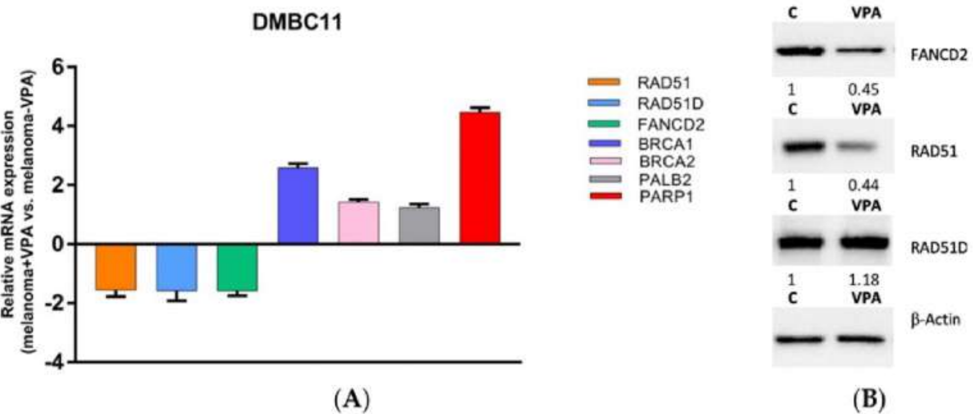

Figure 3. VPA and downregulated RAD51 and FANCD2 in melanoma cells. DMBC11 cells were pretreated with VPA (10 mM) for 178 h. (**A**) Real-time PCR results showing the down and upregulation of the listed genes. (**B**) Western blot analysis of the RAD51, RAD51D and FANCD2 protein levels. β-Actin served as a loading control and the relative expression (R.E.) levels are indicated.

We then confirmed whether the changes in gene expression were reflected at the protein level by analyzing the influence of VPA on the RAD51, FANCD2, and RAD51D protein levels (Figure 3B). The results showed that only the RAD51 and FANCD2 protein levels decreased following HDAC inhibition.

3.4. VPA in Combination with BMN673 and DTIC Reduces Melanoma Growth in NSG Mice

In NSG mice, suboptimal doses of BMN673, VPA, and DTIC did not show any significant reduction in the growth of DMBC11 cells (Figure 4). However, a modest but statistically significant anti-melanoma effect was observed when BMN673 was combined with DTIC, and when BMN673, DTIC, and VPA were used together. It is worth noting that a more potent effect could potentially be achieved through optimization of the treatment protocol.

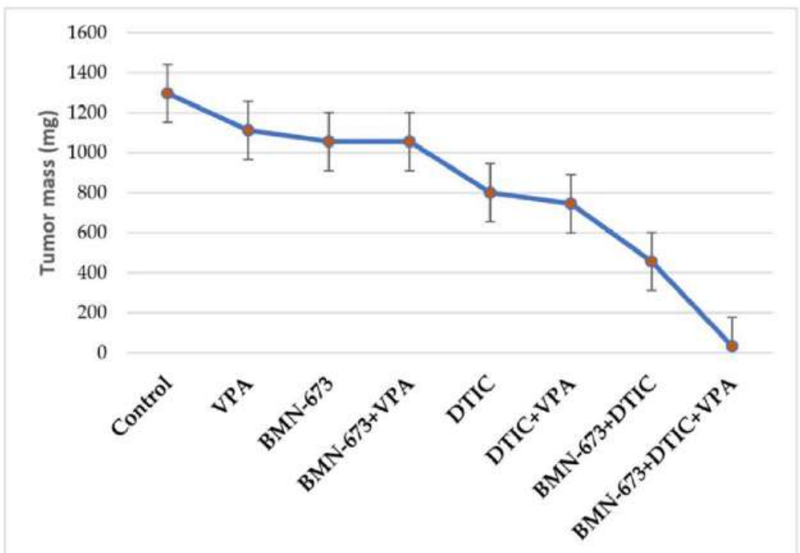

Figure 4. Combination of VPA, DTIC and BMN673 reduced the growth of human melanoma in immunodeficient mice. NAG mice were injected s.c. with DMBC11 melanoma cells followed by treatment with BMN673 (35 mg/kg twice a day), DTIC (8 mg/kg every second day), or BMN673 + DTIC. Data represent the mean ± SD of the tumor mass from 2 independent experiments.

4. Discussion

Recent years have seen significant progress in the development and expanded application of PARP and HDAC inhibitors due to a growing interest in their potential for cancer therapy [13]. Metastatic melanomas are resistant to therapy for a variety of reasons, including their ability to bypass cell-cycle checkpoints [14], express insufficient amounts of critical apoptosis proteins [15], maintain p53 wild-type status, which allows them to upregulate DNA repair genes (DDB2, XPC) [16], and express an oncogenic form of BRAF that gives them an advantage in growth [17]. This may be the reason why melanoma patients have such a poor prognosis.

Before examining whether class I HDACs play a role in melanomas' susceptibility to an alkylating agent and PARP inhibitor, we demonstrated that these HDACs can be suppressed. In order to achieve this, the class I HDAC inhibitor VPA [15], which was utilized, was proven to be functional in melanoma cells. Having established the experimental system, we demonstrated that the inhibition of class I HDACs sensitized melanoma cells to apoptosis induced by dacarbazine. Furthermore, a panel of cell lines in vitro and a melanoma xenograft model in vivo corroborated the protective effect of class I HDAC activity in melanomas.

HDACs have a crucial role in regulating proteins and gene expression. As a result, HDACs can potentially influence the expression of any gene, necessitating the use of screening methodologies to identify the underlying factor responsible for the observed phenotype. This is accomplished by modifying the acetylation of both histones and non-histone molecules [18,19].

In this study, we have identified the combination of an HDAC inhibitor, PARP inhibitor and alkylating agent as a potentially effective therapeutic approach against melanoma cells. Specifically, we found that VPA enhanced the cytotoxicity of DTIC and BMN-673. This increase in cytotoxicity was associated with a stronger induction of DNA damage in cells treated with the HDACi/BMN-673/DTIC combination, likely due to the downregulation of FANCD2 and RAD51 by VPA. The observed stronger cytotoxicity of the combination of an HDACi, PARPi, and alkylating agent may be attributed to the fact that one of the mechanisms underlying tumor resistance to PARP inhibitors is the activation of the HR DNA repair pathway. Romeo et al. obtained similar results, where the HDAC inhibitor VPA, and trichostatin A (TSA), disrupted the interplay between mutp53 and HSP70, resulting in the downregulation of CHK1 and RAD51, sensitizing pancreatic cancer cells to the AZD2461 PARP inhibitor [20].

In this study, a real-time PCR was utilized to investigate the effects of VPA on selected genes' expression, which revealed that VPA impacts genes involved in DNA repair (especially HR). Based on this finding, it is inferred that VPA sensitizes melanoma cells to dacarbazine and talazoparib by either improving apoptosis fidelity or reducing DNA repair effectiveness. To confirm these hypotheses, changes in gene expression were validated using Western blot analysis, revealing RAD51 and FANCD2 as the two proteins affected by VPA. By using an HDAC inhibitor to target specific HDACs, HDAC2 was identified as the class I HDAC responsible for the regulation of RAD51 [1]. Since RAD51 and FANCD2 are critical components of HR, downregulation of these proteins could be responsible for the observed sensitization. Krumm et al. obtained similar results by using various HDAC inhibitors and siRNA to target specific HDACs, identifying class I HDAC as being responsible for regulating RAD51. The role of DNA repair in the observed sensitization to temozolomide was further supported by the results obtained in melanoma cells exposed to fotemustine (alkylating agent) and ionizing radiation (IR) after the inhibition of class I HDAC. Although temozolomide, fotemustine, and IR induce different types of DNA damage, only HR is involved in the repair of all these genotoxins. Our observation of the cells treated with the monotherapy and polytherapy was consistent with previously described studies [1,5,21,22].

The findings presented here point to a scenario in which HDACs, via enhancing the HR-dependent repair of DSBs created during the processing of DNA lesions, are essential

nodes in the resistance of malignant melanoma cells to methylating agent-based therapies. This provides a solid foundation for the targeting of these HDACs during genotoxic agent-based therapy for malignant melanoma and, perhaps, other tumor types as well.

5. Conclusions

This study suggests that VPA synergizes with the PARP inhibitor and alkylating agent to reduce the survival of melanoma cells, due to stronger DNA damage being induced by this combination treatment, while not affecting melanocytes.

Interestingly, the interconnection between the effects induced by an HDACi has never been highlighted before in cancer cells. The findings of this study encourage the use of an HDACi, especially one that inhibits class I HDACs, in combination with a PARP inhibitor in the treatment of melanoma cancer.

Author Contributions: M.D. wrote the manuscript, performed the cell culturing, Western blot, comet assay and phosphorylated histone H2AX experiments, flow cytometry analysis, analyzed the data; A.G.-M. performed the cell culturing; D.J. performed the drug treatment; P.S. and G.B.-P. performed the clonogenic assay; G.H. performed the experiments with mice; J.P., M.R., P.C., M.C., T.Ś. and T.S. conceived the project, designed the experiments, analyzed the data, wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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15.Oświadczenia współautorów w publikacji

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Oświadczam, że w pracy

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mój udział polegał na opracowaniu koncepcji publikacji, tworzeniu manuskryptu oraz wizualizacji. Swój udział w artykule oceniam na 3%.

Gabriela Barszczewska-Pietraszek

mgr inż. Gabriela Barszczewska-Pietraszek

Katedra Genetyki Molekularnej

Uniwersytet Łódzki

ul. Pomorska 141/143

19.06.2023

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy

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. 2023; 14(6):1295.

mój udział wynosił 3% i obejmował współudział w przeprowadzeniu eksperymentów.

Gabriela Barszczewska-Pietraszek

dr Piotr Czarny

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Uniwersytet Medyczny w Łodzi

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

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

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Tomasz Skorski, MD, PhD, DSc

mgr Małgorzata Drzewiecka

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ul. Pomorska 141/143

90-236 Łódź

19.06.2023

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mój udział polegał na prowadzeniu hodowli komórkowych linii glejaków, przeprowadzeniu analizy RealTime PCR oraz Western Blot, analizie danych cytometrycznych, analizie danych. Swój udział w artykule oceniam na 66%

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

Małgorzata Drzewiecka