WYDZIAŁ BIOLOGII
i OCHRONY ŚRODOWISKA
Uniwersytet Łódzki

mgr Monika Toma

Stacjonarne Studia Doktoranckie Genetyki Molekularnej, Cytogenetyki i Biofizyki Medycznej

Syntetyczna letalność w komórkach nowotworowych indukowana inhibitorami białek naprawy pęknięć DNA

Synthetic lethality of cancer cells induced by inhibitors of proteins participating in DNA repair

Praca doktorska wykonana
w Katedrze Genetyki Molekularnej
pod kierunkiem

- Prof. dr. hab. Tomasza Śliwińskiego

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Podziękowania

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Spis treści

Informacje wprowadzające

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Dorobek naukowy

Artykuły naukowe wchodzące w skład rozprawy doktorskiej

Poniższa rozprawa doktorska opiera się na pięciu artykułach opublikowanych w recenzowanych czasopismach: trzy publikacje przeglądowe i dwie doświadczalne.

Publikacje przeglądowe

1. **Toma M**, Skorski T, Śliwiński T. Syntetyczna letalność jako funkcjonalne narzędzie w badaniach podstawowych oraz w terapii przeciwnowotworowej. Postępy Higieny i Medycyny Doświadczalnej. 2014; 68: 1091-1103
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2. **Toma M**, Skorski T, Śliwiński T. DNA Double Strand Break Repair - Related Synthetic Lethality. Current Medicinal Chemistry. 2019; 26(8): 1446-1482(37)
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3. **Toma M**, Sullivan-Reed K, Skorski T, Śliwiński T. RAD52 as a Potential Target for Synthetic Lethality-Based Anticancer Therapies. Cancers 2019; 11, 1561
   140 pkt MNiSW (Według punktacji MNiSW z dnia 31 lipca 2019)**, IF = 6.162**

Publikacje doświadczalne

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   35 pkt MNiSW, IF= 5.168, IF 5-letni = 5.312

2. **Toma M**, Witusik-Perkowska M, Szwed M, Stawski R, Szemraj J, Drzewiecka M, Nieborowska-Skorska M, Radek M, Kolasa M, Matlawska-Wasowska K, Śliwiński T, Skorski

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35 pkt MNiSW

Suma: 265 pkt MNiSW (125 pkt + 140 pkt za publikacje punktowane według listy z dnia 31 lipca 2019)**, IF = 15.797, 5-letni IF = 10.031**

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   25 pkt MNiSW, IF = 1.491, 5-letni IF = 2.059

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19. Skała E, **Toma M**, Kowalczyk T, Śliwiński T, Sitarek P. *Rhaponticum carthamoides* transformed root extract inhibits human glioma cells viability, induces double strand DNA damage, H2A.X phosphorylation, and PARP1 cleavage. Cytotechnology. 2018; 70(6): 1585-1594
20 pkt MNiSW, IF = 1.672, 5-letni IF = 1.891

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100 pkt MNiSW (Według punktacji MNiSW z dnia 31 lipca 2019)

23. Sarnik J, Gajek A, **Toma M**, Pawełczyk J, Rykowski S, Olejniczak A, Śliwiński T, Bielski R, Witczak ZJ, Popławski T. (1-4)-Thiodisaccharides as anticancer agents. Part 5. Evaluation

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70 pkt MNiSW (Według punktacji MNiSW z dnia 31 lipca 2019)**, IF = 2.448, 5-letni IF = 2.352**

24. Sitarek P, **Toma M**, Ntungwe E, Kowalczyk T, Skała E, Wieczfinska J, Śliwiński T, Rijo P. Biological activities of selected abietane diterpenes 3 isolated from *Plectranthus* spp. Biomolecules. 2020; Zaakceptowany do druku
100 pkt MNiSW (Według punktacji MNiSW z dnia 31 lipca 2019)**, IF = 4.694**

Sumaryczny dorobek naukowy: 1085 pkt MNiSW (675 pkt + 410 pkt za publikacje punktowane według listy z dnia 31 lipca 2019)**, IF = 97.734, 5-letni IF = 85.595**

Komunikaty zjazdowe

1. **Toma M**, Gajos-Michniewicz A, Majchrzak K, Wieteska Ł, Szemraj J, Czyż M, Skorski T, Śliwiński T. Personalized synthetic lethality induced by targeting RAD52 and PARP1 in melanomas. 9th International Conference of Anticancer Research. Sithonia, Greece, 6-10 października 2014

2. **Toma M**, Gajos-Michniewicz A, Majchrzak K, Wieteska Ł, Szemraj J, Czyż M, Skorski T, Śliwiński T. Syntetyczna letalność komórek czerniaka wywołana inhibicją białek RAD52 i PARP1. IV Konferencja Biologii Molekularnej. Łódź, Polska 26-28 marca 2015

3. Czarny P, Kwiatkowski D, Korycińska A, **Toma M**, Gałecki P, Śliwiński T. Związek pomiędzy polimorfizmem pojedynczego nukleotydu Ligazy III a depresją. BIOOPEN 2015, Łódź, Polska 20–22 kwietnia 2015

4. Kwiatkowski D, Czarny P, **Toma M**, Sowińska K, Gałecki P, Śliwiński T. Polimorfizm genu XRCC1 a ryzyko wystąpienia choroby Alzheimera. BIOOPEN 2015, Łódź, Polska 20–22 kwietnia 2015

5. Kwiatkowski D, **Toma M**, Korycińska A, Czarny P, Gałecki P, Bachurska A, Talarowska M, Orzechowska A, Śliwińska A, Szemraj J, Śliwiński T. Levels of 8-OxoG Oxidative DNA Lesion and Expression of 8-Oxoguanine DNA Glycosylase 1 (OGG1) in Peripheral Blood Lymphocytes as a Potential Diagnostic Biomarkers of Alzheimer's Disease. CNS DISEASES World Summit 2015. Philadelphia, PA, USA 9–11 września 2015

6. Kwiatkowski D, Czarny P, **Toma M**, Galecki P, Bachurska A, Talarowska M, Orzechowska A, Bobińska K , Śliwińska A, Szemraj J, Śliwiński T. Association between Alzheimer's disease risk and oxidative DNA damage level and/or polymorphisms of the base excision repair genes. Ibero-American Congress on Alzheimer's Disease 14–17 października, 2015, Rio de Janeiro, Brazil

7. Śliwiński T, Kwiatkowski D, Czarny P, **Toma M**, Gałecki P, Bachurska A, Szemraj J. Impaired efficiency of Base Excision Repair system in nuclear and mitochondrial extracts from peripheral blood lymphocytes of patients with Alzheimer's disease. International Stress and Behavior Society "Stress and Behavior" Neuroscience and Biopsychiatry Conference. Rodney Bay, St Lucia 16–18 stycznia, 2016

8. **Toma M**, Śledziński Ł, Śliwińska A, Kolasa M, Radek M, Jeziorski A, Szemraj J, Skorski T, Śliwiński T. Personalized synthetic lethality induced by targeting RAD52 and PARP1 in glioblastomas. Maintenance of Genome Stability. Playa Bonita, Panama 7-10 marca 2016

9. Skała E, Sitarek P, **Toma M**, Szemraj J, Radek M, Nieborowska-Skorska M, Skorski T, Wysokińska H, Śliwiński T. Inhibition of human glioma cell proliferation by altered BAX/BCL-2P53 expression and apoptosis induction by *Rahaponticum carthamoides* root extract. CIPAM 2016 - 6th International Congress of Aromatic and Medicinal Plants. Coimbra, Portugal 29 maja - 1 czerwca 2016

10. Skała E, **Toma M**, Kowalczyk T, Radek M, Wysokińska H, Śliwiński T, Sitarek P. Induction of apoptosis in human glioma cells in grades II and III through the ROS-mediated mitochondrial pathway and caspase activation by *Rhaponticum carthamoides* (Willd.) Iljin transformed root extract. 1st International Congress on Edible Medicinal and Aromatic Plants (ICEMAP 2017). Pisa, Italy 28-30 czerwca 2017

11. Sarnik J, **Toma M**, Czubatka-Bieńkowska A, Macieja A, Śliwiński T, Witczak Z, Bielski R, Popławski T. (1-4)-S-thiodisaccharides: Potential anticancer drug candidates against human malignant gliomas. 255th National Meeting and Exposition of the American-Chemical-Society (ACS) - Nexus of Food, Energy, and Water. New Orleans, LA, USA 18-22 marca 2018

12. Kowalczyk T, Sitarek P, Skała E, **Toma M**, Wielanek M, Pytel M, Śliwiński T. *Menyanthes trifoliata* L *in vitro* and *in vivo* extracts induce apoptosis in human glioma cells. 4th International Mediterranean Symposium on Medicinal and Aromatic Plants. Antalya, Turkey 18-22 kwietnia 2018

13. Skała E, **Toma M**, Kowalczyk T, Śliwiński T, Sitarek P. *Rhaponticum carthamoides* transformed root extract inhibits human glioma cells viability, induces double strand DNA damage, H2A.X phosphorylation, and PARP1 cleavage. 2nd World Congress on Pharmaceutical and Chemical Sciences. Bologna, Italy 23-25 lipca 2018

14. Sitarek P, Kowalczyk T, Santangelo S, Białas AJ, **Toma M**, Kuźma Ł, Wieczfińska J, Śliwiński T. An extract derived from *Leonorus sibiricus in vitro* transgenic roots with AtPAP1 transcriptional factor induces apoptosis via DNA damage in human cancer cells. 2nd

World Congress on Pharmaceutical and Chemical Sciences. Bologna, Italy 23-25 lipca 2018

15. Nieborowska-Skorska M, Podszywałow-Bartnicka P, Maifrede S, Viet Le B, **Toma M**, Valent P, Śliwiński T, Childers W, Piwocka K, Tulin A, Skorski T. PARP1 Inhibitors Eliminated Imatinib-Refractory Chronic Myeloid Leukemia Cells in Bone Marrow Microenvironment Conditions. 60th American Society of Hematology (ASH) Annual Meeting. San Diego, CA, USA 1-4 grudnia 2018

16. Sitarek P, Kowalczyk T, Wielanek M, **Toma M**, Śliwiński T, Skała E. Anticancer effect of *Leonotis nepetifolia* (L.) R.BR. transformed roots and their chemical profile. The 13th World Congress on Polyphenols Applications. Malta, 30 września - 1 października 2019

17. Górski K, Kowalczyk T, Skała E, **Toma M**, Wieczfińska J, Sitarek P. A molecular analysis of the transformed roots of *Leonotis nepetifolia* (L.) R. BR. for the presence of a transgender encoding the gene of DXP reductoisomerase (DXR) and the biological properties of the obtained extracts. I Bioactive Natural Products Research Meeting 2019, Universidade Lusófona/CBIOS-Research Center for Biosciences & Health Technologies, Lisbon, Portugal, 27-28 września 2019

Staże naukowe

1. 02.01.2019 - 03.07.2019 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 ETIUDA6 (UMO-2018/28/ T/NZ7/00105) przyznanego przez Narodowego Centrum Nauki. Temat: Syntetyczna letalność w komórkach nowotworowych indukowana inhibitoram białek naprawy pęknięć DNA. Opiekun: Tomasz Skorski, MD, PhD, DSc

2. 08.01.2018 - 08.04.2018 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 "Grants (Exchange program to the United States)" przyznanego przez The Kosciuszko Foundation. Temat: Induction of PARP1 sensitivity in FLT3(ITD)- positive cells by tyrosine kinase inhibition. Opiekun: Tomasz Skorski, MD, PhD, DSc

3. 01.07.2016 - 01.08.2016 Faculty of Chemistry, **University of Belgrade, Belgrade, Serbia**. Temat: Investigation of thermal stability of major peanut allergens by Molecular Dynamic Simulations and Circular Dichroism. Opiekun: Tanja Cirkovic-Velickovic, PhD

4. 01.08.2014 - 31.08.2014 Institute of Molecular Genetics and Genetic Engineering, Faculty of Biology, **University of Belgrade, Belgrade, Serbia**. Temat: Analysis of molecular pharmacogenetic markers of rare diseases. Opiekun: Sonja Pavlovic, PhD

Streszczenie

Wstęp

Jedną z cech charakterystycznych nowotworów jest ich genetyczna niestabilność, która prowadzi do podwyższonego tempa powstawania mutacji w ich genomach [1]. Z tego też powodu istnieje względnie wysokie ryzyko zajścia mutacji i utraty funkcji w genach, których produkty biorą udział w szlakach kluczowych dla przeżycia komórki - np. mechanizmach naprawy DNA. W takich warunkach przeżycie komórki nowotworowej zostaje uzależnione od znalezienia substytutu utraconego mechanizmu i aktywacji szlaku alternatywnego [2]. Jeśli inaktywujące mutacje powstające w określonych parach genów prowadzą do śmierci komórek, natomiast inaktywacja każdego z nich indywidualnie nie wpływa na przeżycie komórek, mówimy wówczas, że wykazują one interakcje syntetycznej letalności [3]. Zastosowanie inhibitorów lub aptamerów białek w celu zablokowania szlaku alternatywnego stało się w ostatnich latach przedmiotem spersonalizowanej terapii przeciwnowotworowej opartej o syntetyczną letalność. Takie podejście może nie tylko okazać się selektywnym i skutecznym rozwiązaniem w spersonalizowanej terapii przeciwnowotworowej, ale przyczynia się już obecnie do poszerzania wiedzy dotyczącej interakcji genetycznych zachodzących w komórkach [4].

Powstawanie pęknięć dwuniciowych DNA (DSB - ang. *double strand breaks*) w komórkach może być spowodowane ekspozycją na promieniowanie jonizujące, reaktywne formy tlenu czy stres genotoksyczny wywołany np. chemoterapeutykami [5]. Nienaprawione DSB mogą prowadzić do powstawania rearanżacji genetycznych i progresji nowotworu. Przypuszcza się, że mutacje genów biorących udział w naprawie tego rodzaju uszkodzeń oraz aktywacja alternatywnych systemów naprawy może być źródłem oporności komórek nowotworowych na stres genotoksyczny [6]. W komórkach prawidłowych naprawa DSB przebiega poprzez szlak łączenia końców niehomologicznych (NHEJ) w fazie G1/S cyklu komórkowego lub poprzez mechanizm naprawy przez homologiczną rekombinację (HR) w późnej fazie S i G2. Dodatkowo można wyodrębnić dwa podsystemy - podstawowy i alternatywny zarówno dla NHEJ, jak i dla HR. Szlak podstawowy NHEJ - cNHEJ zależny jest od białka DNA-PKcs, natomiast, jeśli nie działa on prawidłowo, jego funkcje są przekierowywane do szlaku altNHEJ opartego na białku PARP1 [7]. Poza naprawą DSB PARP1 bierze udział również w naprawie pęknięć jednoniciowych DNA, a także indukuje HR na zatrzymanych

widełkach replikacyjnych [8]. Naprawa poprzez HR zachodzi przy współdziałaniu białek BRCA1/2 oraz RAD51, jednak zauważono, że w komórkach z mutacjami w genach *BRCA1/2* może dochodzić do aktywacji szlaku alternatywnego, w którym zostają one zastąpione białkiem RAD52 [6]. Mutacje utraty funkcji w genach szlaków podstawowych mogą stwarzać warunki do selektywnej eliminacji komórek nowotworowych wykorzystując inhibitory szlaków alternatywnych. Na obecną chwilę jedynie inhibitory PARP1 znalazły zastosowanie w spersonalizowanej terapii opartej o syntetyczną letalność. Inhibicja PARP1 w komórkach z mutacjami *BRCA1/2* prowadzi do nieskutecznej naprawy uszkodzeń DNA w mechanizmach zależnych od PARP1 i inwersji uszkodzeń do DSB, które z kolei nie mogą zostać wydajnie naprawione przez szlak HR. Może to dalej prowadzić do akumulacji DSB, pogłębiania niestabilności genetycznej i śmierci komórki [9]. Pomimo, że to szlak NHEJ jest głównym szlakiem naprawy w komórkach eukariotycznych, to niektóre typy nowotworów wydają się znacznie bardziej polegać na HR, dlatego jego inaktywacja może wywierać silnie toksyczny wpływ na komórki rakowe [10].

W niniejszej pracy podjęto się poszukiwania kolejnych interakcji syntetycznej letalności w komórkach źle prognozujących guzów litych - glejaka i czerniaka. Pomimo, że czerniak na wczesnych etapach łatwo poddaje się leczeniu, to wyższe stopnie zaawansowania i przerzuty do innych organów zazwyczaj znacząco pogarszają medianę przeżycia poniżej 9 miesięcy [11]. Glejak wielopostaciowy jest natomiast jednym z najczęstszych nowotworów centralnego układu nerwowego, jednak nawet pomimo wykorzystania agresywnych strategii terapeutycznych wykazuje bardzo niski współczynnik przeżywalności (mediana 14.6 miesięcy) [12]. Badania zakładały utworzenie dla każdego badanego przypadku profilu ekspresji genów kluczowych dla naprawy DSB i identyfikację “słabych punktów” naprawy w nowotworze, które mogłyby zostać wykorzystane do stworzenia spersonalizowanego do każdego przypadku zestawu związków celujących w alternatywne szlaki naprawy DSB.

Cel pracy

Celem niniejszej pracy było badanie odpowiedzi linii pierwotnych glejaka i czerniaka wykazujących obniżoną ekspresję białek biorących udział w naprawie DSB na zastosowanie inhibitorów alternatywnych szlaków naprawy. Cel ten osiągnięto poprzez:

1. Wyprowadzanie linii pierwotnych źle prognozujących guzów litych od pacjentów oraz potwierdzanie charakterystyki otrzymanej linii komórkowej poprzez zastosowanie markerów powierzchniowych i genetycznych;

2. Określenie poziomu ekspresji genów, których produkty biorą udział w naprawie DSB (*BRCA1*, *BRCA2*, *PALB2*, homologi *RAD51*, *RAD52* - HR; *DNA-PKcs*, *XRCC5*, *XRCC6*, *LIG4* - cNHEJ; *PARP1*, *LIG3* - altNHEJ) i odniesienie ich poziomu do ekspresji w komórkach prawidłowych, co pozwoli na identyfikację "słabych punktów" w systemach naprawy DSB w komórkach nowotworowych i dobranie inhibitorów, które mogłyby doprowadzić do zajścia syntetycznej letalności;

3. Ocena aspektów odpowiedzi komórek nowotworowych i prawidłowych po zastosowaniu dobranych inhibitorów zastosowanych samodzielnie lub w kombinacji ze związkami alkilującymi stosowanymi obecnie w terapii wybranych do badań typów nowotworów.

Przeprowadzone badania pozwolą na poszukiwanie korelacji pomiędzy profilem ekspresji genów kodujących białka DSB, a wrażliwością na podejście oparte o syntetyczną letalność w komórkach nowotworowych.

Materiały i metody

Materiał do badań stanowiły linie pierwotne czerniaka i glejaka. Wycinki czerniaka sklasyfikowane histopatologicznie jako stadia kliniczne III i IV zostały pobrane od pacjentów Oddziału Chirurgii Onkologicznej Wojewódzkiego Szpitala Specjalistycznego im. M. Kopernika w Łodzi. Komórki czerniaka DMBC 2, DMBC 8, DMBC 10, DMBC 11, DMBC 12 zostały wyizolowane z wycinków w Zakładzie Biologii Molekularnej Nowotworów, Uniwersytetu Medycznego w Łodzi, a obecność komórek nowotworowych w hodowlach została potwierdzona poprzez analizę cytometryczną markerów powierzchniowych charakterystycznych dla komórek czerniaka oraz dla komórek macierzystych nowotworowych [13]. Otrzymane przez zespół linie pierwotne czerniaka zostały nam udostępnione do dalszych badań. Komórki były hodowane w formie trójwymiarowych melanosfer, które w porównaniu do systemu hodowli "*monolayer*", pozwalają na lepsze odwzorowanie heterogenności populacji komórek guza oraz jego trójwymiarowej struktury.

Wycinki glejaka wielopostaciowego sklasyfikowane histopatologicznie jako stadia kliniczne III i IV pobrano od pacjentów Kliniki Neurochirurgii i Chirurgii Nerwów Obwodowych, Uniwersyteckiego Szpitala Klinicznego im. Wojskowej Akademii Medycznej oraz Oddziału Neurochirurgii i Nowotworów Układu Nerwowego, Wojewódzkiego Szpitala Specjalistycznego im. M. Kopernika w Łodzi. Badania uzyskały zgodę Komisji Bioetycznej Uniwersytetu Medycznego w Łodzi (nr zgody RNN/194/12/KE). Komórki glejaka H3, H6 i H7 zostały wyizolowane z wycinków w Katedrze Genetyki Molekularnej Uniwersytetu Łódzkiego. Linie komórkowe zostały poddane sortowaniu z użyciem kulek magnetycznych MACS (ang. *magnetic-activated cell sorting*) wykrywających antygen powierzchniowy CD133, który jest markerem komórek nowotworowych macierzystych. Dodatkowo w celu potwierdzenia obecności komórek glejaka w otrzymanej hodowli przeprowadzona została analiza utraty heterozygotyczności (LOH - ang. *Loss of heterozygosity*) w locus chromosomowych 10q23-24, 10p14 i 22q12.3, których delecje uznawane są za jedne z najczęściej występujących zmian w komórkach glejaków. LOH zostały porównane między próbkami krwi obwodowej pobranej od pacjentów, a pobranymi od nich wycinkami guzów i wyprowadzonymi z nich liniami. LOH10q został wykryty we wszystkich badanych liniach, pokazując ok. 50% spadek w wycinku guza i 65-70% spadek w wyprowadzonej linii komórkowej w porównaniu do wartości w krwi obwodowej, sugerując, że mutacja 10q charakterystyczna dla glejaka została rozpropagowana w hodowli. Kontrole do eksperymentów stanowiły komercyjnie dostępne linie prawidłowych melanocytów (NHEM - ang. *Normal Human Epidermal Melanocytes*) i astrocytów (NHA - ang. *Normal Human Astrocytes*).

Analizę ekspresji genów, których produkty są zaangażowane w naprawę DSB rozpoczęto od izolacji całkowitego RNA z każdej z badanych linii. W następnym etapie został on przepisany na cDNA z wykorzystaniem reakcji odwrotnej transkrypcji. Reakcja łańcuchowej reakcji polimerazy z detekcją w czasie rzeczywistym została przeprowadzona z sondami TaqMan wykrywającymi geny *BRCA1*, *BRCA2*, *LIG3*, *LIG4*, *PALB2*, *PARP1*, *PRKDC*, *RAD51B*, *RAD51C*, *RAD51D*, *XRCC2*, *XRCC3*, *RAD52*, *XRCC5*, *XRCC6*, których ekspresja została znormalizowana do genu referencyjnego *18S rRNA*. *Fold change* w ekspresji genów został obliczony w porównaniu do komórek prawidłowych NHEM (dla linii czerniaków) lub NHA (dla linii glejaków). Badanie Western Blot zostało wykonane dla wybranych z poprzedniego eksperymentu genów w celu potwierdzenia poziomu ich ekspresji na poziomie białka.

Do badań odpowiedzi komórek na zastosowane związki wybrane zostały dwie linie komórkowe każdego typu nowotworu. Decyzja o wyborze linii była podyktowana przede wszystkim poziomem ekspresji genów i białek naprawy DSB, jednak istotna była również łatwość prowadzenia hodowli i tempo wzrostu komórek. Odpowiedź komórek była badana po zastosowaniu inhibitorów PARP1 - olaparybu (AZD2281) w liniach czerniaka i talazoparybu (BMN673) w liniach glejaka, podawanych samodzielnie lub w kombinacji ze związkiem alkilującym stosowanych obecnie w terapii wybranego typu nowotworu - dakarbazyna (DTIC) w przypadku czerniaka lub temozolomid (TMZ) w glejaku. Zastosowanie kombinacji inhibitora PARP1 z DTIC lub TMZ w komórkach z obniżoną ekspresją LIG4 miało na celu zablokowanie szlaku alternatywnego altNHEJ i wytworzenie wrażliwości na uszkodzenia DNA wprowadzane przez związek alkilujący. Komórki inkubowane były ze związkami 48h, po czym część komórek pobierana była do pierwszych analiz, a pozostałe otrzymywały drugą dawkę związków, z którą inkubowane były jeszcze 72h.

Badanie wpływu badanych związków na żywotność komórek czerniaka było prowadzone poprzez wykorzystanie cytometrii przepływowej i barwienia komórek jodkiem propidyny. W komórkach glejaka przeprowadzono analizę cytometryczną po barwieniu komórek jodkiem propidyny i aneksyną V. Wykorzystanie kombinacji barwników oprócz identyfikacji martwych komórek, pozwala również na identyfikację ścieżki śmierci komórkowej. Aneksyna V wybarwia fosfatydyloserynę, która pojawia się na zewnątrz błony komórkowej na wczesnych etapach apoptozy. Jodek propidyny barwi DNA wnikając do wnętrza komórek przez pofragmentowaną błonę komórkową. Jest to zmiana charakterystyczna dla nekrozy i późnych etapów apoptozy.

W badaniach na liniach glejaka przeanalizowane zostały zmiany morfologiczne zachodzące w komórkach po inkubacji z inhibitorem PARP1 ± związkiem alkilującym.W tym celu wykorzystano mikroskopię fluorescencyjną i barwienie komórek kalceiną AM i jodkiem propidyny. Kalceina AM ma zdolność przenikania do wnętrza żywych komórek, gdzie ulega degradacji do kalceiny wykazującej po wzbudzeniu silną, zieloną fluorescencję. Jodek propidyny wybarwia DNA komórek martwych wykazujących niski stopień integralności błony plazmatycznej. By przeanalizować wpływ związków na rozkład komórek linii glejaka i czerniaka w cyklu komórkowym przeprowadzono analizę cytometryczną po barwieniu jodkiem propidyny z dodatkiem RNazy.

Zdolność komórek nowotworowych do proliferacji była badana z wykorzystaniem testu klonogenności. Metoda testuje zdolność każdej pojedynczej komórki do przejścia podziałów i utworzenia kolonii. Komórki prawidłowe NHEM i NHA nie mają zdolności wzrostu w miękkim agarze, dlatego kontrolę stanowiły komórki nowotworowe niepotraktowane związkami.

Analiza akumulacji DSB w komórkach czerniaka została wykonana z wykorzystaniem testu ELISA wykrywającego ufosforylowany histon γH2A.X poprzez zastosowanie specyficznych przeciwciał. W badaniach na liniach pierwotnych glejaka poziom ufosforylowanego histonu γH2A.X był badany poprzez identyfikację cytometryczną utrwalonych komórek, które związały przeciwciało Alexa Fluor 647 Mouse Anti-H2A.X (pS139). Wyniki badań wykrywających fosforylację histonu γH2A.X zostały potwierdzone przez wykonanie neutralnego testu kometowego, który rozpoznaje DSB.

W celu potwierdzenia hipotezy projektu komórki linii glejaka i czerniaka z obniżoną ekspresją LIG4 zostały poddane transfekcji plazmidem pCMV6-AC-GFP-LIG4, przenoszącym cDNA dla LIG4 w celu podniesienia wewnątrzkomórkowego poziomu tego białka. Kontrolę stanowiły komórki poddane transfekcji pustym plazmidem. Komórki GFP^+ zostały wysortowane i poddane działaniu badanych związków przez 48h. Żywotność komórek była liczona po barwieniu błękitem trypanu. Kolejnym modelem były linie NALM6 rodzicielska i NALM6 LIG4-/-, które zostały poddane działaniu olaparybu, a żywotność została policzona po barwieniu błękitem trypanu. Komórki glejaków z obniżoną ekspresją LIG4 i z prawidłowym poziomem LIG4 były poddawane wyciszaniu PARP1 z wykorzystaniem siRNA. Kontrolę negatywną stanowiły komórki poddane transfekcji plazmidem kontrolnym (ang. *siRNA non-targeting control*). Kontrolę pozytywną stanowił zestaw do wyciszania ekspresji genu *GAPDH* w komórkach. Po transfekcji komórek badanymi plazmidami przeanalizowany został spadek ekspresji badanych mRNA, a następnie komórki zostały poddane inkubacji z temozolomidem (TMZ) przez 48h. Komórki glejaka zostały także poddane transfekcji plazmidem pMIG-mCherry-PARP1(E988K) kodującym dominujący negatywny mutant (E988K)PARP1 nieaktywny katalitycznie. Komórki $mCherry^+$ zostały wysortowane i poddane 48h inkubacji z TMZ. Żywotność komórek po traktowaniu związkami była analizowana przez barwienie błękitem trypanu i porównanie do kontroli negatywnej.

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. Efekt synergistyczny związków został przeanalizowany stosując podejście addytywności odpowiedzi dwuczynnikowej analizy wariancji ANOVA (ang. *two-way ANOVA*) [14].

Przeprowadzone zostały również wstępne badania *in vivo* na myszach NSG (NOD scid gamma) ksenograftach linii pierwotnej czerniaka DMBC11. Komórki zostały podane podskórnie, a kiedy zaobserwowano wzrost guzów, rozpoczęto podawanie związków. Badania zostały przeprowadzone na 4 grupach oznaczających warianty podawanych związków (kontrola, olaparyb, DTIC, olaparyb + DTIC) - po 6 myszy na grupę.

Wyniki

Analiza ekspresji genów, których produkty biorą udział w naprawie DSB wykazała obniżony poziom *LIG4* w komórkach czerniaka, w porównaniu do komórek prawidłowych melanocytów NHEM. LIG4, wraz z partnerem w oddziaływaniach - XRCC4, przeprowadza łączenie końców w cNHEJ. Jej obniżony poziom może prowadzić do niewydajnej naprawy DSB przez szlak kanoniczny i aktywacji szlaku altNHEJ.

W celu zablokowania mechanizmu alternatywnego wykorzystany został inhibitor PARP1 - olaparyb. Kombinacja inhibitorów PARP1 i DTIC - miała natomiast na celu wzmocnienie efektu zablokowania altNHEJ i wytworzenia wrażliwości na uszkodzenia wprowadzane przez związek alkilujący. Do badań odpowiedzi komórek wybrane zostały dwie linie - DMBC 11 i DMBC 12. Barwienie komórek jodkiem propidyny i ich analiza cytometryczna wykazały specyficzną eliminację komórek czerniaka po zastosowaniu olaparybu razem z DTIC. Po 48h inkubacji jedynie kombinacja związków powodowała spadek żywotności komórek czerniaka. Podanie drugiej dawki i inkubacja przez kolejne 72h spowodowała spadek żywotności po samodzielnym zastosowaniu olaparybu lub DTIC, jak i prowadziło do dalszego spadku liczby żywych komórek po dodaniu kombinacji. Takie samo traktowanie prawidłowych melanocytów NHEM nie przyniosło efektu toksycznego nawet po 5-dniowej inkubacji.

Badanie rozkładu komórek w fazach cyklu komórkowego nie wykazało znaczących zmian w jego przebiegu po inkubacji z badanymi związkami, za wyjątkiem niewielkiego wzrostu populacji komórek w fazie G2/M po traktowaniu kombinacją związków.

Badanie zdolności komórek czerniaka do proliferacji wykazało niemal zupełne zatrzymanie podziałów komórek potraktowanych inhibitorami PARP1 w kombinacji z DTIC. Związki zastosowane samodzielnie prowadziły jedynie do obniżenia podziałów o ok. 30-45%.

Poziom ufosforylowanego histonu γH2A.X, który jest markerem przebiegającej naprawy DSB, nie ulegał zmianie w komórkach prawidłowych melanocytów NHEM potraktowanych inhibitorem PARP1 i DTIC samodzielnie i w kombinacji, jednak w komórkach linii czerniaka DMBC11 i DMBC 12 kombinacja prowadziła do odpowiednio 5- i 2-krotnego wzrostu liczby DSB. Akumulacja DSB w komórkach czerniaka była jeszcze bardziej znacząca w wyniku testu kometowego w warunkach neutralnych.

Badania *in vivo* przeprowadzone na ksenograftach pierwotnej linii czerniaka w myszach NSG pozwoliły zaobserwować niewielką, jednak istotną statystycznie redukcję masy guza po zastosowaniu związków. Badania te zostały potraktowane jako wstępne, a otrzymanie silniejszego efektu może wymagać dalszej optymalizacji protokołu.

Założenie, że eliminacja komórek czerniaka jest spowodowana przez interakcje syntetycznej letalności między LIG4 a PARP1, zostało zweryfikowane poprzez podwyższenie ekspresji LIG4 w komórkach czerniaka z wykorzystaniem wektora plazmidowego przenoszącego cDNA dla LIG4. Komórki wzbogacone w LIG4 wykazywały obniżoną wrażliwość na olaparyb w porównaniu do komórek poddanych transfekcji plazmidem kontrolnym. Dodatkowo, komórki białaczkowe NALM6 z knockout'em LIG4-/- wykazywały znacznie większą wrażliwość na zastosowanie olaparybu niż komórki rodzicielskie posiadające LIG4.

Badanie poziomu ekspresji genów naprawy DSB w komórkach linii pierwotnych glejaka wielopostaciowego również pozwoliła zaobserwować spadek ekspresji *LIG4* w badanych komórkach. Obniżony poziom LIG4 został potwierdzony także na poziomie białka poprzez wykonanie analizy Western Blot. W badaniach odpowiedzi komórek na celowanie w szlak alternatywny wykorzystany został inhibitor PARP1 - talazoparyb. Związek podawano samodzielnie oraz w kombinacji ze związkiem alkilującym stosowanym w terapii glejaka - TMZ.

Zastosowanie w komórach linii glejaka talazoparybu w kombinacji z TMZ wykazało znacznie bardziej toksyczny efekt, niż każdy ze związków zastosowany samodzielnie. Toksyczność dla prawidłowych astrocytów ludzkich była nieznaczna, nawet po inkubacji z drugą dawką związków (48h+72h). Badanie cytometryczne po barwieniu aneksyną V i jodkiem propidyny wykazało, że śmierć komórek nowotworowych z obniżoną ekspresją LIG4 po inkubacji z inhibitorem PARP1 i związkiem alkilującym zachodzi poprzez apoptozę. Barwienie komórek kalceiną AM i jodkiem propidyny wykazało w mikroskopie fluorescencyjnym uszkodzenia błony komórkowej oraz obkurczanie i fragmentację komórek nowotworowych po zastosowaniu badanych związków.

Badanie rozmieszczenia komórek w fazach cyklu komórkowego wykazało akumulację potraktowanych związkami komórek glejaka w fazie subG1 odpowiadającej komórkom ulegającym śmierci, których DNA uległo fragmentacji przez endonukleazy. Nie obserwowano znaczących zmian w rozmieszczeniu prawidłowych astrocytów w fazach cyklu komórkowego.

Test klonogenności wykazał zupełne zatrzymanie podziałów komórek linii glejaka z obniżoną ekspresją LIG4 po zastosowaniu kombinacji talazoparybu i TMZ. Jedynie TMZ zastosowany samodzielnie był w stanie znacząco obniżyć potencjał proliferacyjny linii glejaka wielopostaciowego.

Inkubacja komórek glejaka z kombinacją inhibitora ze związkiem alkilującym prowadziła ponadto do akumulacji toksycznych DSB.

Rola obniżonej ekspresji LIG4 na wrażliwość komórek glejaka na inhibitor PARP1 została określona poprzez poddanie komórek jednej z linii transfekcji wektorem przenoszącym cDNA dla LIG4. Podwyższenie wewnątrzkomórkowego poziomu LIG4 spowodowało uzyskanie oporności na zastosowanie kombinacji talazoparybu i TMZ.

Dodatkowo, wyciszono ekspresję PARP1 w wybranych liniach z obniżoną ekspresją LIG4, a komórki poddano następnie inkubacji ze związkiem alkilującym. Tak potraktowane komórki były bardziej wrażliwe na zastosowanie TMZ, niż komórki poddane transfekcji plazmidem kontrolnym (wyniki badań wykonanych w zagranicznym ośrodku przedstawione zostały w danych uzupełniających). Przeanalizowano również odpowiedź linii pierwotnych glejaka wykazujących poziom ekspresji LIG4 zbliżony do komórek prawidłowych astrocytów.

Po wyciszeniu ekspresji PARP1 nie obserwowaliśmy znaczącego spadku żywotności komórek po inkubacji ze związkiem alkilującym.

Komórki glejaka z niskim i prawidłowym poziomem LIG4 zostały również poddane transfekcji plazmidem kodującym dominujący negatywny mutant białka PARP1 (E988K) nieaktywny katalitycznie. Tak potraktowane komórki z obniżoną ekspresją LIG4 były bardziej wrażliwe na zastosowanie TMZ w porównaniu do komórek z prawidłowym poziomem LIG4, jak i w porównaniu do komórek poddanych transfekcji plazmidem kontrolnym (wyniki przedstawione w danych uzupełniających).

Podsumowanie i wnioski

Zważywszy na rosnącą wiedzę na temat zmian genetycznych i epigenetycznych zachodzących w komórkach nowotworowych, spersonalizowane podejście do terapii przeciwnowotworowej i terapia oparta o syntetyczną letalność zyskują sobie w ostatnich latach uwagę licznych grup badawczych. Chociaż w obecnej chwili jedynie zdolność inhibitorów PARP1 do eliminacji komórek z mutacjami *BRCA1/2* znalazła zastosowanie kliniczne, to inhibitory PARP1 mogłyby zostać wykorzystane w terapii spektrum innych typów nowotworów z defektami w szlakach naprawy DNA.

By przeanalizować terapeutyczne działanie strategii przeciwnowotworowej opartej o syntetyczną letalność w komórkach źle prognozujących guzów litych, od pacjentów wyprowadzono linie pierwotne czerniaka i glejaka. Wykrycie w nich obniżonej ekspresji LIG4 pozwoliło nam postawić hipotezę, iż zastosowanie inhibitorów szlaku altNHEJ w kombinacji ze związkiem alkilującym doprowadzi do specyficznej eliminacji komórek nowotworowych, bez toksycznego efektu na komórki prawidłowe, w których prawidłowo funkcjonuje szlak podstawowy NHEJ. LIG4 jest elementem biorącym udział w naprawie cNHEJ, a jej obniżona ekspresja może być źródłem nieefektywnej pracy tego szlaku i aktywacji alternatywnych ścieżek naprawy. Źródła obniżonej ilości LIG4 w komórkach nie są jeszcze dobrze poznane, jednak wstępne wyniki badań sugerują m.in. niewydajną pracę szlaków JAK2-STAT5 lub PI3K-AKT [15]. Niska ekspresja LIG4 była wcześniej wykrywana w liniach nerwiaka zarodkowego i korelowała z wyższym stadium choroby oraz niższym prawdopodobieństwem przeżycia pacjenta [16].

Zastosowanie kombinacji związku alkilującego i inhibitora PARP1 - olaparybu lub talazoparybu było skuteczne w selektywnej eliminacji komórek czerniaka i glejaka. Inkubacja z badanymi związkami doprowadzała do akumulacji DSB ponad naprawialny próg, zahamowania proliferacji komórek nowotworowych, zmian morfologicznych, a końcowo do zajścia apoptozy. Obniżony poziom LIG4 i zablokowanie alternatywnych ścieżek naprawy były bezpośrednimi czynnikami odpowiedzialnymi za powstanie wrażliwości na uszkodzenia wprowadzane przez związek alkilujący do komórek glejaka i czerniaka, gdyż przywrócenie ekspresji LIG4 powodowało podniesienie oporności komórek na dodawane związki. Zastosowanie inhibitora PARP1 ze związkiem alkilującym nie dawało efektu toksycznego w prawidłowych melanocytach i astrocytach.

Analiza bazy danych TCGA (ang. *The Cancer Genome Atlas*) wykazała, że obniżona ekspresja i mutacje *LIG4* są wykrywane w średnio 7% przypadków czerniaka i 4% glejaka wielopostaciowego, jednak całkowity udział mutacji czynników biorących udział w szlaku podstawowym NHEJ był znacznie większy. Może to sugerować, że potencjalnie znacznie szersza grupa pacjentów mogłaby skorzystać z terapii spersonalizowanej opartej o syntetyczną letalność między inhibitorami PARP1 a defektami szlaku cNHEJ.

Przeprowadzone badania sugerują możliwość zastosowania terapii opartej o syntetyczną letalność w celu selektywnej eliminacji komórek czerniaka i glejaka z obniżoną ekspresją LIG4. Niski poziom LIG4 i inhibicja PARP1, który bierze udział w szlaku alternatywnym NHEJ, naprawie pojedynczych pęknięć DNA oraz indukcji HR na zatrzymanych widełkach replikacyjnych, jest przyczyną akumulacji DSB ponad próg, który komórka jest w stanie naprawić. W komórkach prawidłowych funkcjonował szlak kanonicznej naprawy cNHEJ, redukujący liczbę uszkodzeń wprowadzanych przez związki alkilujące.

Wnioski

- Obniżony poziom ekspresji LIG4 w komórkach linii pierwotnych czerniaka i glejaka wiązał się z ich wrażliwością na zastosowanie inhibitorów PARP1, szczególnie w kombinacji ze związkiem alkilującym, wzmacniającym efekt zahamowania alternatywnego szlaku naprawy;

- Badane związki prowadziły do gromadzenia DSB ponad naprawialny próg, znacząco obniżały potencjał proliferacyjny komórek oraz prowadziły do zmian morfologicznych i apoptozy;
- Przywrócenie ekspresji LIG4 w komórkach powodowało utratę wrażliwości na inhibitory;
- Zastosowane związki nie dawały efektu toksycznego w prawidłowych melanocytach i astrocytach, gdyż prawidłowo funkcjonował w nich mechanizm cNHEJ redukujący liczbę wprowadzanych przez związki alkilujące uszkodzeń.

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Summary

Introduction

One of the hallmarks of cancer cells is their genetic instability which might lead to increased generation of mutations in their genomes [1]. For that reason, there is an increased risk that loss of function mutations will arise in genes whose products are a part of mechanisms crucial for cell survival, e.g. DNA repair systems. Under such conditions, cancer cell's survival depends on finding a substitute for the lost pathway [2]. If inactivation of a specific set of genes leads to cell death, whereas inactivation of each of these genes individually does not affect cell functioning and survival, then these genes are considered to exhibit "synthetic lethal" interactions [3]. Targeting the alternative pathways using inhibitors and aptamers has become an attractive strategy gaining increasing interest in recent years. Synthetic lethality approach might not only prove to be a selective and effective solution in personalized anticancer therapy, but it is already contributing to expanding the knowledge about genetic interactions occurring in cells [4].

Double strand breaks (DSBs) might arise due to an exposition to factors like ionizing radiation, reactive oxygen species or genotoxic stress e.g. due to chemotherapy [5]. Unrepaired DSBs can lead to genetic rearrangements and tumor progression. It has been suggested that inactivating mutations of genes involved in DSB repair and activation of a back-up pathway is a source of the therapy-refractory character of some cancer cells [6]. In normal cells in G1/S phase of the cell cycle DSBs are repaired via non-homologous end joining (NHEJ), whereas homologous recombination (HR) mechanism repairs DSBs during G2 and late S phase. What is more, two subsystems (canonical and alternative) can be distinguished for both NHEJ and HR. The canonical NHEJ (cNHEJ) pathway depends on DNA-PKcs protein, however, if any protein crucial for cNHEJ is absent and the mechanism is not able to perform efficiently, its functions are redirected to the alternative pathway (altNHEJ) basing on PARP1 [7]. PARP1 is involved not only in DSB repair but also participates in repair of DNA single-strand breaks and induces HR at stalled replication forks [8]. Repair by HR bases on interactions between BRCA1/2 and RAD51, however, it has been suggested that cells carrying inactivating *BRCA1/2* mutations rely on alternative pathway depending on RAD52 and RAD51 [6]. Loss of function mutations in canonical pathway genes can create conditions for the selective elimination of cancer cells with alternative pathway inhibitors. At

present, only PARP1 inhibitors have found an application in personalized therapy based on synthetic lethality. PARP1 inhibition in cells carrying *BRCA1/2* mutations lead to inefficient DNA damage repair by PARP1-dependent mechanism and leads to the inversion of lesions to DSBs, which in turn cannot be repaired efficiently due to the mutations inactivating BRCA1/2-HR pathway. Such conditions may further lead to the accumulation of toxic DSB, aggravation of genetic instability and cell death [9]. Although NHEJ system is the predominant DSB repair mechanism in eukaryotic cells, some types of cancer seem to rely mostly on HR, therefore its inactivation can exert a strongly toxic effect on cancer cells [10].

The aim of this research was to identify new synthetic lethality interactions within primary cell lines of melanoma and glioblastoma. Although at the early stages melanoma can be successfully cured, cancer progression and especially metastasis to other organs usually significantly worsen median survival (< 9 months) [11]. Glioblastoma multiforme on the other hand is one of the most commonly occurring cancer types in the central nervous system, and often despite the use of aggressive therapeutic approach survival rate median stays bellow 14.6 months [12]. The aim of the research presented in this thesis was to analyze the expression level of DSB repair genes in each glioblastoma and melanoma case. Basing on the created profile ‘repair mechanism vulnerabilities' were identified which could be utilized to create a personalized approach using inhibitors targeting alternative DSB repair pathways.

Aim of the study

The main aim of this study was to investigate the response of primary glioblastoma and melanoma cell lines expressing reduced level of proteins involved in DSB repair, to the treatment with inhibitors of the alternative DSB repair pathways. This aim was achieved by:

1. Establishment of primary cell lines from surgical specimens of solid tumors and confirmation of the obtained cell line’s characteristics using surface and genetic markers;

2. Determination of the expression level of genes whose products are involved in DSB repair systems (*BRCA1*, *BRCA2*, *PALB2*, homologs of *RAD51*, *RAD52* - HR; *DNA-PKcs*, *XRCC5*, *XRCC6*, *LIG4* - cNHEJ; *PARP1*, *LIG3* - altNHEJ) and comparison of their level to expression in normal cells. Identification of the "weak points" in DSB repair systems in cancer cells and selection of the inhibitors that could lead to cell’s synthetic lethality;

3. Evaluation of cancer and normal cell's response to the treatment with selected inhibitors applied alone or in combination with the alkylating compounds currently used in the therapy of selected types of cancer.

The conducted research will let us identify correlations between the expression profile of genes encoding DSB proteins and cancer cell's sensitivity to the approach based on synthetic lethality strategy.

Materials and methods

The subject of this study were glioblastoma and melanoma primary cell lines. Melanoma specimens classified as clinical stages III and IV were acquired from patients of the Clinic for Oncological Surgery, Copernicus Memorial Hospital, Medical University of Lodz. DMBC 2, DMBC 8, DMBC 10, DMBC 11, DMBC 12 melanoma cells were isolated from samples in the Department of Cancer Molecular Biology, Medical University of Lodz, and the presence of cancer cells in the established cultures was confirmed by cytometric analysis of surface markers specific for melanoma cells and cancer stem cells [13]. The primary melanoma cell lines obtained by the team were shared with us for further research on personalized anticancer therapy. The cells were grown in the form of three-dimensional melanospheres, which, compared to the monolayer culture system better reconstitute the structure of the tumor and the heterogeneity of its cell population.

Samples of glioblastoma multiforme classified as clinical stages III and IV were obtained from patients of the Department of Neurosurgery, Copernicus Memorial Hospital, Medical University of Lodz and Department of Neurosurgery, Surgery of Spine and Peripheral Nerves, University Hospital WAM-CSW, Medical University of Lodz. The research gained approval from the Bioethical Commission of the Medical University of Lodz (approval No. RNN / 194/12 / EC). H3, H6 and H7 glioblastoma cells were isolated from surgical specimens at the Department of Molecular Genetics at the University of Lodz. Cell lines were sorted using magnetic beads MACS (Magnetic-Activated Cell Sorting) detecting the surface antigen CD133, which is a marker of the cancer steam cells. In addition, to confirm the presence of glioblastoma cells in the obtained culture, an analysis of loss of heterozygosity (LOH) at the chromosomal locus 10q23-24, 10p14 and 22q12.3 was performed. Detection of the genetic changes in at least one of these locations is considered a common change characteristic for

glioblastomas. LOH were compared between peripheral blood samples, tumor bulk obtained from patient and established cell line. LOH10q was detected in all tested lines, showing an approximately 50% decrease in the tumor samples and a 65-70% decrease in the derived cell lines compared to the peripheral blood. This suggests that the 10q mutation characteristic for glioblastoma was propagated in the established culture. Normal human epidermal melanocytes (NHEM) and normal human astrocytes (NHA) commercial cell lines were used as a control for the experiments.

Analysis of the expression of the genes whose products are involved in DSB repair began with the isolation of total RNA from each of the tested lines. In the next step, reverse transcription was performed in order to translate mRNA into cDNA. Polymerase chain reaction with real-time detection was carried out using TaqMan probes detecting genes *BRCA1*, *BRCA2*, *LIG3*, *LIG4*, *PALB2*, *PARP1*, *PRKDC*, *RAD51B*, *RAD51C*, *RAD51D*, *XRCC2*, *XRCC3*, *RAD52*, *XRCC5*, *XRCC6*. Their expression was normalized to the *18S rRNA* reference gene. Fold change of the gene expression was calculated as compared to NHEM (for melanoma lines) or NHA (for glioma lines). Western Blot was performed for genes selected from the previous experiment in order to confirm their expression at the protein level.

Two cell lines of each tumor type were selected for the experiments of the cell response to the compounds utilizing the synthetic lethality approach. The decision was made basing majorly on the level of expression of DSB repair genes and proteins, however, the ease of culture and the rate of cell divisions were also considered. Cell response was studied after using PARP1 inhibitors - olaparib (AZD2281) in melanoma and talazoparib (BMN673) in glioblastoma cell lines, alone or in combination with an alkylating compound currently used in the treatment of the selected cancer type - dacarbazine (DTIC) for melanoma or temozolomide (TMZ) in glioblastoma. The use of the PARP1 inhibitor in combination with DTIC or TMZ in cells with reduced LIG4 expression was intended to block the altNHEJ alternative pathway and to generate sensitivity to the DNA damage introduced by the alkylating compound. The cells were incubated with the treatment variants for 48h, after which some cells were collected for the first experiments, and the remaining cells received a second dose of compounds with which they were incubated for 72h more.

The study of the therapeutic effect of the tested compounds on melanoma cell viability was conducted using flow cytometry and propidium iodide staining. In glioblastoma

cells, a cytometric analysis was performed after cell staining with propidium iodide and annexin V. The use of a combination of fluorescent dyes not only allowed identification of dead cells but also allowed to distinguish cell death pathway. Annexin V stains phosphatidylserine, which appears outside the cell membrane in the early stages of apoptosis. Propidium iodide stains DNA by penetrating cells through the fragmented cell membrane. This is a characteristic change occurring in necrosis and late stages of apoptosis.

In glioblastoma primary cell lines fluorescent microscopy was used to analyze the morphological changes occurring in the cells after incubation with the compounds. Cells were stained with calcein AM and propidium iodide. Calcein AM can penetrate living cells, where it is degraded into calcein, which gives strong green fluorescence when excited. Propidium iodide stains the DNA of dead cells that demonstrate low plasma membrane integrity. To analyze the effect of compounds on the distribution of glioma and melanoma cell lines in the cell cycle phases, the cytometric analysis was performed after propidium iodide + RNase staining.

The ability of tumor cells to proliferate was tested using clonogenic assay. The method tests the ability of each individual cell to undergo divisions and form a colony. NHEM and NHA normal cells do not have the ability to grow in soft agar, therefore the untreated cancer cells constituted control for the experiment.

The analysis of DSB accumulation in melanoma cells was performed in ELISA assay detecting phosphorylated histone γH2A.X by specific antibodies. In studies on primary glioblastoma cell lines, the level of phosphorylated histone γH2A.X was tested by cytometric identification of Alexa Fluor 647 Mouse Anti-H2A.X (pS139) antibody - stained cells. The results of tests detecting phosphorylation of γH2A.X histone were confirmed by performing a neutral comet test that recognizes DSB.

To confirm the hypothesis and increase intracellular level of LIG4, glioma and melanoma cell lines with reduced LIG4 expression were transfected with plasmid pCMV6-AC-GFP-LIG4. The cells transfected with an empty plasmid constituted a control. GFP^+ cells were sorted and exposed to the tested compounds for 48 hours. Cell viability was calculated after trypan blue staining. The next model were the parental NALM6 and NALM6 LIG4 -/- lines, which were treated with olaparib and viability was counted after trypan blue staining. Glioblastoma cells with reduced LIG4 expression as well as cells with normal LIG4 level were

subjected to PARP1 silencing using siRNA. The cells transfected with control plasmid (siRNA non-targeting control) constituted a negative control for the experiment. A kit for silencing GAPDH gene expression in cells was used as a positive control. After transfection of cells with given plasmids, the decrease in expression of the tested mRNA was analyzed. Cells were incubated with temozolomide (TMZ) for 48 hours. Glioblastoma cells were also transfected with plasmid pMIG-mCherry-PARP1(E988K) encoding catalytically inactive dominant negative mutant (E988K) of PARP1. $mCherry^{+}$ cells were sorted and incubated 48h with TMZ. Cell viability after treatment with compounds was analyzed by trypan blue staining and compared to a negative control.

The results were obtained in triplicates and presented as mean ± SD. The results were compared using an unpaired Student's t-test. P values <0.05 were considered statistically significant. The synergistic effect of compounds has been analyzed using the additivity approach - two-way ANOVA [14].

Preliminary *in vivo* studies have also been performed on NSG (NOD scid gamma) mice xenografts of the primary DMBC11 melanoma cell line. The cells were administered subcutaneously, and when tumor growth was observed, the compounds were administered. The studies were carried out on 4 groups denoting variants of the administered compounds (control, olaparib, DTIC, olaparib + DTIC) - 6 mice per group.

Results

Analysis of the expression profile of genes whose products are involved in DSB repair showed a reduced level of *LIG4* in melanoma cells compared to normal melanocyte cells. LIG4, together with its interaction partner - XRCC4, performs end joining in cNHEJ pathway. Its reduced level may lead to inefficient DSB repair by cNHEJ and activation of the alternative pathway - altNHEJ.

PARP1 inhibitor olaparib was used in order to suppress the alternative mechanism. The alkylating agent DTIC was administered in combination with PARP1 inhibitor to strengthen the toxic effect of altNHEJ inactivation, creating sensitivity to damage introduced by the alkylating compound. Two primary melanoma cell lines - DMBC 11 and DMBC 12 were selected for the analysis of cell response. Cytometric analysis after propidium iodide staining revealed specific elimination of melanoma cells when olaparib was used together with DTIC.

After 48 hours of incubation, only the combination of compounds reduced the viability of melanoma cells, however, administration of a second dose and incubation for another 72h resulted in a further decrease in cell viability. The same treatment of normal NHEM melanocytes did not exert a toxic effect even after a 5-day incubation.

The examination of cell distribution in the phases of the cell cycle did not show significant changes in its course after incubation with tested compounds. A slight increase in the cell population in the G2/M phase was only observed after treatment with the combination of compounds.

Analysis of the proliferative potential of melanoma cells showed almost complete arrest of cell divisions after treatment with PARP1 inhibitor in combination with DTIC. Compounds used alone led only to average 30-45% reduction of divisions.

The level of phosphorylated histone γH2A.X, which is a marker of ongoing DSB repair, did not increase in normal NHEM melanocytes cells treated with PARP1 inhibitor and DTIC alone and in the combination, however in DMBC11 and DMBC12 melanoma cell lines combination of the compounds resulted in respectively 5- and 2-fold increase in DSB amount. Accumulation of DSB in melanoma cells was even more significant in performed comet assay under neutral conditions.

In vivo studies conducted on primary DMBC11 melanoma cell line xenografts in NSG mice showed a slight but statistically significant reduction in tumor mass after combination of PARPi and alkylating agent was administered for 24 days. These results are considered to be only preliminary, and further protocol optimization might allow obtaining a stronger effect.

The hypothesis that melanoma cell elimination is caused by synthetic lethality interactions between LIG4 and PARP1 has been verified by increasing LIG4 expression in melanoma cells using a plasmid vector carrying cDNA for LIG4. LIG4-enriched cells exhibited reduced sensitivity to olaparib compared to cells transfected with the control plasmid. Also, NALM6 leukemia cells with the LIG4-/- knockout were much more sensitive to olaparib than parental cells expressing LIG4.

Analysis of the expression level of DSB repair genes in the primary glioblastoma multiforme cell lines also demonstrated a decreased expression of LIG4 in the tested cells.

The reduction of the LIG4 level was also confirmed by performing Western Blot analysis. PARP1 inhibitor - talazoparib was used to investigate cell response to the alternative pathway targeting. The compound was administered alone and in combination with an alkylating agent - TMZ used in the treatment of glioblastoma patients.

The use of talazoparib in combination with TMZ exerted significantly more toxic effect than any of the compounds used alone. Toxicity to normal human astrocytes was slight to none, even after incubation with the second dose of compounds (48h + 72h). Cytometric analysis after staining with annexin V and propidium iodide showed that the death of analyzed cancer cells after incubation with a PARP1 inhibitor + TMZ occurs through apoptosis. Fluorescent microscopy after staining with calcein AM and propidium iodide showed cell membrane damage, shrinking and fragmentation of tumor cells occurring after the application of tested compounds.

Analysis of glioblastoma cells distribution in the cell cycle phases showed the accumulation of compound-treated glioblastoma cells in the subG1 phase corresponding to dead cells whose DNA was fragmented by nucleases. No significant changes in the distribution of normal astrocytes in the phases of the cell cycle were observed.

Clonogenic assay demonstrated the complete abolishment of glioblastoma cell division after treatment with a combination of talazoparib and TMZ. Only TMZ used alone was able to significantly reduce the proliferative potential of glioblastoma multiforme primary cell line.

Incubation of glioblastoma cells with the combination of the inhibitor and the alkylating compound also led to a significant accumulation of the toxic DSBs.

The role of reduced LIG4 expression on the sensitivity of glioblastoma cells to the PARP1 inhibitor was determined by cell transfection with an expression vector carrying cDNA for LIG4. Increasing intracellular LIG4 levels resulted in resistance to the combination of talazoparib and TMZ.

Also, PARP1 expression was silenced in selected lines with reduced LIG4 expression, and the cells were then incubated with the alkylating compound. Cells with PARP1 silenced were more sensitive to TMZ than cells transfected with the control plasmid (results in the supplementary data). The response of glioma primary cell lines exhibiting a level of LIG4

similar to normal astrocyte cells was also analyzed. After silencing PARP1 expression we did not observe a significant decrease in cell viability after incubation with the alkylating compound.

Glioblastoma cells with low and normal LIG4 expression were also transfected with a plasmid coding dominant-negative PARP1 (E988K) mutant. Cells with reduced LIG4 expression which were expressing catalytically inactive PARP1 were more sensitive to TMZ when compared to cells with normal LIG4 levels (results in the supplementary datas).

Resume and conclusions

Growing knowledge of genetic and epigenetic changes occurring in cancer cells resulted in an increasing interest in synthetic lethality and a personalized approach to anticancer therapy. Although at the present moment only ability of PARP1 inhibitors to eliminate BRCA1/2-deficient cells found clinical application, PARP1 inhibitors could be potentially utilized for the treatment of different cancer types expressing defects in DNA repair pathways.

In order to analyze the therapeutic effect of an anticancer therapy based on synthetic lethality in cells of solid tumors, glioblastoma and melanoma primary cell lines were derived from surgical specimens. Detection of low LIG4 expression in given cell lines allowed us to hypothesize that the use of altNHEJ inhibitors in combination with an alkylating compound will lead to selective elimination of cancer cells, with no toxic effect on normal melanocytes and astrocytes in which cNHEJ functions properly. LIG4 is involved in the end ligation step in cNHEJ and its reduced level might be a reason for ineffective repair by cNHEJ and activation of a back-up repair mechanism. The sources of the reduced level of LIG4 in cells are not yet well understood, however, preliminary research results suggest inefficient JAK2-STAT5 or PI3K-AKT pathway activity [16].

The combination of alkylating agent and PARP1 inhibitors - olaparib or talazoparib was effective in the selective elimination of melanoma and glioblastoma cells. Treatment with the tested compounds led to the accumulation of DSBs above the repairable threshold, inhibited proliferative potential of tumor cells, resulted in morphological changes and apoptosis. Reduced expression of LIG4 and inhibition of the alternative repair pathway were directly responsible for the emergence of the cellular sensitivity to damage induced by an

alkylating agent to glioblastoma and melanoma cells. Restoration of LIG4 expression resulted in increased resistance to analyzed compounds. Using PARP1 inhibitor with an alkylating compound did not have a toxic effect on normal melanocytes and astrocytes.

The analysis of the TCGA database (The Cancer Genome Atlas) shows that reduced expression and mutations of LIG4 are detected in average in 7% melanoma and 4% glioblastoma cases, however, the total percentage of general cNHEJ mutation was significantly higher. This discovery might suggest that potentially a wider group of patients could benefit from personalized anticancer therapy based on synthetic lethality between PARP1 inhibitors and cNHEJ pathway defects.

The results of the conducted research suggest the therapeutic effect of the approach based on targeting PARP1 in order to selectively eliminate melanoma and glioma cells with reduced LIG4 expression. Downregulation of LIG4 and inhibition of PARP1 which is involved in altNHEJ, DNA single-strand break repair and induction of HR at stalled replication forks, caused DSB accumulation and led to cancer cell death. In normal cells, cNHEJ repair pathway operated properly reducing the number of DNA lesions introduced by an alkylating agent.

Conclusions

- The decreased expression level of LIG4 in melanoma and glioblastoma primary cell lines was associated with their sensitivity to PARP1 inhibitors, especially when applied in combination with an alkylating compound which enhanced the result of alternative pathway inhibition;
- Compounds led to the accumulation of DSBs above the repairable threshold, significantly reduced cell proliferative potential, and led to morphological changes and apoptosis;
- Restoration of LIG4 expression in cells resulted in the loss of sensitivity to the treatment;
- Used compounds did not affect normal melanocytes and astrocytes, because the cNHEJ mechanism which functioned correctly, reduced the number of damage introduced by alkylating compounds.

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Syntetyczna letalność jako funkcjonalne narzędzie w badaniach podstawowych oraz w terapii przeciwnowotworowej*

Synthetic lethality as a functional tool in basic research and in anticancer therapy

Monika Toma[1], Tomasz Skorski[2], Tomasz Śliwiński[1]

[1] Katedra Genetyki Molekularnej Uniwersytetu Łódzkiego
[2] Department of Microbiology and Immunology, and Fels Institute for Cancer Research, Temple University, School of Medicine, Philadelphia, PA, USA

Streszczenie

Z roku na rok liczba odnotowanych zachorowań na nowotwory drastycznie wzrasta. Poszukuje się narzędzi, które będą pozwalały eliminować zmienione komórki o fenotypie nowotworowym, nie tylko szybko, ale również bardzo specyficznie, nie uszkadzając przy tym komórek prawidłowych. Takie podejście terapeutyczne niesie za sobą zmniejszenie liczby jej działań niepożądanych, a więc także poprawę rekonwalescencji chorego. Nową nadzieją w stworzeniu skutecznej spersonalizowanej terapii przeciwnowotworowej, stało się odkrycie zjawiska syntetycznej letalności. Zauważono, że jednoczesne mutacje niektórych par genów mogą prowadzić do śmierci komórki, podczas gdy każdy z nich zmutowany indywidualnie nie wywołuje efektu letalnego. Ponieważ w komórkach nowotworowych dochodzi do licznych zmian w materiale genetycznym, odnajdując pary oddziałujących ze sobą w ten sposób genów, można się przyczynić do powstania potencjalnej terapii przeciwnowotworowej. Przypuszcza się, że proces taki wyewoluował, by uodpornić komórkę na uszkodzenia pojedynczych genów – dany szlak nie zależy wówczas od pojedynczego wariantu. Takie procesy komórkowe doprowadziły jednak do powstania chorób o złożonej etiologii, takich jak nowotwory. Obecnie prężnie rozwijająca się technika badania z użyciem iRNA, shRNA czy małocząsteczkowych inhibitorów, pozwala na odnajdywanie oddziaływań międzygenowych, mających znaczenie w powstawaniu zjawiska syntetycznej letalności. Umożliwia to nie tylko wykorzystanie tego zjawiska jako potencjalnej terapii przeciwnowotworowej, ale również jako narzędzia do identyfikowania funkcji nowo poznanych genów. Syntetyczna letalność pozwala także na poszerzenie wiedzy na temat biologii molekularnej nowotworów, która jest wykorzystana do stworzenia nowych i bardzo skutecznych spersonalizowanych terapii przeciwnowotworowych.

Słowa kluczowe: **syntetyczna letalność • terapia przeciwnowotworowa • oddziaływania międzygenowe • iRNA • shRNA • małocząsteczkowe inhibitory**

Summary

Nowadays, cancer and anticancer therapy are increasingly mentioned topics. Groups of researchers keep looking for a tool that will specifically and efficiently eliminate abnormal cells without any harm for the normal ones. Such method entails the reduction of therapy's side effects, thus also improving patient's recovery. Discovery of synthetic lethality has become

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a new hope to create effective, personalized therapy of cancer. Researchers noted that pairs of simultaneously mutated genes can lead to cell death, whereas each gene from that pair mutated individually does not result in cell lethality. Cancer cells accumulate numerous changes in their genetic material. By defining the pairs of genes interacting in cell pathways we are able to identify a potential anticancer therapy. It is believed that such a process has evolved to create cell resistance for a single gene mutation. Proper functioning of a pathway is not dependent on a single gene. Such a solution, however, also led to the evolution of multifactorial diseases such as cancer. Research techniques using iRNA, shRNA or small molecule libraries allow us to find genes that are connected in synthetic lethality interactions. Synthetic lethality may be applied not only as an anticancer therapy but also as a tool for identifying the functions of recently recognized genes. In addition, studying synthetic lethality broadens our understanding of the molecular mechanisms governing cancer cells, which should be helpful in designing highly effective personalized cancer therapies.

Keywords: **synthetic lethality • anticancer therapy • gene-gene interactions • iRNA • shRNA • small molecule inhibitors**

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Adres autora: dr hab. inż. Tomasz Śliwiński, prof. nadzw. UŁ; Katedra Genetyki Molekularnej, Uniwersytet Łódzki, ul. Pomorska 141/143, 90-236 Łódź; e-mail: tomsliw@biol.uni.lodz.pl

Wykaz skrótów: **8-okso-dG** – 7,8-dihydro -8- okso- deoksyguanina; **AKT** – serynowo-treoninowa kinaza białkowa; **AP** – miejsce apurynowe/apirymidynowe; **ATM, ATR** – białka uczestniczące w odpowiedzi komórki na pęknięcia nici DNA; **BCR-ABL** – fuzyjna kinaza powstała w wyniku translokacji; **BER** – naprawa DNA przez wycinanie zasad; **BRCA1, BRCA2** – białka uczestniczące w odpowiedzi komórki na dwuniciowe pęknięcia nici DNA; **CIP/KIP** – rodzina inhibitorów kinaz cyklinozależnych; **CML** – przewlekła białaczka szpikowa; **DSB** – pęknięcia dwuniciowe DNA; **E2F1** – czynnik transkrypcyjny; **EGFR** – receptor czynnika wzrostu naskórka; **GFP** – białko zielonej fluorescencji; **GMP** – monofosforan guanidyny; **HER2/neu** – onkogen; **HPRT1** – fosforybozylotransferaza hipoksantynowo-guaninowa; **HR** – naprawa DNA przez homologiczną rekombinację; **IMP** – inozynomonofosforan; ***MLH1, MSH2, MSH6, PMS2*** – geny kodujące białka naprawy błędnie sparowanych zasad DNA; **MMR** – naprawa błędnie sparowanych zasad DNA; **MTOR** – kinaza biorąca udział w odpowiedzi komórki na stres; **NHEJ** – naprawa DNA przez niehomologiczne łączenie końców; **PARP1** – polimeraza poli-ADP-rybozy; **PCC** – przedwczesna kondensacja chromatyny; **PDGF** – płytkopochodny czynnik wzrostu; **PINK1** – mitochondrialna kinaza serynowo-treoninowa; **PIP3** – trójfosforan fosfatydyloinozytolu; ***PTEN*** – gen supresorowy; **RAD51, RAD52** – białka naprawy DNA przez homologiczną rekombinację; **RB1** – gen supresorowy, negatywny regulator cyklu komórkowego; **RPA** – białko wiążące jednoniciowy DNA; ***XP*** – rodzina genów związanych z chorobą *xeroderma pigmentosum*.

Wstęp

Syntetyczna letalność jest zjawiskiem zachodzącym w komórce, oznaczającym jednoczesne zaburzenia dwóch lub więcej genów, powodujące śmierć tej komórki lub nawet całego organizmu, w skład którego wchodzi. Produkt jednego z tych genów jest istotny w procesie przeżycia komórki. Gdy ulega uszkodzeniu, jego funkcja jest zastępowana przez drugi gen, uczestniczący w szlaku alternatywnym dla procesu, w którym uczestniczył pierwszy. Uszkodzenie bądź celowe wyłączenie obu genów kończy się śmiercią komórki lub organizmu. W komórce nowotworowej utrata funkcji genów jest bardzo prawdopodobna ze względu na występowanie licznych rearanżacji w jej genomie. Przeżycie komórki jest zatem bezpośrednio uzależnione od działania takich alternatywnych genów. Ze względu na skutek, który jest wywoływany w komórce, zagadnienie syntetycznej letalności wydaje się wyjątkowo atrakcyjne i budzi coraz większe zainteresowania naukowców. Jest też przedmiotem coraz liczniejszych badań obejmujących m.in. wykorzystanie leków nowej generacji stosowanych w terapiach przeciwnowotworowych, opierających się na zjawisku syntetycznej letalności. Genetycy zainteresowali się syntetyczną letalnością również dlatego, ponieważ może dostarczyć istot-

nych informacji o interakcjach między genami, czy też ich produktami. Ponadto zakrojone na szeroką skalę badania z wykorzystaniem tego zjawiska stwarzają możliwość m.in. poszerzenia wiedzy na temat działania obecnie wykorzystywanych leków oraz poznania punktów docelowych terapii.

Dwa geny (A i B) można uznać, że są syntetycznie letalne, jeśli mutacja w każdym z genów oddzielnie nie ma wpływu na żywotność komórki, natomiast jednoczesne mutacje zarówno w genie A, jak i B prowadzą do śmierci komórkowej. Komórka jest zdolna do utrzymania równowagi częściowo przez uniezależnienie aktywności metabolicznej od pojedynczego czynnika, który może łatwo ulec zaburzeniu przez mutacje wywołane przez czynniki endo- lub egzogenne. Obecnie uważa się, że taka „odporność" komórek na uszkodzenie pojedynczych genów mogła odgrywać główną rolę w powstawaniu i ewolucji chorób wieloczynnikowych, takich jak nowotwory. Odporność genetyczna jest zapewniana m.in. przez swoistą funkcjonalną „nadmierność genetyczną", np. przez posiadanie dwóch alleli jednego genu. Innym przykładem mogą być tzw. białka kondensatory (np. białko szoku cieplnego Hsp90), które pozwalają na maskowanie fenotypu nieprawidłowego zwinięcia „zmienionych" białek [37].

Termin „syntetyczna letalność" zaproponował w 1946 r. Theodor Dobzhansky. Przymiotnik „syntetyczna" pochodzi z języka greckiego i oznacza mieszaninę dwóch podmiotów, z której powstaje coś zupełnie nowego. Po raz pierwszy proces ten opisał na początku XX w. Amerykański genetyk Calvin Bridges, który podczas krzyżowania *Drosophila melanogaster*, spostrzegł, że kombinacja pewnych nieallelicznych genów była letalna, mimo iż homozygotyczne osobniki rodzicielskie były żywotne i funkcjonalne. Geny te były dla siebie alternatywą, więc dopiero kombinacja ich rearanżacji, powodowała śmierć osobnika, ze względu na to, iż mutacji uległy geny biorące udział w procesie koniecznym do jego przeżycia [4]. Podobne zjawisko zaobserwował Theodor Dobzhansky u *Drosophila pseudoobscura* [14].

Chcąc poznać procesy molekularne zachodzące w komórkach ludzkich naukowcy skupili się na początku na prostszym i tańszym modelu badawczym, jakim są komórki drożdżowe, których genomy składają się z wielu genów będących ludzkimi ortologami. Badania nad syntetyczną letalnością w drożdżach pozwoliły naukowcom otrzymać złożone mapy oddziaływań zachodzących miedzy genami. Analiza takich sieci powiązań w komórkach ludzkich wydaje się niezwykle atrakcyjnym kierunkiem badań, głównie dlatego, że wiele ludzkich genów wciąż nie ma przypisanych im funkcji, mimo iż upłynęła już dekada od poznania sekwencji ludzkiego genomu. Obecnie, przy wykorzystaniu metod analizy syntetycznej letalności, takich jak biblioteki interferencyjnego RNA, możliwym staje się poznawanie procesów, a nawet całych szlaków komórkowych, których wcześniej nie znano [22]. Oddziaływania fizyczne między genami, wykazywanie ich mniej lub bardziej zbliżonych funkcji, ich jednoczesna ekspresja, czy też kolokalizacja, okazują się elementami pozwalającymi dokładnie poznać interakcje międzygenowe [43]. Jednak bez wykonania odpowiednich badań funkcjonalnych nie można jednoznacznie stwierdzić, czy oznaczane dwa geny stanowią kompensację względem siebie, pod kątem pełnionej przez nie funkcji w komórce. Po bliższym poznaniu genomów drożdżowych naukowcy zaczęli się interesować tym, jaki jest stopień zachowania ewolucyjnego tych oddziaływań między gatunkami. *S. cerevisiae* i *S. pombe*, które uległy rozdzieleniu 400 mln lat temu i współdzielą niespełna 30% interakcji genetycznych. Poddano tym samym w wątpliwość użyteczność modelu drożdżowych interakcji do przewidywania zjawiska syntetycznej letalności u wyższych kręgowców. Uzyskane wyniki badań z wykorzystaniem tego modelu nie są jednoznaczne, co mogło być spowodowane ówczesnymi ograniczeniami technicznymi, bądź też wynikało z preferencyjności zjawiska syntetycznej letalności tylko w niektórych procesach komórkowych [13,38,40]. Najlepszym sposobem na badanie tego procesu u ludzi są badania właśnie na komórkach ludzkich, a nie próby korelacji z wynikami uzyskanymi na organizmach modelowych.

Syntetyczna letalność w komórkach ludzkich

Badania syntetycznej letalności na komórkach ludzkich oparte są przede wszystkim na wykorzystaniu jej potencjału, jako przyszłościowej terapii przeciwnowotworowej, narzędzia do poznawania funkcji genów oraz oddziaływań między nimi. Wiedza o sieciach molekularnych, które przebiegają w komórkach prawidłowych, czy też nowotworowych jest jeszcze ograniczona. Nie pozwala to na wytypowanie genów, stanowiących dla siebie alternatywę, czy też zastępstwo. Istnieje wiele pomysłów na to, jak wykorzystać efekt syntetycznej letalności do tego celu. Zdarzyć się może, że jeden gen koduje produkty spełniające wiele funkcji w komórce. Jest tak w przypadku onkoproteiny E2F1 (E2F transcription factor 1), która odpowiada zarówno za modulowanie cyklu komórkowego, zdolności proliferacyjnych, a także za przebieg apoptozy. Produkt genu E2F1 pozwala komórkom ssaczym na wejście w fazę S cyklu komórkowego, podczas której dochodzi do tworzenia przez niego kompleksu z cykliną A/CDK2 (cyclin-dependent kinase 2). W takim kompleksie zupełnie zniesiona jest zdolność E2F1 do wiązania z DNA. Inhibitorem E2F1, blokującym wejście komórki w fazę S jest białko RB, produkt genu *RB1* retinoblastoma 1; gen supresorowy, negatywny regulator cyklu komórkowego. Natomiast zdolność blokowania RB, ma kompleks cyklina A/CDK2. Zaproponowano model opierający się na zwiększeniu ekspresji takich białek jak E2F1, które pozostają wysoce aktywne w komórkach nowotworowych, wywołującym tym samym przekroczenie progu ekspresji, wymaganego do zajścia apoptozy. Zatrzymanie aktywności RB w celu zajścia dodatniego sprzężenia zwrotnego, w którym E2F1 aktywowałby swój własny promotor, może pozwolić na uśmiercanie komórek transformowanych, pozostawiając nietknięte komórki prawidłowe [1,23]. W badaniach przeprowadzonych na myszach wykazano, że osobniki pozbawione genu *Cdk2* rozwijały się prawidłowo. Zależność przytoczona wcześniej nie funkcjonuje w przypadku inhibicji działania partnera cykliny A - CDK2. Dzieje się tak prawdopodobnie dlatego, że obecny jest inny part-

ner katalityczny, który potrafi zastąpić CDK2 w tych interakcjach [3].

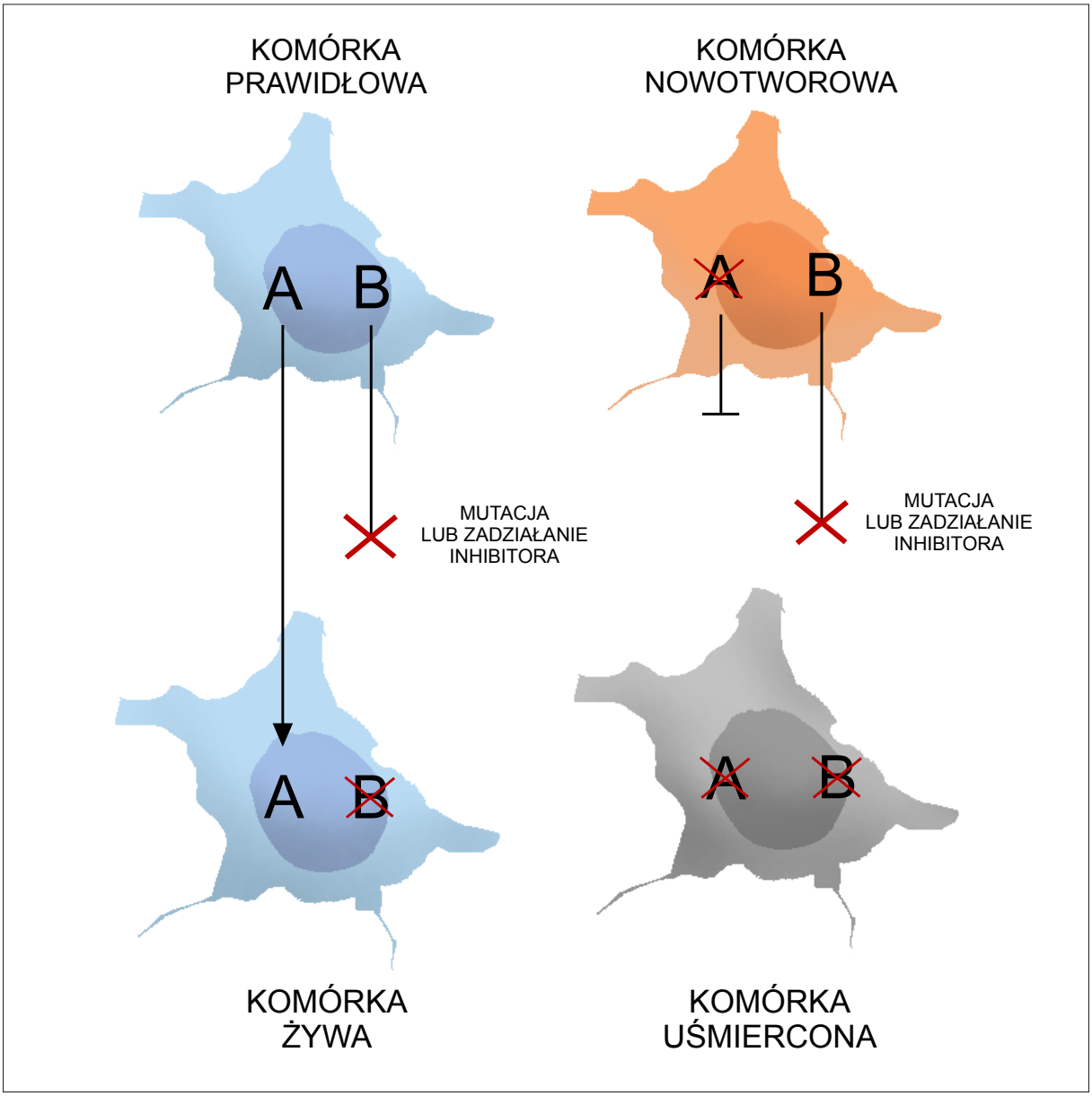
Zjawiska syntetycznej letalności mogą być także wykrywane na podstawie obserwacji poszczególnych punktów kontrolnych cyklu komórkowego. Przedwczesna kondensacja chromatyny PCC (prematurely condensed chromatin) jest zjawiskiem występującym podczas fazy S w komórkach ssaków. Zachodzi, gdy podział rozpoczęty zostanie przed zakończeniem replikacji DNA. Aby zapobiegać takim zdarzeniom, wykształcone zostały punkty kontrolne cyklu komórkowego, w których główną rolę odgrywają białka, takie jak ATR (ataxia telangiectasia and RAD3-related protein) czy p53. Ich zadaniem jest powstrzymanie komórki przed nieprawidłowym podziałem i zatrzymanie jej aktywności. Aktywność genu *ATR* może być inhibowana przez zastosowanie kofeiny. Kiedy dodatkowo pozbawimy komórkę aktywności p53, dojdzie do przedwczesnej, letalnej kondensacji chromatyny [33].

Pojęcie syntetycznej letalności może nieco przypominać zjawisko „uzależnienia od onkogenu", które przejawiają komórki nowotworowe stając się zależnymi od swoich zmian genetycznych, a więc stają się podatne na nagłe zmiany w swoich onkogenach lub odzyskanie funkcji przez geny supresorowe. Pojawienie się konkretnego fenotypu nowotworu jest uzależnione od pojawiających się w komórce mutacji. Wystąpienie niektórych typów nowotworów jest zależne od pojawienia się mutacji w zaledwie pojedynczym genie. Przykładowo siatkówczak, uwarunkowany jest mutacją w genie *RB1*. Pojedyncza mutacja rzadko może spowodować przemiany komórki prawidłowej w nowotworową, a tym bardziej w jej zezłośliwienie. Natępne mutacje spowodowane postępującym obniżaniem stabilności genetycznej powodują stopniową progresję nowotworu. Nowotwory, a zwłaszcza ich wzrost i podział stają się uzależnione od ekspresji niektórych genów, które podlegały zmianom w toku transformacji. Istnieje obecnie wiele przykładów nowotworów, które wydają się być „uzależnione" od niektórych onkogenów, dodatkowo wyniki badań sugerują również, że komórki nowotworowe mogą być „uzależnione" od inaktywacji genów supresorowych [20]. Termin „uzależnienie onkogenu" zaproponował po raz pierwszy Bernard Weinstein. Domniemywał, że komórka żyje i dzieli się tak długo, dopóki sygnał proliferacyjny jest otrzymywany od onkogenu, a może to być związane ze zdolnością takich onkogenów do zawiązywania skomplikowanych sieci molekularnych, które mogą dostarczać sygnałów proliferacyjnych i antyproliferacyjnych. Jeśli onkogen ulegnie wyciszeniu lub dojdzie do przywrócenia funkcji genu, przewagę osiągną sygnały antyproliferacyjne, co doprowadzi do zahamowania wzrostu lub śmierci komórki. Uzależnienie od onkogenu może leżeć u podstaw skuteczności terapii inhibitorem kinaz - Imatinibem, który powstrzymuje hiperaktywność fuzyjnej kinazy BCR-ABL (BCR - breakpoint cluster region, ABL - c-abl oncogene 1, non-receptor tyrosine kinase) w przewlekłej białaczce szpikowej CML (chronic myeloid leukemia) [9,20,41].

Nowotwory powstają na skutek zmian genetycznych zachodzących etapowo. Z każdą kolejną modyfikacją fenotyp komórki płynnie przechodzi z prawidłowego w nowotworowy. Każde następne zaburzenie zwiększa również prawdopodobieństwo zajścia uzależnienia od onkogenu. Fenotyp nie jest sumą pojedynczych mutacji, gdyż zmienione geny niejednokrotnie biorą udział w wielu złożonych procesach integrując złożone sieci metaboliczne. W komórkach nowotworowych onkogen może odgrywać odmienną i bardziej istotną rolę w porównaniu z jego rolą w komórce prawidłowej, stąd nowotwór może być bardziej zależny od aktywności konkretnego genu. Zmienione geny podlegają ciągłej presji selekcyjnej [42]. Zachowaniu ulegają jedynie mutacje, które są dla nowotworu neutralne lub korzystne względem wariantu dzikiego genu, jedynie wówczas presja selekcyjna działa na ich korzyść. Możliwe jest również, że mutacja pierwotna, która zaszła w jednym z genów, może zmniejszać skutki mutacji, która nastąpiła w innym genie, a nawet nie pozwalać na śmierć komórki w wyniku syntetycznej letalności. Mutacja w genie *RB1* prowadzi do powstania sprzężenia zwrotnego, w którym czynnik E2F1 aktywuje sam siebie. Może to stymulować komórkę zarówno do wejścia w fazę S, a także może promować wejście jej na drogę apoptozy zależnej od białka p53. Jeśli jednak w pierwszej kolejności doszłoby do mutacji w białku p53, niemożliwa stanie się apoptoza komórki. Nie będzie możliwym również zajście efektu syntetycznej letalności w komórkach z mutacją w genie *RB1*. Powstała mutacja może się okazać więc korzystna dla komórki nowotworowej, gdyż spowoduje jej proliferację. Wszystko to będzie jednak uwrażliwiało ją również na utratę takiej wersji genu [20].

Zjawisko uzależnienia od onkogenu może przypominać syntetyczną letalność, gdyż komórka uzależniona zostaje ostatecznie od produktu pojedynczego, zmutowanego genu. Jednak właśnie jednym z mechanizmów tłumaczących zajście uzależnienia komórek nowotworowych od onkogenu jest proces syntetycznej letalności. Na przykład komórka nowotworowa może zostać uzależniona od jakiegoś onkogenu, ponieważ w czasie swojego rozwoju doszło do utraty funkcji innego genu, o podobnej funkcji. Sprowadza się to do wniosku, że syntetyczna letalność może zostać wykorzystana jako potencjalna terapia przeciwnowotworowa. Podejrzewa się jednak, że ze względu na mnogość zmian w komórkach nowotworowych pary genów wykazujące interakcje w komórkach prawidłowych nie zawsze mogą korespondować z istniejącymi w komórkach nowotworowych, co może powodować jeszcze większe ich uzależnienie od danego onkogenu [42].

Uzależnienie od onkogenu może być spowodowane utratą dodatkowych szlaków sygnalizacyjnych. Niestabilność genomu nowotworowego i nieobecność presji selekcyjnej działającej w celu utrzymania dodatkowych dróg sygnalizacyjnych w procesie komórkowym wywołuje efekt zwany „optymalizacją genomu". Jest to zjawisko, któremu podlega informacja genetyczna mająca dla komórki nieznaczną wartość adaptacyjną. Elementy te muszą być zależne od metabolizmu komórkowego, co stanowi dla komórki dodatkowe obciążenie. Racjonalnym rozwiązaniem wydaje się więc usunięcie ich z genomu [15,21]. Szlaki, które aktywowane zostają na wczesnych etapach



Ryc. 1. Schemat syntetycznej letalności. Komórki prawidłowe mają dwa funkcjonalne, alternatywne wobec siebie szlaki, w procesie niezbędnym do jej przeżycia (np. zależne od genów A i B, istotnych dla tych szlaków). W komórkach nowotworowych, w których często dochodzi do licznych rearanżacji materiału genetycznego, w wyniku której jeden z istotnych szlaków stanowiących alternatywę względem siebie, zostaje wyłączony (brak funkcjonalnego genu, np. A). Powoduje to uzależnienie komórki od szlaku alternatywnego. W chwili zablokowania tego szlaku w wyniku mutacji genu np. B, lub za pomocą np. małocząsteczkowego czynnika chemicznego, takiego jak inhibitor czy aptamer, oddziałującego na jego produkt, dochodzi do śmierci komórki nowotworowej. Mutacja lub zahamowanie genu B w komórce prawidłowej nie wpływa znacząco na jej przeżywalność, ponieważ wciąż obecny jest funkcjonalny gen A

rozwoju nowotworu, mogą się okazać jednocześnie jego słabym punktem. Z czasem szlaki alternatywne zostaną usunięte w wyniku optymalizacji genomu, a cały szlak oparty będzie na pojedynczym czynniku.

Badania syntetycznej letalności w komórkach ludzkich skupiają się obecnie przede wszystkim na możliwości wykorzystania tego procesu w celach terapeutycznych. Być może niedługo możliwe stanie się spersonalizowanie terapii dla konkretnego profilu choroby, a obecnie wiadomo, że niestabilność komórek nowotworowych jest zarówno ich mocną stroną, jak i potencjalną szansą uzyskania przez naukowców skutecznych, celowanych terapii przeciwnowotworowej. Przez liczne mutacje powstające w komórkach nowotworowych, powstaje wiele nierozróżnianych jeszcze do końca typów nowotworów. Ta zmienność dostarcza jednak również możliwości dokładnego nakierowania terapii przeciwnowotworowej na jak najbardziej wybiórcze, celowane działanie, wywołujące jak najmniej działań niepożądanych.

Potencjalne terapie przeciwnowotworowe

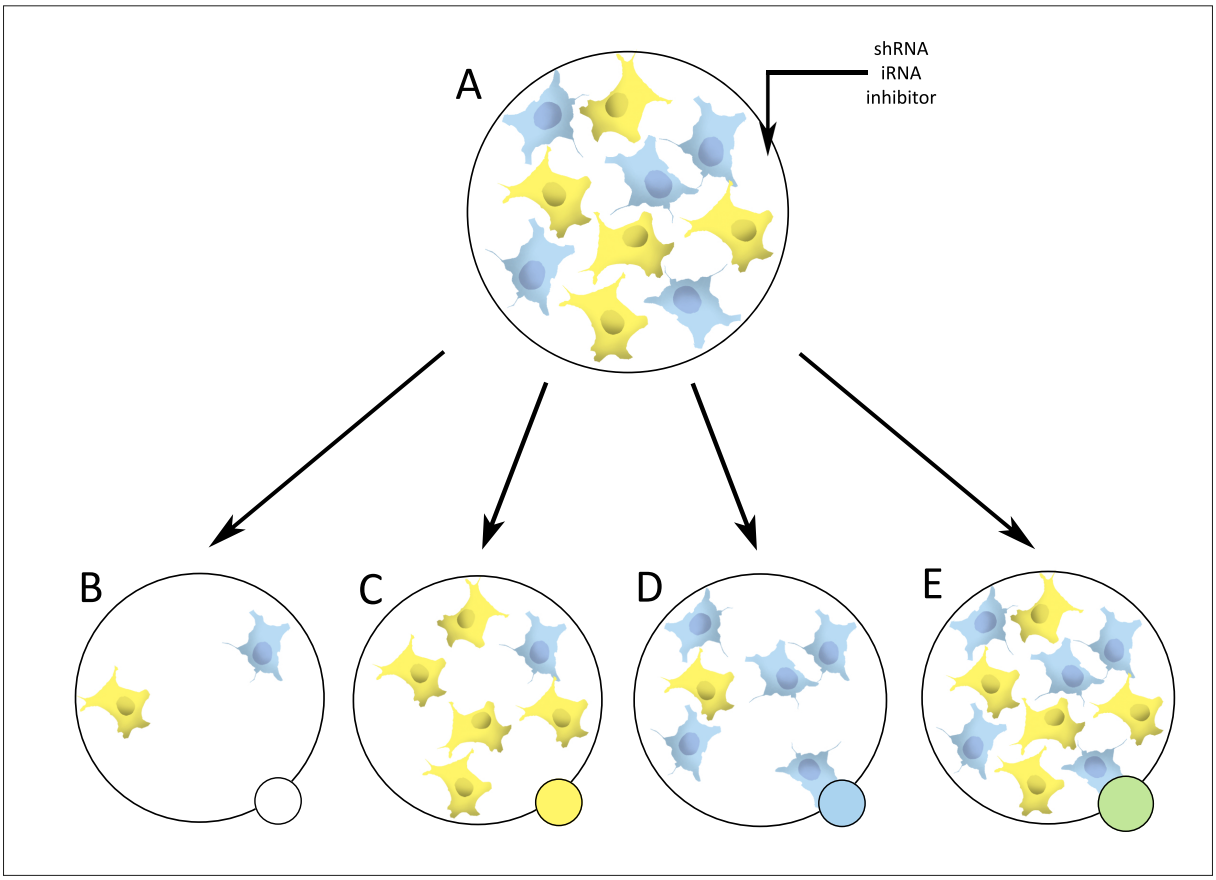
Standardowe związki stosowane w chemioterapii odkryto dzięki ich zdolnościom do eliminowania szybko dzielących się komórek. Niestety, nie są to tylko komórki nowotworowe, ale m.in. również szybko dzielące się komórki w cebulkach włosów, błonach śluzowych układu pokarmowego czy w szpiku kostnym. Ich działania niepożądane, takie jak utrata włosów, nudności czy obniżenie odporności, są ściśle związane z niewybiórczym działaniem chemioterapii. Związki te mogą być toksyczne dla komórek prawidłowych o obniżonym stopniu proliferacji, np. deksorubicyna (działa szkodliwie na serce), bleomycyna (płuca), cytarabina (móżdżek) [20]. Słabym punktem obecnych poszukiwań bezpiecznej i zarazem skutecznej terapii jest nie tyle niezdolność do odnalezienia związku, który zniszczy komórki nowotworowe, bo przecież takich odkryto wiele w ostatnich 50 latach, lecz odnalezienie związków, które zniszczą nowotwór w stężeniu nieszkodliwym dla pacjenta. Wiele z chemioterapeutyków używanych obecnie charakteryzuje się niskim indeksem terapeutycznym (stosunkiem dawki, która wywołuje objawy toksyczne, do dawki dającej efekt terapeutyczny) i wąskim oknem terapeutycznym (stężeniem leku, w którym można przewidzieć i opanować skutki zastosowania go w terapii – stężenie, w którym działanie terapeutyczne jest najwyższe, a działania niepożądane najmniejsze). Na wielkość tych czynników wpływ ma wiele zmiennych, np. niezwykle istotna jest swoistość działania leku, czyli to, jak wiele jest miejsc docelowych leku, które jednak nie są celem stosowanej terapii. Niezamierzone punkty działania terapii mogą być przyczyną pojawienia działań niepożądanych, a nawet mogą być antagonistami zamierzonych miejsc docelowych leku. Brak wybiórczości w działaniu chemioterapii stał się głównym czynnikiem limitującym wysokość dawki stosowanej podczas leczenia. Zaistniała więc wyraźna potrzeba odnalezienia kuracji, która skutecznie i selektywnie będzie eliminowała wyłącznie komórki nowotworowe, a jednocześnie nie będzie wykazywała działania toksycznego na komórki prawidłowe.

Zmiany genetyczne leżące u podstaw niestabilności genetycznej są niezwykle ważne w badaniach nad nowymi terapiami, ponieważ wszystkie nowotwory są genetycznie niestabilne, a zmiany te są prawdopodobnie niezbędne w procesie progresji i zezłośliwienia nowotworów. Dobrze wiadomo również, że defekty w wielu genach naprawy DNA, stwarzają możliwość wykorzystania w terapii konkretnego czynnika uszkadzającego DNA. Na przykład, mutacje w genach z rodziny *XP* (*Xeroderma pigmentosum*) uwrażliwiają komórki na światło ultrafioletowe, a mutacje w genach *ATM* (ataxia telangiectasia mutated) bądź *BRCA2* (breast cancer 2) uwrażliwiają komórki na promieniowanie jonizujące. Chociaż strategie te pozwalają przekształcić niestabilność genetyczną w potencjalną terapię, zróżnicowanie komórek nowotworowych, wynikające z ich niestabilności genetycznej, może jednocześnie zmniejszać skuteczność zaprojektowanych na tej podstawie leków [18,28].

Obecne badania są skupione na procesach i czynnikach pozwalających na odróżnienie komórek nowotworowych od pozostałych, prawidłowych komórek organizmu. Są to przede wszystkim czynniki, które biorą udział w przemianie komórki prawidłowej do zmienionej nowotworowo, przerzutującej komórki. Kluczowym jest również poznawanie potencjalnych miejsc działania terapii. Jednak ich mnogość jest obecnie czynnikiem zarówno dostarczającym materiału badawczego naukowcom, jak i utrudniającym identyfikację poszczególnych typów i podtypów nowotworów. Dotąd badania były nakierowane na swoiste cechy molekularne komórek nowotworowych i przyniosły kilka dobrze zapowiadających się terapii.

Muromonab-CD3 zapisał się na kartach historii jako pierwsze przeciwciało zastosowane w terapii. Stosowany był jako czynnik zapobiegający odrzuceniu przez biorcę przeszczepu. Jest to mysie przeciwciało monoklonalne swoiste dla antygenu CD3 limfocytów T. Wiąże się specyficznie do łańcucha glikoproteiny CD3, która jest zaangażowana w rozpoznawanie antygenów i stymulację limfocytu. Jednak okazało się, że niemodyfikowane przeciwciała mysie wywołujące odpowiedź ze strony układu odpornościowego człowieka, były nietrwałe i na ogół nie działały wydajnie. Pierwszym przeciwciałem zastosowanym przeciwko komórkom nowotworowym był rituximab (Rituxan), który został użyty w terapii nieziarniczego chłoniaka B-komórkowego. W 1998 r. jako potencjalną terapię przeciwko rakowi piersi z wykrytą nadekspresją onkogenu HER2/neu (v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2) (prawie 25% wszystkich nowotworów piersi) wyprowadzono trastuzumab (Herceptin). Takie podwyższenie ekspresji HER2/neu jest związane z bardziej agresywnym fenotypem nowotworu oraz gorszym prognozowaniem. Trastuzumab wiąże zewnętrzne domeny receptora HER2/neu (należy do rodziny receptorów czynników wzrostowych, jest receptorem kinazy tyrozynowej), zapobiegając tym samym powstawaniu kaskady sygnalizacyjnej i zwiększając stężenie białka p27 (należy do rodziny inhibitorów kinaz cyklinozależnych zwanej CIP/KIP – CDK interacting protein/Kinase inhibitory protein), co ostatecznie doprowadza do zatrzymania cyklu komórkowego. Stąd trastuzumab jest czynnikiem, który jest naprowadzony na szczególną cechę określonego rodzaju nowotworu [8,41].

Dalsze próby stworzenia swoistego leku przeciwnowotworowego zaowocowały zidentyfikowaniem i rozwojem małocząsteczkowych związków chemicznych (inhibitorów, aptamerów). Jednym z obecnie stosowanych leków tego typu jest opracowany już na początku lat 90 XX w. imatinib, który znalazł zastosowanie głównie w leczeniu przewlekłej białaczki szpikowej. Niekorzystne działanie na aktywność hybrydowej kinazy tyrozynowej powstałej w wyniku translokacji bcr-abl (tzw. chromosom Philadelphia), która jest odpowiedzialna za fenotyp przewlekłej białaczki szpikowej jest związana z konkurencyjnym wiązaniem się leku do domeny wiążącej ATP takiego zmienionego białka, co zaburza jej funkcjonalność. Ponieważ lek nie działał na wszystkie analizowane kinazy tyrozy-



Ryc. 2. Schemat badań przesiewowych pozwalających na identyfikację czynników wywołujących syntetyczną letalność. A – mieszanina komórek prawidłowych (niebieskie) i komórek nowotworowych izogenicznej linii (żółte) wyznakowanych fluorescencyjnie. Inkubacja mieszaniny z czynnikiem mającym wywołać w komórkach syntetyczną letalność, tj. biblioteka siRNA, shRNA czy małocząsteczkowy związek chemiczny (aptamer, inhibitor); B-E – analiza wybiórczości działania badanego czynnika; B – wywołanie syntetycznej letalności w obu typach komórek (znikoma fluorescencja); C – wybiórcze działanie na komórki prawidłowe (fluorescencja komórek nowotworowych); D – wybiórcze działanie na komórki nowotworowe (fluorescencja komórek prawidłowych); E – brak działania czynnika na komórki (fluorescencja zarówno komórek prawidłowych, jak i nowotworowych)

nowe, wyprowadzono wniosek, iż muszą istnieć różnice w ich domenach wiążących ATP, które stanowią zawadę przestrzenną do wiązania się go. Stwierdzono również, że imatinib działa hamująco na receptory płytkopochodnego czynnika wzrostu PDGF (platelet-derived growth factor), co prowadzi do zablokowania podziałów komórkowych [6].

Niestabilność genetyczna jest cechą typową dla komórek nowotworowych, a zmiany w materiale genetycznym są charakterystyczne dla takich właśnie komórek, zwłaszcza zmiany w genach naprawy DNA, które mogą uwrażliwiać nowotwór na działanie pewnych czynników uszkadzających. Jednym z pierwszych naukowców, którzy zajęli się wykorzystaniem syntetycznej letalności w poszukiwaniu nowych leków przeciwnowotworowych był Leland Hartwell. Jego prace oparte były na modelu drożdżowym. Sugerował, że czynnikiem limitującym rozwój nowoczesnych terapii przeciwnowotworowych i identyfikację nowych leków, oprócz problemu określenia bezpiecznej dawki terapeutycznej, jest również identyfikacja selektywnych cech nowotworów. Stąd przedmiotem jego badań stały się mutacje utraty funkcji genów, takich jak geny naprawy DNA, czy geny supresorowe. Badania w poszukiwaniu syntetycznej letalności mogą być prowadzone w dwojaki sposób: przez analizę genów, które potencjalnie mogą wykazywać interakcje z powstawaniem tego zjawiska bądź też przez analizę całego genomu. Wybór potencjalnych celów wymaga wielu wcześniejszych analiz, niezbędnych do poznania dróg i możliwości wywołania specyficznych mutacji w genach, koniecznych do analizy ostatecznych skutków funkcjonalnych w komórce, wywołanych kombinacją zmutowanych genów. Zespół Hartwella wyprowadził 70 linii izogenicznych *Saccharomyces cerevisiae*, każda z mutacją w pojedynczym genie, będącym elementem odpowiedzi na uszkodzenia, tj. naprawy DNA, czy też genów kodujących białka odpowiedzialne za regulację cyklu komórkowego. Mutanty te mogły więc być uważane za modele komórek nowotworowych. Badania przesiewowe w poszukiwaniu leków, które wywołują wyższą śmiertelność u mutantów, w stosunku do komórek typu dzikiego, pozwoliły na wyłonienie leków o cechach terapeutycznych, zarówno w interakcji z DNA, jak i z białkami odpowiedzialnymi za oddziaływanie z nim. Dobrym

przykładem takiego związku chemicznego jest cisplatyna, która powoduje wiele rodzajów mutacji, co odzwierciedlała mnogość wrażliwych mutantów drożdżowych. Co istotne natomiast, odznaczała się dużą swoistością działania na komórki, w których uszkodzeniu ulegały geny naprawy postreplikacyjnej. Natomiast mitoksantron, będący inhibitorem topoizomerazy II, wykazywał specyficzne działanie na linie drożdżowe z defektami w genach naprawy pęknięć dwuniciowych DNA. Możliwe staje się więc wykorzystanie cisplatyny i mitoksantronu w terapii nowotworów z mutacjami odpowiednio w genach naprawy poreplikacyjnej czy naprawy pęknięć dwuniciowych DNA. Wykazano ponadto potencjalną możliwość wykorzystania tego typu badań do identyfikacji oddziaływań międzygenowych, które mogą zostać wykorzystane do wywołania syntetycznej letalności [18].

Prawdopodobnie największą słabością współczesnych terapii jest niewielka skuteczność działania na zaawansowane postaci nowotworów, ze względu na ich złożoność, wynikającą z wieloetapowej i złożonej drogi ich przejścia od komórki prawidłowej do nowotworu złośliwego. Mimo że wiele wiadomo na temat ogromnej liczby aberracji zachodzących w komórkach nowotworowych, trudno przełożyć tę wiedzę na stworzenie skutecznej, swoistej terapii i minimalizację działań niepożądanych. Niewątpliwą zaletą terapii z wykorzystaniem syntetycznej letalności wydaje się właśnie potencjalna możliwość użycia jej w terapii początkowych, jak i zaawansowanych stadiów nowotworów, zarówno w terapii kombinowanej jak i jednoczynnikowej. Dużą skutecznością może cechować się terapia przez podawanie czynnika biorącego udział w wywoływaniu syntetycznej letalności z czynnikiem cytotoksycznym. Komórka nie będzie zdolna do naprawiania powstających uszkodzeń DNA, a ich nagromadzenie wprowadzi ją na ścieżkę programowanej śmierci.

Możliwości wykorzystania badań nad syntetyczną letalnością

W przeszłości badania syntetycznej letalności ograniczały się wyłącznie do organizmów modelowych, takich jak muszka owocowa czy nicień *Caenorhabditis elegans* [11,28]. Analizie podlegał nie tylko profil genetyczny, ale także jego wpływ na fenotyp osobnika, odzwierciedlający wpływ zastosowanego czynnika na organizm. Podobnie model drożdżowy okazał się nieoceniony w wyjaśnieniu zjawiska syntetycznej letalności u człowieka. Okazuje się jednak, że nie wszystkie geny ludzkie znajdują swoje ortologi w organizmach modelowych. Obecnie badania syntetycznej letalności nie ograniczają się wyłącznie do organizmów modelowych bądź też tylko do wybranych genów podstawowych dla komórek nowotworowych. Odnajdowane są wciąż nowe sposoby analiz tego zjawiska, takie jak wykorzystywanie bibliotek siRNA, shRNA, czy małocząsteczkowych związków chemicznych. Podejście takie pozwala na odnajdywanie w krótkim czasie genów biorących udział w interesujących badacza interakcjach. Prowadzenie badań z wykorzystaniem RNA wiąże się z wykorzystywaniem izogenicznych linii komórkowych, o ściśle zdefiniowanym genotypie, wykorzystywanym w badaniu oraz czy i jak komórki zareagują na analizowany czynnik. Dokładna analiza, nawet najbardziej nieoczekiwanego wyniku badań, może pozwolić na szczegółowe poznanie właściwości badanego czynnika, a przede wszystkim zwiększyć wiedzę na temat biologii nowotworów przez poznawanie punktów docelowych terapii, jak i swoistych sieci powiązań między genami w tych komórkach [7].

Wykorzystując biblioteki oparte na interferencyjnym RNA można się dowiedzieć, które geny są istotne w kontekście określonego szlaku. Pozwala to na odnajdywanie nieznanych wcześniej szlaków oddziaływań między genami, co nie zawsze pozwala na odnajdywanie potencjalnych kandydatów na czynniki terapeutyczne, ale niemal zawsze dostarcza istotnej wiedzy na temat biologii tych oddziaływań i czynników biorących w nich udział. Decydując się na wykorzystanie w badaniach bibliotek małocząsteczkowych związków chemicznych, takich jak inhibitory czy aptamery, celem eksperymentu może się stać wytypowanie czynników o właściwościach terapeutycznych dla konkretnego genotypu nowotworu (mających wywoływać syntetyczną letalność przy zadanym genotypie) [24]. Istnieją pewne przesłanki, kiedy do poszukiwań syntetycznej letalności powinno używać się iRNA, a kiedy bibliotek inhibitorów czy aptamerów. Zastosowanie poszczególnych schematów niesie ze sobą różne wady i zalety użycia metody. Przy wykorzystaniu bibliotek RNA działa się czynnikiem „odgórnie", czyli na jednostkę strukturalną, której funkcji nie zna się, po to, by poszukiwać oddziaływań, co pozwala na dokładną identyfikację funkcji genów i interakcji gen-gen. Wadą metody jest to, iż odnalezione interakcje nie zawsze prowadzą do wyprowadzenia potencjalnej terapii. Związane jest to z tym, że można nie znać czynnika, który mógłby być zastosowany do modulowania tych interakcji. Ponadto nieswoista toksyczność iRNA może prowadzić do fałszowania wyników. Zauważono np., że inhibitor receptora czynnika wzrostu naskórka EGFR (epidermal growth factor receptor) - erlotinib, zwiększał nieznacznie przeżycie w grupie pacjentów ze zdiagnozowanym rakiem trzustki. W celu zidentyfikowania czynnika, którego inhibicja będzie wzmacniała skutki blokowania EGFR, potraktowano komórki interferencyjnym RNA działającym na niemal 800 zaproponowanych genów, w obecności erlotinibu. Badania wyłoniły kilka kinaz, których zablokowanie poskutkowało obniżeniem przeżywalności komórek raka trzustki [30].

Zaletą metody przeszukiwania bibliotek małocząsteczkowych związków chemicznych jest to, iż bezpośrednio wyłaniają spośród potencjalnych kandydatów te, które istotnie wpłynęły na komórki. Podejście „oddolne" oznacza tyle, że aby zastosować metodę, trzeba mieć zidentyfikowany cel dla tego czynnika. Nie pozwala to natychmiast wyciągnąć jakichkolwiek wniosków na temat biologii, czy genetyki oddziaływań. Podejście takie okazuje się przydatne do analizy powiązania struktury białek i aktywności czynników, co może się stać pomocne przy optymalizacji działania tych czynników, czy identyfikacji metod zwalczania oporności na leki [7].

Zastosowanie metody genetycznej badania syntetycznej letalności, czyli bibliotek iRNA czy shRNA, jest oparte na prostej zasadzie transfekcji materiału genetycznego z zewnątrz komórki do jej wnętrza, gdzie ulega ekspresji lub interferencji. Dokonywać tego można za pomocą różnych podejść eksperymentalnych. Stosując biblioteki iRNA można umieścić komórki na płytkach, w których jest już obecny iRNA komplementarny do odcinka materiału genetycznego, w który się celuje. Do płytek dodany musi zostać również czynnik transfekcyjny. Obce RNA ze środowiska do wnętrza komórki można przetransportować również w wyniku elektroporacji – metoda opiera się na potraktowaniu komórki polem elektrycznym o odpowiednio wysokim napięciu, w celu odwracalnego zwiększenia przepuszczalności błony komórkowej i wniknięcia obcego materiału genetycznego do jej wnętrza. Metoda jest stosowana również do przenoszenia leków do wnętrza komórek. Tak potraktowane komórki poddawane są inkubacji, podczas której ma dojść do interferencji. Następnym etapem jest analiza przeżywalności komórek.

Transfekcji można też dokonywać wykorzystując wektory plazmidowe przenoszone np. przez *Escherichia coli,* czy w wyniku infekcji retrowirusowej [2,34]. Zaletą płynącą z wykorzystania plazmidów, jest możliwość dodatkowego zastosowania tzw. „kodów kreskowych", czyli niezmiennych, charakterystycznych sekwencji flankujących dodawany shRNA. Do linii izogenicznej, u której zmutowano gen A, transfekowany zostaje plazmid, w którym jest obecny gen shRNA blokujący gen B. Gen B jest prawdopodobnie „partnerem" dla genu A w kontekście pojawienia się syntetycznej letalności. Na plazmidzie gen shRNA został oflankowany charakterystyczną sekwencją swoistego „kodu kreskowego". Komórki poddaje się inkubacji w wybranych przez siebie warunkach, a następnie za pośrednictwem ilościowej łańcuchowej reakcji polimerazy qPCR (z zastosowaniem starterów komplementarnych do sekwencji kodu kreskowego) bądź też z użyciem mikromacierzy analizowana jest liczba poszczególnych sekwencji. Ma ona odzwierciedlać skuteczność działania zadanego shRNA, a więc też to, czy rzeczywiście zachodzi zjawisko syntetycznej letalności w wyniku interakcji między genami A i B [35]. W przypadku próby stworzenia potencjalnej terapii skierowanej przeciwko jednemu z tych genów, niezbędne będzie wykonanie drugiej analizy, w poszukiwaniu czynnika, który zablokuje gen bądź jego produkt. W tym celu przeprowadza się analizę metodą chemiczną, czyli z zastosowaniem np. małocząsteczkowych związków chemicznych, których zadaniem jest inhibicja wybranego czynnika i zablokowanie szlaku. Eksperymentami tego rodzaju były te przeprowadzone przez Hartwella na izogenicznych mutantach drożdży [18]. Do przeprowadzenia analizy tego typu potrzebna jest znajomość interakcji zachodzących między wybranymi genami oraz produktów, które kodują. Badania takie są przydatne nie tylko do poszukiwania potencjalnych terapii przeciwnowotworowych, ale również w przypadku prób ich optymalizacji oraz modyfikacji czynników terapeutycznych (np. odnalezienie najwydajniejszej postaci leku wykorzystywanego w terapii).

Przykładem takiego eksperymentu mogą być badania wykonane przez Arnolda Simonsa, których celem było ustalenie metody chemicznej do analizy zjawiska syntetycznej letalności w komórkach ludzkich. Jako mechanizm modelowy wybrana została biosynteza monofosforanu guanidyny (GMP - guanosine monophosphate), ponieważ składa się z kilku alternatywnych szlaków, które mogą się okazać idealne do przeprowadzenia analizy takich oddziaływań. W warunkach prawidłowych, GMP jest wytwarzane w procesie syntezy *de novo*. Synteza alternatywna przewiduje natomiast konwersję hipoksantyny do inozynomonofosforanu (IMP – inosine monophosphate) lub guaniny bezpośrednio do GMP. Do przejścia szlaków konwersji jest wymagana w obydwu przypadkach, fosforybozylotransferaza hipoksantynowo-guaninowa HPRT1 (hypoxanthine-guanine phosphoribosyl transferase), która nie jest niezbędna do przeżycia komórki w warunkach prawidłowych, natomiast w warunkach stresowych, kiedy zablokowane przez inhibitory zostaną szlaki syntezy *de novo*, może się stać istotna. Eksperyment zaprojektowany przez zespół Simonsa niejako integruje podejście genetyczne i chemiczne. Zakłada bowiem umieszczenie w komórkach pozbawionych HPRT1 i hodowanych z inhibitorami szlaku *de novo* (takimi jak kwas mykofenolowy) plazmidu, na którym oprócz białka HPRT1 umieszczony zostanie również gen wytwarzający białko zielonej fluorescencji GFP (green fluorescent protein). Przyrost ilości fluorescencji w detektorze po inkubacji będzie oznaczał wzrost transkrypcji genów *HPRT1*, a zatem również prawidłowe działanie inhibitorów szlaku *de novo*. Takie wysoko wydajne badania przesiewowe czynników, które potencjalnie wywołują w komórce syntetyczną letalność, mogą pomóc w tworzeniu terapii działających na wybraną parę genów, wykazujących opisane wyżej interakcje [39].

Celem przeprowadzania takich analiz jest personalizacja terapii przeciwnowotworowej, w której po przeanalizowaniu profilu ekspresji genów pacjenta cierpiącego z powodu konkretnego typu nowotworu, możliwym będzie przypisanie mu indywidualnego sposobu leczenia, który będzie dla niego wybiórczy i skuteczny. Możliwa dzięki takiemu podejściu stanie się minimalizacja działań niepożądanych, a więc również jakość rekonwalescencji osoby chorej.

Białka naprawy DNA a syntetyczna letalność w komórkach nowotworowych

Doskonałym celem do wywołania skutku syntetycznej letalności mogą zostać czynniki biorące udział w naprawie DNA. Systemy naprawy DNA składają się z wielu szlaków alternatywnych. Naprawa dwuniciowych pęknięć DNA DSBs (double strand breaks) odbywa się za pomocą – naprawy DNA przez rekombinację homologiczną HR (homologous recombination) oraz niehomologicznego łączenia końców NHEJ (non-homologous end joining). Komórki nowotworowe mają często mutacje w jednym ze szlaków naprawy DNA, alternatywnych względem siebie, powodując jego unieczynnienie. Sytuacja ta może być przydatna w przypadku wyłączenia pozostającego szlaku funkcjonal-

nego, w celu wywołania syntetycznej letalności w tych komórkach. Niezwykle istotnym czynnikiem naprawy DNA przez HR jest białko RAD52, którego rolą jest rozpoznanie uszkodzenia, wiązanie pojedynczej nici DNA i pośrednictwo w łączeniu homologicznych końców DNA. Ponadto wiąże ono białko RAD51 i stymuluje je do homologicznego łączenia nici. Szczególnie istotną rolę odgrywa w komórkach z defektem białka BRCA2 odpowiadającego zarówno za transport RAD51 do jądra komórkowego, jak i za jego umiejscowienie podczas przebiegu naprawy DNA. BRCA2 i RAD52 współdzielą wiele funkcji, takich jak wiązanie jednoniciowego DNA, oddziaływanie z RAD51 i białkami RPA (replication protein A). Pozwala to domniemywać, że możliwe jest wywołanie efektu letalnego w komórkach nowotworowych mających mutację w genie *BRCA2*, przez zastosowania inhibicji białka RAD52 [12,17].

Jeden z eksperymentów, wykorzystujący wyżej opisane geny naprawy DNA, przeprowadzono na dwóch liniach komórkowych, z których jedna (Capan-1 – komórki ludzkiego raka trzustki) miała tylko jeden gen BRCA2, który dodatkowo zawierał delecję, co skutkowało tworzeniem krótszej postaci białka. Druga linia (EUFA423 – komórki wyprowadzone od pacjenta z anemią Fanconiego) wytwarzała natomiast dwie zmutowane wersje tego białka. W pierwszej linii określono niższy poziom ekspresji białka RAD52 i, jak oczekiwano, nie wykryto białka BRCA2 o prawidłowej długości. W przypadku EUFA423 wykrywano jedynie niewielkie ilości zmutowanego BRCA2 zarówno w jądrze, jak i w cytoplazmie, co pozwalało domniemywać, że taka postać białka może być niestabilna i szybko ulega degradacji. Białko RAD51 jest obecne w strukturach subjądrowych w postaci tzw. skupisk jądrowych, które nie tylko pozwalają na wykrycie obecności tego czynnika, ale także odzwierciedlają intensywność przebiegu naprawy przez rekombinację homologiczną. Obydwie linie przeanalizowano pod kątem obecności takich miejsc w jądrze przed i po stymulacji promieniowaniem jonizującym. W pierwszej z linii nie wykryto istotnych zmian, natomiast w drugiej zaobserwowano 15% wzrost liczby skupisk RAD51. Ponieważ niewiele cząsteczek BRCA2 w drugiej z linii osiąga jądro komórkowe, podejrzewa się, iż taki wzrost był spowodowany zwiększoną aktywnością RAD52, co mogło być spowodowane niedoborem funkcjonalnej wersji białka BRCA2. Analizowano również oddziaływanie białek RAD52 i RAD51 przez ich kolokalizację. W komórkach, na które nie podziałano promieniowaniem jonizującym, kolokalizacja osiągała niski poziom. Po zastosowaniu czynnika uszkadzającego, oprócz przyrostu w czasie skupisk obydwu tych białek obserwowano również ich umiejscowienie w podobnych miejscach jądra, co potwierdza tezę, że RAD52 funkcjonuje jako regulator funkcji RAD51 pod nieobecność BRCA2. Obniżenie poziomu RAD52 za pomocą siRNA doprowadziło do zmniejszenia zdolności komórek ze zmutowaną wersją genu *BRCA2*, do tworzenia skupisk jądrowych z RAD51, a więc i skuteczności naprawy przez homologiczną rekombinację [17]. Mutacje w genie *BRCA2* nie są jednak często badane, stwierdzano natomiast obniżenie ekspresji tego czynnika np. w sporadycznym raku piersi i jajnika. Potencjalnie toksyczność zastosowanych inhibitorów białka RAD52 w komórkach z defektem w genie BRCA2 może się przyczynić do powstania strategii działania przeciwko tej grupie nowotworów. W układzie takim RAD52 jest syntetycznie letalne względem BRCA2.

Inny podejściem było wykorzystanie mutagenezy oraz aptameru, co pozwoliło wskazać fenyloalaninę 79 w domenie I białka RAD52 odpowiedzialnej za wiązanie DNA (RAD52-fenyloalanina 79 aptamer [F79]), jako ważny cel do indukcji syntetycznej letalności w komórkach nowotworowych z niedoborem BRCA1 i/lub BRCA2, bez wpływu na komórki i tkanki prawidłowe. Połączenie aptameru F79 z białkiem RAD52 zakłóca jego oddziaływanie z DNA, co prowadzi do akumulacji pęknięć dwuniciowych tylko w komórkach nowotworowych, w przeciwieństwie do komórek prawidłowych. Ujęcie takie może zainicjować spersonalizowane podejście terapeutyczne u wielu pacjentów z nowotworami wykazujących niedobór BRCA na poziomie genetycznym i funkcjonalnym [10].

Zadaniem polimerazy poli(ADP-rybozy) PARP1 (poly (ADP-ribose) polymerase 1) jest umożliwianie przebiegu naprawy pęknięć jednoniciowych DNA w systemie naprawy DNA przez wycinanie zasad azotowych BER (base excision repair), przez wiązanie się do jednoniciowego DNA i rekrutację białek naprawy do miejsca uszkodzenia. Inhibicja tego enzymu w komórkach z mutacjami w genach *BRCA1* (breast cancer 1) i *BRCA2* prowadzi do niestabilności chromosomowej, zablokowania cyklu komórkowego, a ostatecznie do śmierci komórki. Zahamowanie działania PARP1 powoduje zatrzymanie naprawy BER i konwersję pęknięcia jednodo dwuniciowego. Naprawą tego typu uszkodzeń zajmuje się natomiast w głównej mierze wcześniej wspomniany szlak HR (szlak NHEJ działa mniej wydajnie w przypadku uszkodzeń z lepkimi końcami). Jeśli w komórce będzie niski poziom białek naprawy DNA przez homologiczną rekombinację, takich jak BRCA1 i BRCA2, dojdzie do gromadzenia pęknięć dwuniciowych, na skutek niewydajnie zachodzącego procesu ich naprawy. Doprowadzi to do zatrzymania cyklu komórkowego i śmierci takiej komórki. Inhibitory PARP1 są jedną z pierwszych zidentyfikowanych grup niskocząsteczkowych czynników, które biorą udział w wywołaniu syntetycznej letalności. Hipotezę potwierdziły badania prowadzone przez zespół H. E. Bryanta. Początkowo były prowadzone na komórkach chomika chińskiego z defektem w naprawie DNA przez HR (dwie linie komórkowe pozbawione XRCC2 (X-ray repair complementing defective repair 2) lub XRCC3 (X-ray repair complementing defective repair 3), które to linie okazały się wrażliwe na działanie inhibitorów PARP. Następnie badania przeprowadzono na modelu bardziej odpowiednim dla komórek ludzkich. Ponieważ defekty genu *BRCA2* są charakterystyczne dla ludzkiego raka piersi, to właśnie takie linie wytypowano do eksperymentu. Przeżywalność obydwu linii znacząco zmniejszyła się po zastosowaniu inhibitorów polimerazy poli(ADP-rybozy). Do komórek dodawano również siRNA, którego zadaniem była minimalizacja ekspresji *BRCA2*. Utrata BRCA1 lub BRCA 2 i zadziałanie na komórkę

nowotworową inhibitorami PARP1 jest świetnym sposobem zaaranżowania potencjalnej, nowatorskiej terapii przeciwnowotworowej, opartej na wywołaniu zjawiska syntetycznej letalności, powstałego w wyniku defektu w naprawie DNA [5,16]. Inhibitory tego typu mogą się okazać nieocenione w walce z nowotworami piersi i jajników, a wiele leków tego typu znajduje się na etapie badań klinicznych [19]. Doskonale rokują również połączenia inhibitorów wywołujących syntetyczną letalność z cytostatykami, takimi jak cisplatyna, karboplatyna czy temozolomid. Dodatkowe uszkodzenia DNA wywoływane przez te cytostatyki, nie są skutecznie usuwane przez niefunkcjonalne, w komórkach nowotworowych, szlaki naprawy DNA. Dochodzi do kumulacji uszkodzeń oraz stopniowego obniżania przeżywalności komórek nowotworowych. Terapia taka działa selektywnie, pozostawiając nienaruszone komórki prawidłowe [31,36].

Naprawa błędnie sparowanych zasad DNA MMR (mismatch repair) jest innym z systemów usuwania uszkodzeń DNA, który odgrywa główną rolę w ustalaniu homeostazy genetycznej komórki. Zadaniem MMR jest m.in. poreplikacyjne usuwanie błędów popełnianych przez polimerazę DNA, ale również naprawa zasad zawierających 8-oxo-guaninę, która powstaje w komórkach na skutek kontaktu guaniny z reaktywnymi formami tlenu. Mutacje w genach MMR, takich jak *MLH1* (MutL homolog1), *MSH2* (MutS Homolog2), *MSH6* (MutS Homolog 6) czy *PMS2* (postmeiotic segregation increased 2) skutkować mogą m.in. przyspieszonym starzeniem, powstawaniem nowotworów, czy tzw. syndromu Lyncha (dziedziczny rak jelita grubego niezwiązany z polipowatością) [25]. Gen *PINK1*(*PTEN* induced putative kinase 1) koduje natomiast białko kinazy serynowo-treoninowej, która jest umiejscowiona w mitochondriach. Jej główną rolą jest ochrona komórki przed stresem oksydacyjnym, który może być powodem nieprawidłowego działania zarówno tych organelli, jak i całej komórki. Niedobór tego białka prowadzi do akumulacji reaktywnych form tlenu i powstawania oksydacyjnych uszkodzeń DNA, takich jak 8-okso-dG (8-oxoguanine) w genomie komórek z mutacjami w genach naprawy MMR. Podczas replikacji dochodzi do nieprawidłowego parowania zmienionej guaniny z adeniną, co powoduje później powstawanie miejsca AP (apurinic/apyrimidinic site), ma to na celu ograniczenie transwersji GC → TA. W kolejnych cyklach replikacyjnych powoduje to akumulację pęknięć jednoniciowych DNA, co może znacząco wpływać na prawidłowe funkcjonowanie komórki, a także na jej przeżywalność [26]. Wykazano ponadto, iż możliwe jest wywołanie syntetycznej letalności przez zastosowanie inhibitorów polimerazy DNA β w komórkach z niedoborem białka MSH2 oraz inhibitorów polimerazy DNA γ w komórkach z niedoborem aktywności MLH1. Zastosowanie ich, podobnie jak we wcześniejszym, przypadkowo prowadziło do gromadzenia 8-oxo-G w genomie jądrowym w przypadku pary MSH2/pol β lub w genomie mitochondrialnym w MLH1/pol γ [26].

Gen *PTEN* (phosphatase and tensin homolog) to gen supresorowy, do utraty funkcji, którego dochodzi w licznych przypadkach nowotworów, zarówno sporadycznych, jak i dziedzicznych. Jego mutacje są często wykrywane w przypadkach nowotworów piersi, macicy, stercza, tarczycy, skóry czy też w glejakach. *PTEN* wykazuje dwojaką naturę – fosfatazy białkowej i lipidowej. Gen *PTEN* koduje lipidową fosfatazę fosfatydyloinozytolu 3, która odpowiada za defosforylację trójfosforanu fosfatydyloinozytolu PIP3 (phosphatidylinositol (3,4,5)-triphosphate). PIP3 działa stymulująco na szlak kinazy AKT (*v*-Akt murine thymoma viral oncogene homolog 1), który zapobiega przebiegowi programowanej śmierci komórki i stymuluje syntezę białek. PTEN przez inaktywację PIP3 wpływa na zatrzymywanie cyklu komórki w fazie G1 i doprowadza do jej wejścia na drogę programowanej śmierci. Komórka pozbawiona prawidłowo funkcjonującego genu *PTEN* wymyka sie spod kontroli i podlega niekontrolowanym podziałom. Białko MTOR (mechanistic target of rapamycin) bierze udział w mechanizmach regulujących przeżycie komórki w warunkach niedoboru substancji odżywczych. Ponieważ uczestniczy ono w szlaku zależnym od Akt i kinazy PI3, postanowiono przeanalizować interakcje genetyczne zachodzące między MTOR a PTEN w sytuacji obniżonej ekspresji tego drugiego. Wykorzystano w tym celu pochodną rapamycyny, która powoduje zatrzymywanie cyklu komórkowego w fazie G1. Działa na MTOR i inhibuje jego fragment odpowiedzialny za fosforylację substratów, takich jak kinaza S6 czy 4E-BP1. Jak się okazało zarówno linie ludzkich komórek nowotworowych, jak i komórki mysie, pozbawione genu PTEN były wrażliwe na działanie rapamycyny. Utrata funkcjonalnego PTEN może predysponować komórki do podatności na zahamowanie działania MTOR nawet niskimi dawkami pochodnej rapamycyny. Wynik potwierdzała również analiza poziomu kinazy S6 [32]. Gen *PTEN* jest niezwykle istotny do utrzymania stabilności genetycznej komórki. Występujące w nim mutacje mogą zupełnie pozbawić komórkę naprawy DNA przez rekombinację homologiczną. W takiej sytuacji możliwe jest wykorzystanie inhibitorów skierowanych przeciwko białkom szlaku BER, takim jak PARP1. W przypadku niefunkcjonalności tego szlaku, nienaprawione uszkodzenia zostaną przekonwertowane do uszkodzeń dwuniciowych. Niefunkcjonalny szlak HR nie będzie zdolny do naprawy tych uszkodzeń, co doprowadzi do śmierci komórki [29].

Opisane badania są przykładami wykorzystania syntetycznej letalności w badaniach podstawowych, jak i próbach stworzenia spersonalizowanej terapii przeciwnowotworowej. Badania te pozwalają odnaleźć cechy odróżniające komórkę nowotworową od prawidłowej, co powoduje, że potencjalna terapia przeciwnowotworowa może się stać ściśle celowana i wywołać możliwie najmniej działań niepożądanych. Użyteczność wykorzystania narzędzia syntetycznej letalności nie kończy się tylko na identyfikacji potencjalnych terapii przeciwnowotworowych. Niezwykle istotnym celem tego typu analiz jest bowiem poznanie biologii molekularnej nowotworów i interakcji międzygenowych, czy też ich produktów, zachodzących w toku ich rozwoju.

Podsumowanie

Syntetyczna letalność jest niezwykle użytecznym narzędziem zarówno w procesach definiowania funkcji genów, jak i do poznawania biologii nowotworów i poszukiwania potencjalnych terapii przeciwnowotworowych. Postęp technologiczny i możliwość wykorzystania bibliotek iRNA, shRNA, jak i małocząsteczkowych związków chemicznych, takich jak inhibitory czy aptamery, okazują się niezwykle użyteczne do poznawania natury syntetycznej letalności i próby jej wykorzystania u ludzi. Chemioterapia jest metodą walki z nowotworami stosowaną od kilku dekad, jednak wciąż nie zmieniały się priorytety jej działania. Stosowane są głównie leki eliminujące szybko dzielące się komórki. Takie podejście jest przyczyną pojawiania się u pacjentów wielu działań niepożądanych oraz znacząco utrudnia ich rekonwalescencję. Syntetyczna letalność może w obecnej sytuacji okazać się złotym środkiem na selektywne zabijanie wyłącznie komórek nowotworowych. Wykorzystanie jej w połączeniu z radioterapią, czy czynnikami cytotoksycznymi może dodatkowo zwiększać skuteczność tego typu terapii. Badania syntetycznej letalności znajdują się jednak w początkowych etapach i wymagane jest jeszcze przeprowadzenie wielu analiz tak, by dokładnie poznać to zjawisko. Jednak potencjalne, przyszłościowe korzyści dla człowieka, jakie będzie można uzyskać w rezultacie z przeprowadzonych badań, z pewnością zrekompensują poświęcone środki.

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REVIEW ARTICLE

DNA Double Strand Break Repair - Related Synthetic Lethality

Monika Toma[1], Tomasz Skorski[2,*] and Tomasz Sliwinski[1,*]

[1]*Laboratory of Medical Genetics, Faculty of Biology and Environmental Protection, University of Lodz, Pomorska 141/143, 90-236 Lodz, Poland;* [2]*Department of Microbiology and Immunology, 3400 North Broad Street, Temple University Lewis Katz School of Medicine, Philadelphia, PA 19140, USA*

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Abstract: Cancer is a heterogeneous disease with a high degree of diversity between and within tumors. Our limited knowledge of their biology results in ineffective treatment. However, personalized approach may represent a milestone in the field of anticancer therapy. It can increase specificity of treatment against tumor initiating cancer stem cells (CSCs) and cancer progenitor cells (CPCs) with minimal effect on normal cells and tissues. Cancerous cells carry multiple genetic and epigenetic aberrations which may disrupt pathways essential for cell survival. Discovery of synthetic lethality has led a new hope of creating effective and personalized anti-tumor treatment. Synthetic lethality occurs when simultaneous inactivation of two genes or their products causes cell death whereas individual inactivation of either gene is not lethal. The effectiveness of numerous anti-tumor therapies depends on induction of DNA damage therefore tumor cells expressing abnormalities in genes whose products are crucial for DNA repair pathways are promising targets for synthetic lethality. Here, we discuss mechanistic aspects of synthetic lethality in the context of deficiencies in DNA double strand break repair pathways. In addition, we review clinical trials utilizing synthetic lethality interactions and discuss the mechanisms of resistance.

Keywords: DNA repair, double strand breaks (DSB), synthetic lethality, anticancer therapy, radiotherapy, PARP.

1. INTRODUCTION

Most of the anticancer therapeutics currently in use lack specificity of their actions in that the number of their off-targets is still too high and this remarkably influences a patient's convalescence. Their ability to kill rapidly proliferating cells also goes against normal cells with high division rate. In addition, many drugs, including bleomycin, cytarabine or doxorubicin are toxic also to normal tissues (namely to lungs, cerebellum and heart), hence are of low therapeutic index. Taking all that into consideration, the vulnerability of current cancer treatment is not in its inability to find a compound that will be able to kill cancer cells, but in finding one that will work specifically and eliminate cancer cells at a dose that will not cause toxic effect to other tissues [1, 2]. For the last two decades, the efforts of researchers have been focused on finding the tools that would be able to eliminate cancer cells not only quickly but also as pointedly as possible. Nowadays, the need for rationally designed selective drugs has a great chance of achievement due to multiple studies being carried out on personalized anticancer therapy. Such approach takes into account molecular uniqueness of each patient's cancer cells and attempts to find a treatment that would most effectively influence them, while the primary goal of personalized therapy is to select an individual specific treatment with the least side effects. Carcinogenesis is a long-term complex mechanism involving accumulation of multiple changes in a cell's genome. Therefore, we are looking for changes in the DNA of cancer cell that would let us distinguish them from normal cells and create an innovative treatment that specifically target them. Carrying out a panel analysis (e.g. gene and protein expression analysis, oncogene detection) and obtaining patient-specific results could allow us to aim molecular changes for a specific patient tumor.

*Address correspondence to these authors at the 3400 N. Broad Street, MRB 548, Philadelphia, PA 19140, USA; Tel: +1 215-707-9157, Fax: +1 215-707-9160 e-mail: tskorski@temple.edu; 141/143, 90-236 Lodz, Poland; Tel: +48 42-635-44-86; Fax: +48 42-635-44-84; E-mail: tomasz.sliwinski@biol.uni.lodz.pl

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Cancerous cells develop genomic instability responsible for tumor progression and accumulation of numerous modifications in their genome [3]. Changes in the functioning of essential processes for cell survival (e.g. DNA repair mechanisms) could be exploited as a novel tool for personalized anticancer therapy. In tumor cells, loss of one of the genes, the products of which are crucial for such pathways, is highly probable due to the enormous variety of rearrangements in their genomes. Under such conditions, in order to survive, cancer cells must find a substitute of the lost pathway, ipso facto, becoming "addicted" to it. Targeting such alternative mechanisms could potentially sensitize malignant cells, subsequently resulting in their death (Fig. **1**). The phenomenon described above is termed "synthetic lethality" and has lately become a novel tool for personalized anticancer therapy. Lethal effect is caused by the loss of function of two and more genes, whereas individual mutation in each of these genes does not cause any changes in cells viability [4, 5]. Researches over this phenomenon are conducted within many cellular pathways. New trends in developing a synthetic lethality based on alterations in mechanisms like DNA repair are very likely to have a great impact on specificity and efficiency of novel anticancer therapies. Cancer-specific abnormalities and malignant cells' reliance on compensatory pathway create an opportunity to induce synthetic lethality through targeting a gene (or its product) that is essential for proper functioning of back-up pathway. This approach will enable us selectively eliminate cancer cells without harming the functions of normal cells, because even after inhibition of alternative mechanism, proper activity of basic pathway will rescue them from the effects of the inhibitor [6]. It is believed that such "resistance" of cells to mutations of some genes by redirecting crucial processes to alternative pathways could play a major role in the evolution of multifactorial diseases such as cancers [7, 8]. Cell survival is ensured by functional "genetic excessiveness", e.g. by evidence of two pathways complementing each other or having two alleles of one gene [9, 10]. Synthetic lethality is already contributing to a broader understanding of associations occurring between pairs of genes in malignant cells.

Neoplastic diseases are extremely heterogeneous. Moreover, the bulk of each tumor itself does not consti-

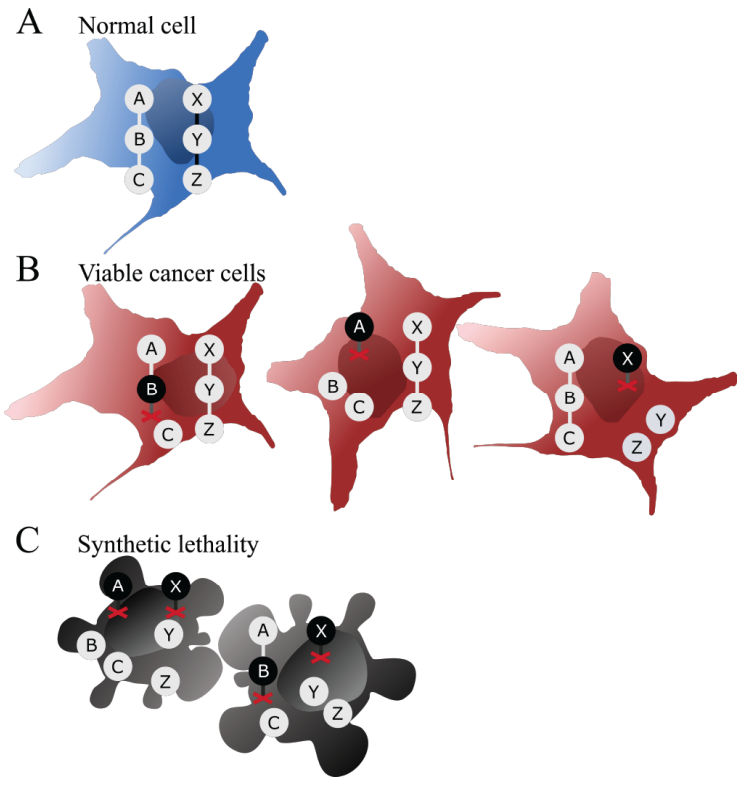

Fig. (1). Synthetic lethality scheme. A. Normal cells possess two functional, alternative pathways of the process crucial for their survival (depending on the genes marked with either ABC or XYZ). B. In tumor cells, genomic instability often results in loss of function mutation in one of such genes eliminating one of the pathways arising cell "addiction" to the alternative route. C. When one of the genes of both ABC and XYZ pathway is impaired due to the loss of function mutation or to the targeting it with inhibitor, cancer cell dies. Treatment with inhibitors affecting one of the pathways would not influence normal cells, since basic pathway remains functional in them.

tute a homogenous structure but consist of cancer progenitor cells (CPCs) and a small cohort of quiescent and proliferating cancer stem cells (CSCs), which act as the therapy refractory and tumor initiating cells that are usually responsible for tumor relapse [11-15]. Synthetic lethality, in contrast to currently available therapies, offers the opportunity to eliminate drug-resistant CSCs and CPCs. A correlation between disrupted DNA repair mechanisms and survival of cancer stem and progenitor cells undergoing genotoxic stress induced by reactive oxygen species (ROS) and cytotoxic treatment has been established [16]. CSCs and CPCs "addicted" to their DNA repair pathways could be eliminated by targeting these mechanisms.

The idea of synthetic lethality is gaining increasing attention each year, with researchers continuously designing new methods helpful in discovering synthetic lethality interactions - screens with the use of CRISPR method (Clustered Regularly Interspaced Short Palindromic Repeats) [17], shRNA (short hairpin RNA) [18, 19], small-molecule libraries [20], or reported this year MiSL (Mining Synthetic Lethals) [21]. Additionally, original comprehensive methods are being invented to detect abnormalities in pathways of cancer cells. At present, it is recommended to use next-generation sequencing (NGS) including analysis of genetic instability markers like loss of heterozygosity (LOH) or telomeric allelic imbalance (TAI) for identification of patients carrying abnormalities [22, 23]. Recent novel methods, like GEMA (Gene Expression and Mutation Analysis), have the potential of finding a clinical use through detecting abnormalities that could be exploited in anticancer therapy based on synthetic lethality [24]. Factors that could become targets for such approach can be found easily in the first synthetic lethality interaction predicting database - SynLethDB, which collects information from published data, multiple screens, *in silico* predictions and related databases [25]. Efforts of researchers are also focused on finding new compounds and new approaches that could be used to inhibit activity of alternative pathways. The US Food and Drug Administration (FDA) approval for PARP (Poly [ADP-ribose] polymerase) inhibitor - olaparib (Lynparza™) in monotherapy of patients with BRCA-depleted ovarian cancer is a good evidence of the progress made in this field [26]. Hopefully, further progress will allow us exploit a broad range of compounds and target molecular uniqueness of broader spectrum of tumor types.

2. SYNTHETIC LETHALITY – A NOVEL APPROACH TO CANCER TREATMENT IN PERSONALIZED ANTICANCER THERAPY

The concept of a clinical therapy based on death induction in cancer cells carrying mutations impairing activity of the mechanisms essential for cell survival, by inhibiting activity of back-up path that the tasks were rewired to, is termed "synthetic lethality". This phenomenon owes its name to Theodor Dobzhansky, however, it was observed for the first time in 1922 in a macro scale on a fruit fly by Calvin Bridges [27, 28]. Early studies over synthetic lethality interactions were performed on yeasts as a great number of their genes have human orthologs [29, 30]. Studies over synthetic lethality interactions contribute to the increasing knowledge on genes function, molecular biology of the cancer and mechanisms of action of anticancer compounds [31]. Revolutionary idea of using such approach in anticancer therapy was first published in 1997 by a Nobel Prize winner Leland H. Hartwell. He described it as a method of a great therapeutic potential that could also help us identify webs of interactions underlying malignant phenotype [32].

Carcinogenesis seamlessly changes normal cells into cancerous cells and to highly invasive derivatives at the final phase. Genetic and epigenetic deregulations are the hallmarks of tumor cells which let them exhibit increased proliferative potential, evade apoptosis and growth suppressing signals, ensure self-sufficiency in growth signaling as well as metastatic and tissue-invasive character at its final stages [33-36]. Extensive sequencing studies revealed landscape of over 140 more ("mountains") and less ("hills") often altered genes promoting tumorigenesis. Although the general number of mutations occurring in a typical cancer is outstanding, only two to eight of them are essential for tumor development and progression [37-39]. Such changes can become the source of the so called "oncogenic addiction" of malignant cells viability to mutations and to pathway redirection after loss or reduction of functions critical for their cell survival mechanism. Also a separate name – "non-oncogenic addiction" – has been proposed for similar interactions occurring in genes that are not themselves classified as oncogenes, but are crucial for cancer cells survival and support malignant phenotype [40-42]. Interactions of both kinds bring to light new vulnerabilities which make cancer cells more dependent on the functions of some oncogenes and hypersensitive to reactivation of suppressor genes [43].

The changes arising in the genome of cancer cells constitute unique features that help distinguish them from their normal analogues. Targeting back-up pathways, which buffer the loss of mechanisms crucial for cancer cell survival, with genetic or chemical methods, should offer a specific and efficient therapeutic solution. Many examples can be given to demonstrate the potential of anticancer therapy based on synthetic lethality. Multiple genes involved in many cellular functions including metabolism or signaling pathways have been reported to demonstrate such interactions with one or more partner genes [44-49]. Furthermore, DNA repair mechanisms appear to be the ideal target for developing the therapy because of the tumor-specific changes which were found in multiple genes crucial for these pathways. Moreover, some proteins involved in repair create a web of interactions between multiple pathways. A good example is PARP1 directly involved in base excision repair (BER), alternative non-homologous end joining repair (altNHEJ) and promoting homologous recombination (HR) [50]. Because of the wide variety of functions it exhibits, PARP1 is lately being extensively examined as a target for synthetic lethality-based anticancer therapy in tumors with detected abnormalities in DNA repair pathways like HR and classical non-homologous end joining (cNHEJ). All mechanisms that restore the original DNA sequence share similarities in the order of their stages, e.g. damage detection and strand binding by repair machinery, gap filling and ends ligation, meaning that there could be many potential synthetic lethality interactions occurring and many potential targets for the therapy [51]. Synthetic lethality-based therapy can be used to enhance the efficiency of currently used agents e.g. cytotoxic drugs or radiotherapy. Inhibition of essential for cell survival alternative pathways may cause sensitization to such agents. For instance, deactivation of double strand breaks repair pathways may create the environment suitable for the cells sensitivity to factors giving rise to DNA lesions.

3. DOUBLE STRAND BREAK CLASSICAL AND ALTERNATIVE REPAIR PATHWAYS

Our genetic material is exposed to the numerous endogenous and exogenous damaging factors including ROS generated during cell's metabolism, unusual DNA structures, collision of transcription and replication machinery, radiation, hypoxia, carcinogens delivered with food or medicines including chemotherapeutics [52-54]. Every compound of a DNA structure can be influenced by such agents. For this reason, DNA repair systems are critical to maintain the stability of the genome and prevent cell from malignant transformation or death. Cell physiological aging occurs by accumulation of oxidative DNA damage caused by free radicals having their origin in the cell's metabolism. Each day, some 10^5 oxidative DNA lesions per cell appear but are rapidly repaired in our genome by complicated DNA repair machinery. Some of them are converted to single strand DNA breaks (SSBs) occurring approximately 5000 times/cell daily [55, 56]. The most toxic DNA lesions are double strand breaks (DSBs) which are defined as complete disruptions in chromosome integrity [57, 58]. In comparison to the SSBs, where the original sequence can be easily restored based on access to the uninterrupted, complementary strand, faithful rebuilding of both chromosome integrity and order of the sequence after DSB occurrence could by an uphill task for repair machinery. Pathological DSBs arise as a result of ionizing radiation (IR), response to the clastogen (i.e. temozolamide, cisplatin, mitomycin C, bleomycin), as well as from endogenous processes like replication on the template including unrepaired lesion or from disrupted repair processes [59]. DSBs can be the reason of cell death and contribute to genomic instability by causing mutations, genome rearrangements, neoplastic transformation and LOH [60]. About 2-3% of malignant cells chromosomes seams to demonstrate the "scars" appearing after numerous DSBs, deletions or inversions it suffered from [61]. To prevent such hazardous effects of DNA lesions, organisms have developed a wide range of proteins taking part in DSB repair pathways.

DSBs are extremely toxic lesions that are repaired in cells either by HR repair or NHEJ and their two sub pathways. HR system is able to restore the original sequence based on the template of sister chromatid or homologous chromosome. That mechanism has its pros and cons – though it is error-free, for its activity it requires short distance to the homologous sequence, meaning it can be active during G2/S phases of the cell cycle due to the short distance from homologous sequence [62]. On the other hand, NHEJ is a fast repair mechanism that doesn't require extensive homology. It can work directly on the blank ends or use one or several base pairs of microhomology between short single stranded DNA (ssDNA) overhangs that arise due to the chemical nature of DSB or due to the activity of template-independent polymerases taking part in NHEJ. However, because this repair system may cleave the DSB in search of microhomology between overhangs or to achieve DSBs form it will be able to ligate, some part of the genetic information can get lost in the process. Moreover, polymerases that can take part in NHEJ

mechanism can cause insertions due to their template-independent activity [63]. What is more, the partner strands for NHEJ repair are not always chosen properly what makes this system being considered error-prone [64].

The cell cycle phase and the chemical nature of the DNA ends are the factors that play a crucial role in selection of machinery to approach lesion. It is very unlikely for DSBs to have ends that can be directly connected. In most cases they are incompatible due to the overhangs and chemical modifications at their termini, meaning such elements must be removed by resection process which ensures that both ends have structures that can be processed during the repair. Canonical NHEJ is able to work on ligatable, blunt or near-blunt DSBs that are not damaged or don't include any modifications. Resection in this process involves small regions [65]. HR on the other hand can perform more extensive end-resection led by the MRN complex (MRE11/RAD50/NBS1) [66]. Early stage of resection termed "end clipping" removes relatively short fragment of one of the strands predisposing the ends to be processed by altNHEJ, further "extensive resection" by helicases and exonucleases leads to creation of long ssDNA overlaps that are the substrates for HR [67]. Competition between DSB repair processes settles between Ku and MRN complexes as well as between antagonistic p53 binding protein 1(53BP1) and BRCA1 proteins. The first one accumulates in the vicinity of DSB by recognizing dimethylation of H4 histone (H4Lys20) [68]. It promotes NHEJ repair by blocking BRCA1 – mediated end resection which would have promoted HR [69, 70]. Lack of H4Lys20 methylation after replication weakens the affinity of 53BP1 to the lesion and promotes BRCA1-mediated end resection and HR [71, 72]. Moreover, it has also been proposed that BRCA1 may play a role in promoting cNHEJ pathway and not altNHEJ by inhibiting the end-resecting activity of MRN complex which acts at the initial stage of altNHEJ [73].

Although NHEJ system may constitute one of the reasons of genomic instability, it is still the major DSB repair mechanism in human and other higher eukaryotes cells. It can be divided into two subpathways – classical or canonical NHEJ (cNHEJ) and back-up NHEJ (bNHEJ) also termed alternative (altNHEJ) or microhomology-mediated end joining (MMEJ) (Fig. **2**). Both classical and alternative paths are genetically independent of each other, and there seems to be no considerable crosstalk between them [74-76]. First step of cNHEJ repair process is damage recognition and binding by Ku heterodimer (Ku70/Ku80) which creates a ring around each end of a lesion and protects them from nucleases. Ku rings exhibit barely any contact with helix and their interactions are based more on proper steric adaptation of the Ku complex to the shape of DNA grooves [77, 78]. What is more, Ku heterodimer has been lately identified as an effective 5' dRP/AP lyase [79-81]. Ku performs a primary role in promoting and proper functioning of NHEJ repair, not only by detecting the DSBs, protecting them and removing nucleotide damage near DSBs ends, but also by serving as a kind of protein loading factor which recruits other core repair proteins required lesion repair [82]. When bound to DNA, Ku complex exhibits a high affinity to the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) and stimulates its activity, resulting in formation of DNA-PK complex at each end of the damage [83, 84]. The primary function of this compound is tethering the ends of the lesion and recruiting different proteins depending on the phosphorylation of its different residues. DNA-PKcs protein contains two clusters of conserved sites (ABCDE and PQR) that, upon activation of a protein, can be autophosphorylated or phosphorylated by other kinases. Modification in ABCDE cluster on both sites of DSB results in opening lesion conformation, leaving it available for ligases. Ends-processing mechanisms are promoted when phosphorylation of the ABCDE sites occurs only on one side. Modification in the second cluster of DNA-PK protein – PQR results in more sealed conformation of the synapse, which blocks nucleolytic end-edition activities. Instead, it allows ligation and polymerization processes to take place. It has been proposed that phosphorylation in this cluster occurs after microhomology appears between the opposite ends of DSB. Its role is to hold such a structure in place, stabilize it and open it for polymerases and ligases. DNA-PK inactivation and dissociation is mediated by phosphorylation in T site and other places [85]. It has been suggested that blunt ends are less likely to activate DNA-PKcs activity than single strand overhangs [86]. When Ku-bound, DNA-PKcs can recruit and activate Artemis nuclease which exhibits both exo- and endonucleolytic properties and can modify a wide variety of DNA substrates at the border between double-stranded DNA and single strand overhangs. Artemis requires phosphorylation of ABCDE cluster of DNA-PKcs to access the DNA [87]. End-editing during NHEJ repair can be performed by a group of different nucleases, including apraxin (APTX), PNKP-like factor (APLF), CtBP-interacting protein (CtIP) or previously mentioned MRN complex [88-90]. Ku complex

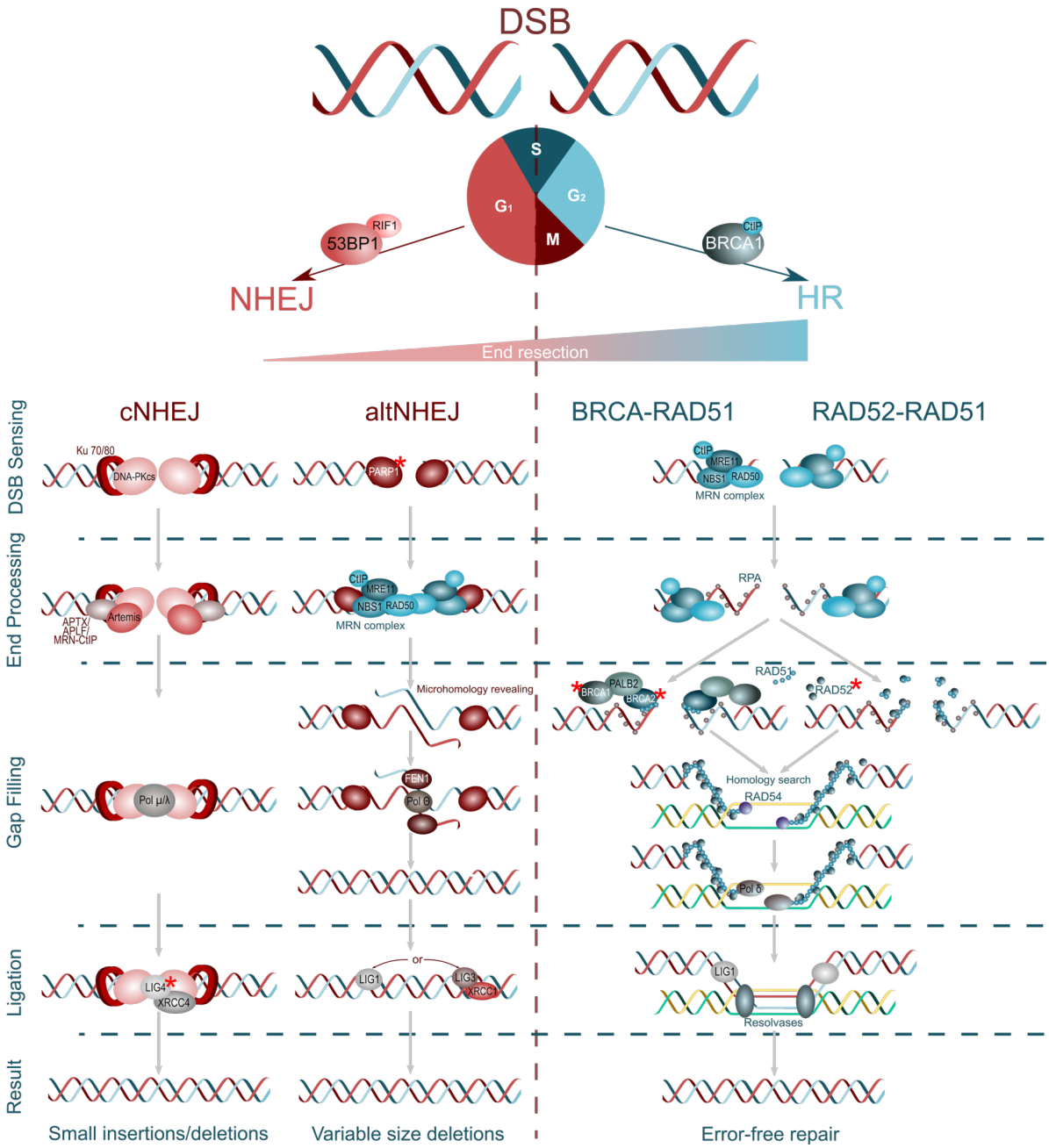

Fig. (2). DNA DSBs repair in mammalian cells with current synthetic lethality targets marked with red stars. DSBs repair occurs either by HR repair or NHEJ and their two sub pathways. The choice is determined basing on the cell cycle phase and the length of 5' end resection which is promoted by BRCA1/CtIP and inhibited by 53BP1/RIF1. In cNHEJ Ku heterodimer creates a ring around each end of a lesion and protects them from nucleases and resection. When bound to DNA, Ku exhibits a high affinity to the catalytic subunit DNA-PKcs and stimulates its activity, resulting in formation of DNA-PK complex at each end of the damage. Phosphorylation of different residues of DNA-PKcs is required for recruitment of many other repair proteins including end modifying proteins (Artemis/APTX/APLF/ MRN/CtIP) or polymerases (Pol λ/μ). The final step of cNHEJ is performed by LIG4 which in cooperation with XRCC2 ligates ends of DSB. AltNHEJ plays minor role in DSB repair under physiological conditions, however disturbance in the activity of cNHEJ proteins, like LIG4 causes inactivation of the classical pathway and redirects the process to the altNHEJ. Process requires "end-clipping" which reveals regions of microhomology between DSB termini. Homology annealing is catalyzed by Pol Θ and residual flap structures are removed by FEN1. Ligation is carried out by either LIG1 or LIG3 in cooperation with XRCC1. Extensive resection and close proximity of sister chromatid during G2/S phases of cell cycle predisposes DSB to be repaired by HR. MRN complex stimulated by CtIP reveals long ssDNA overhangs at the DSB termini. RPA proteins cover ssDNA tails and protect them. Recombination factors like BRCA1/PALB2/BRCA2 complex and/or RAD52 mediate at RAD51 localization at ssDNA overhangs and creation of recombination filament. With the support of RAD54, filament invades sister chromatid and searches for homology. After strand elongation by Pol δ junction is resolved and ligated by LIG1.

interacts also with polymerases - polμ and polλ which can work in both dependent and independent manner from the template [91, 92]. The crucial element of cNHEJ repair is ligation by a complex of ATP-dependent DNA ligase IV (LIG4), X-ray repair cross-complementing protein 4 (XRCC4), Cerunnos (also known as XRCC4-like factor; XLF) and paralog of XRCC4 and XLF (PAXX) [93]. At first, XRCC4-Cerunnos complex encircles DNA duplex, then aligns and stabilizes the position of ends. PAXX interacts with that complex and with Ku heterodimer to mediate DNA repair [94]. LIG4 merges with XRCC4 *via* BRCT domains at its C-terminal region and secure ligation of a DNA terminus [95].

Disturbance in the activity of cNHEJ proteins, like Ku, DNA-PKcs or LIG4 causes inactivation of the classical pathway and redirects the process to the altNHEJ mechanism. Though altNHEJ is not significant for DSB repair under physiological conditions, it has been shown that altNHEJ can play a major role in damage repair at collapsed replication forks [96]. In comparison to the canonical system, altNHEJ relays much more on microhomology between DNA termini and works slower, what may cause migration of DSB termini and selection of wrong repair partners. The microhomology-prone character of this repair system makes it look for complementarities in the distant sequences from where actual DSB occurred, causing relatively extensive end-resection and loss of DNA fragments between complementary sequences [97]. What is more, due to its lower kinetics, this system is considered one of the reasons of extensive genome rearrangements [98]. AltNHEJ involves activity of CtIP, MRN complex, PARP1, polΘ and DNA ligase I (LIG1) or III (LIG3), which act as the substitutes of the crucial cNHEJ proteins [99, 100]. At the first step, PARP1 recognizes DSB and binds both ends leading to its activation and to modification of various proteins [101]. PARP1 competes with Ku complex for binding to the lesion, however it exhibits lower affinity to DSB so its binding in the presence of functional cNHEJ system is less likely to happen [102]. Its role in altNHEJ is to participate in synapsis creation and recruitment of other repair factors like polymerases, ligases or nucleases [103]. End-processing and microhomology revealing is managed by MRN complex which binds DNA ends and resects them in 5' to 3' manner, thus exposing complementary sequences [104, 105]. MRN activity and the process of resection are stimulated by CtIP protein which itself exhibits nucleolytic activities that are not required for altNHEJ end-resection [106, 107]. Once ends have been edited by the protein complex and the complementary sequences have been revealed and connected, altNHEJ proceeds to the stage of microhomology annealing being held by polymerase Θ [108]. In the latest studies, polymerase Θ has been shown to exhibit a number of unique features, hence determining its major role in altNHEJ repair [109]. It can interact with DNA in three ways – template-dependent polymerase using ssDNA tails of both DSB ends for synthesis of complementary strand, template-independent polymerase adding ssDNA overhangs or it can "snap back" an overhang in a hairpin-shape to be able to continue synthesis of complementary sequence on its own template. When homology isn't present polΘ has an ability to create short complementary regions by extending ssDNA overhangs *via* its terminal transferase activity, which may give rise to nucleotide insertion [110]. Polymerase Θ has been reported to efficiently align two 6-15 nt long 3' overhangs sharing 4 bp of homology but struggling while working on longer ssDNA termini, this may suggest involvement of other polymerase after the stage is initiated by polymerase Θ [111-113]. It has been suggested that polymerase Θ forms a tetrameric structure that is able to bind ssDNA inside and fold it back as well as bind both ends of DSB and screen it for a homology, thanks to its helicase activity [114]. The flap structure created after connection of the deep-strand homologous regions is removed by nucleolytic activity of ERCC1/XPF complex or by endonucleolytic activity of flap structure-specific endonuclease 1 (FEN1) [115-119]. Subsequently, a stable, annealed and ligatable polymerization product has to be sealed by LIG1 or a complex of LIG3α and XRCC1 that stabilizes it [120, 121].

Microhomology-based NHEJ is similar to the process of single strand annealing (SSA). Human genome contains many direct sequence repeats. DSBs occurring in such regions have a possibility of being easily repaired by short end-resection and revealing homology found in the repeats continuation on the partner strand. The process is terminated by nucleolytic removal of flap structures and ligation [122].

As it has been previously mentioned, HR repair is mostly active during mid S and G2 phase of the cell cycle, due to the close proximity to the sister chromatid (Fig. **2**). Also homologous chromosomes can be partners for this system, however, it may lead to the loss of heterozygosity in HR which uses such donors [123]. Furthermore, HR requires prolonged 5' to 3' end resection in order to perform extensive homology search. The unique character of this pathway manifests itself in

the high accuracy in reconstructing the original sequence and its ability to work on stalled replication forks [124]. HR involves steps of end processing revealing long ssDNA overhangs, search for complementarity between revealed fragment and homologous sequence, invasion of recombinase-ssDNA complex onto a homologous sequence and formation of D-loop, template-dependent synthesis extending 3' end, formation of two Holliday junctions, separation of the structure and ligation [125]. Initial step of end processing requires cooperation between CtIP protein and MRN complex and helicase activity of BLM (Bloom syndrome RecQ like helicase) protein [126]. MRN (MRE11/RAD50/NBS1) catalyzes initial 5' to 3' end resection. MRE11 exhibits both ssDNA endonucleolytic and exonucleolytic activity. Both get stimulated when protein is in conjunction with RAD50 forming $MRE11_2RAD50_2$ core [127, 128]. Further resection of DSB ends is maintained by EXO1 (exonucleases 1) exonucleolytic activity [129]. When ssDNA tail is being formed, RPA proteins (replication protein A) bind it covering like beads and protect from creating the secondary structures as well as from nucleolytic attack. However, the protective effect of RPA proteins at 3' termini has to be abolished later, to allow for recombinase binding [130]. Once ssDNA tail is ready, recombinase filament has to be formed at the end of an overhang in order to support the invasion on the homologous sequence. RAD51 is a recombinase which plays an essential role in HR. Proper activity of RAD51 is provided by a group of proteins including its 5 paralogs – RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3, all of which have been suggested to have a part in assembling of a filament [131]. Paralogs are able to form different complexes like BCDX2 (RAD51B, RAD51C, RAD51D, XRCC2), CX2 (RAD51C and XRCC2), BC (RAD51B, RAD51C), etc. and participate in various processes [132, 133]. For instance, BCDX2 and CX2 act at the different stages of HR, recruiting and stabilizing RAD51 filament [134]. Other proteins crucial for filament formation are BRCA1 and BRCA2 - tumor suppressor proteins the loss of which has already been widely correlated with increased risk of breast and ovarian cancer [135]. BRCA1 promotes HR repair and interacts with a number of proteins involved in DSB repair including resection MRN-CtIP complex [136]. BRCA2 contains ds- and ssDNA binding sites as well as a few RAD51 binding motifs which may indicate the important role of BRCA2 in the filament formation at the ssDNA termini [137-139]. Partner and localizer of BRCA2 (PALB2) as a scaffold for both BRCA proteins and serves ensures their proper localization and stable binding during HR repair [140, 141]. In BRCA1-PALB2-BRCA2 complex, BRCA1 is responsible for directing the remaining two to the damaged region [142, 143]. Lastly, RAD54 protein cooperates with RAD51 filament at many stages of its action by supporting it in homology search, D-loop creating as well as catalyzing RAD51 dissociation from DNA heteroduplex [144]. The main responsibility for proper DSB repair in HR repair relies on RAD51 recombinase. With the assistance of previously mentioned proteins - BRCA2, RAD51 paralogs and RAD52, RAD51 assembles on the previously isolated and RPA–protected DNA 3' overhangs and forms presynaptic filament which is stabilized by RAD54. In the next step, the filament penetrates homologous dsDNA particle and examines it looking for complementarity. RAD51 is then removed from heteroduplex to provide access of DNA polymerase [145]. Currently two models – Double Strand Break Repair (DSBR) and Synthesis-Dependent Strand Annealing (SDSA) explain how recombination process may occur. In the first model proposed by Szostak *et al*., one of the 3' tails invades a homologous dsDNA fragment creating a D-loop and each 3' end of DSB is annealed on the template of complementary strand of homologous DNA sequence. Such model provides for creation of two Holliday junctions which can get resolved by cutting either outer or inner strands of each of the junctions. If both junctions are cut the same way, the process won't result in crossover in the final products. However, if junctions are resolved differently, such that one has its outer strand cut while the other has its inner strand cut, the process generates crossovers [146]. The second model is SDSA. This provides for migration of a "bubble" or a D-loop caused by 3' end annealing machinery, traveling along one of the strands of homologous DNA fragment. Synthesis of only one 3' ssDNA overhang of the lesion is led on a template of homologous DNA sequence and as soon as this process is finished, the product is displaced and paired with its partner basing on the sequence homology between freshly synthesized sequence and partners ssDNA overhang. Any excessive 3' flaps are then eliminated to allow for ligation. A gap in the strand which had not been involved in heteroduplex formation is filled basing on the template of complete complementary strand. This model does not provide for generating Holliday's junctions or crossover products [147-149]. SDSA model has also many variations that include replication-like repair with synthesis of leading strand using an unbroken homologous template and lagging-strand on the template of freshly synthesized partner strand.

Another example is a model providing for involvement of both strands of homologous section in annealing process of damaged strands, but without junction cutting or possibility of crossover products [150].

RAD52 protein is one of the factors taking part in DSB repair *via* HR. It is able to bind RPA coated ssDNA and promote interactions of RAD51 with target substrate by directing it to RPA-ssDNA complex [151]. RAD51 recruitment to the termini of ssDNA strongly depends on RAD52 because it is able to abolish the RAD51-binding inhibitory effect of RPA and promote recombinase binding and filament creation [152, 153]. RAD52 suppresses RPA in an immediate protein-protein interaction. Synthesis takes place once a filament is created. During the whole process, RAD52-RPA complexes remain spread along the invading RAD51-coated tail which may point to stabilizing function of this element [154]. Moreover, RAD52 is a protein involved in previously mentioned SSA repair system. Interestingly, yeast cells deficient in Rad52 exhibit highly increased sensitivity to genotoxic stress [155]. However, this phenomenon is unlikely to occur in mammalian cells, where RAD51 loading is mainly performed by BRCA proteins. RAD52 knockout mice are viable, fertile and do not express any changes in phenotype. In vertebrates, absence of RAD52 also results in no obvious changes in HR functioning [156, 157]. For that reason, it has been proposed that BRCA and RAD52, which both are recombination mediators, take part in two separate HR subpathways – BRCA1/2-RAD51 dependent and backup, RAD52-RAD51 dependent (Fig. **2**). It has been discovered that tumors carrying reduction- or loss-of-function mutations in BRCA1/2 suppressor genes are sensitive to RAD52 deprivation [158]. On the other hand, mutation in RAD51 in mice resulted in early embryonic lethality, while RAD51-defficient vertebrate cells reacted with accumulation of chromosomal breaks and cell death, suggesting that alternative pathway does not go beyond RAD52 activity [159-161].

4. ALTERATIONS IN DSB REPAIR SYSTEMS AS A CHANCE FOR PERSONALIZED ANTICANCER THERAPY BASED ON SYNTHETIC LETHALITY

Changes occurring in cancer cells genomes and alterations in repair systems, in particular, offer an opportunity of designing a therapy that would pointedly and efficiently eliminate malignant derivatives. Addiction to the genes which products are involved in DSB repair mechanisms is a common phenomenon in tumor cells deprived in factors crucial for basic pathways. These genes (or their products) can potentially become targets for synthetic lethality-based anticancer therapy, which will result in cell sensitivity to DNA damage. Inefficient DSB repair will result in lethal lesion accumulation and cell death. First and foremost, this simple, yet elegant approach, targets only cancer-specific vulnerabilities, as a result will not affect normal cells. For this reason, synthetic lethality-based therapy should also exhibit limited number of side effects and lower doses of drugs should give satisfactory results [162].

Extensive studies over factors involved in DSB repair systems have made it possible to predict potential targets for synthetic lethality. Different approaches consider both complementary nature between HR and NHEJ system during the cell cycle as well as existence of alternative HR and NHEJ pathways activated in the event of basic pathway failure.

5. PARP INHIBITION IN CELLS DEFICIENT IN HR COMPONENTS

For the present moment, only interaction of synthetic lethality between PARP1 and BRCA genes has found application in clinical therapy. Evidence of correlation between mutation in BRCA1/2 genes and response to PARP inhibitor were for first time reported in 2005 [163, 164]. PARP proteins are involved in multiple cellular pathways, including repair of base modifications and SSBs in BER/SSBR system where it interacts with main proteins of this system such as XRCC1, OGG1 (8-oxoguanine DNA glycosylase 1), DNA polymerase β or LIG3 [165, 166]. The important role of PARP in the DSB repair has also been revealed recently. Besides previously explained function of this protein in PARP1-dependent altNHEJ, this factor also plays a crucial role in HR induction at stalled replication forks. Such obstacle triggers poly[ADP-ribosyl]ation, resulting in recruitment and activation of MRN complex at the disrupted replication region and further repair by HR system [101]. Despite the multifunctional character of PARP proteins, mice which had been deprived of it stayed viable and fertile, however, they displayed enhanced sensitivity to genotoxic stress induced by factors such as alkylating agents or radiation, this was manifested by increased number of gene amplifications, sister chromatid exchanges (SCEs) and recombination in general [167, 168]. Although effectiveness of anticancer therapy based on targeting PARP in BRCA-deficient cells has been confirmed in multiple clinical trials, the mechanism of inhibition remains unclear [169-171]. The fact whether PARP inhibitors only cause catalytic inhibition that decreases the ability of PARP to perform its function or they also result in

PARP trapping at the DNA strand during an ongoing repair, due to impaired ability of autoADP-ribosilation, is still being researched. Such failure in PARP dissociation from DNA sites may result in stalling replication forks which needs HR pathway for work resumption [162, 172, 173]. Ineffective repair of SSBs under absence of PARP results in their conversion to DSBs. In cells with detected mutations in BRCA1/2 such situation leads to the accumulation of toxic lesions, sensitivity to genotoxic stress, further genomic instability and cell death [174].

Despite the fact that NHEJ is a major repair system in mammalian cells, some cancer types seem to rely mostly on extensive homology dependent pathway [175]. Mutations in BRCA1/2 have been associated with the elevated risks of breast and ovarian cancer. It has been established that about 11% to 15% of the cases of epithelial ovarian cancer (EOC) are correlated with inherited mutations in BRCA1/2 genes [176, 177]. Similarly, extensive studies on the frequency of mutations in BRCA1/2 in breast cancer patients have shown that the majority of cases are correlated with mutation in this pair of genes [178]. Mutations of BRCA1 and/or BRCA2 have been found also in numerous cases of melanoma, pancreatic and prostate cancer [179-181]. Interestingly, in leukemia cells, BCR-ABL1 fusion has been associated with a decreased expression of BRCA1 protein to nearly undetectable level. Downregulation in protein activity was not due to lower gene expression, but to repressed translation [182, 183]. Also chronic hypoxia in cancer cells leads to defects in HR proteins such as RAD51, RAD54, BRCA1/2 and XRCC3 [184]. Moreover, while many sporadic cancer cases do not carry germline mutations in BRCA genes, they still share pathological phenotype typical for BRCA-mutated cells. Such phenomenon, termed "BRCAness", may occur, for instance, due to epigenetic gene silencing [185]. The listed conditions create an excellent opportunity for personalized anticancer therapy based on targeting PARP1 in cells with disrupted HR pathway. It has been also proposed that in BRCA-proficient cells, environment appropriate for synthetic lethality-based therapy could be provided by thermal degradation of BRCA2 with local mild hyperthermia (40-43°C) [186-189].

In order to exploit the strategy of synthetic lethality in phenotype of ineffective HR repair in wider group of patients, other factors crucial for this pathway, along with PALB2 [190, 191], BARD1 (BRCA1 associated RING domain) [192, 193], RAD51C [194, 195], and RAD51D [196], have also been screened in search of interactions which could increase clinical relevance of PARP1 inhibitors [178, 197]. Besides, RAD54B - a mammalian paralog of RAD54 is a HR repair protein whose mutations have been detected in numerous types of cancer including colorectal, breast, lung cancer and primary lymphoma [198, 199]. RAD54B interacts in a highly specific manner with RAD51 which stimulates its ATPase and helix-opening activities. The efficiency of HR is significantly reduced in the absence of RAD54 and RAD54B [200]. Decreased level of RAD54B has also resulted in chromosomal instability and increased number of chromosomes [201]. It has been reported that PARP inhibition results in specific elimination of RAD54B-deficient cells and elevated level of DSBs, hence suggesting synthetic lethal interactions between these two genes [199]. Furthermore, LIG4 and FEN1 deficiency has also been found to be correlated with synthetic lethal killing of RAD54B-deprived cells [201, 202].

6. PARP INHIBITION IN CELLS DEFICIENT IN NHEJ COMPONENTS

PARP inhibitors may also find clinical use under conditions of ineffective NHEJ repair resulting from mutation or downregulation of its crucial components. It has been lately reported that targeting PARP in LIG4 deficient melanoma cell lines results in their highly specific elimination due to the accumulation of toxic DSBs. Reduced LIG4 expression resulted in inefficient effectiveness of cNHEJ and redirecting of repair to an alternative pathway. Additionally, targeting PARP1 protein responsible for BER, altNHEJ and HR induction at stalled replication fork resulted in accumulation of lethal DSBs and cell death. In fact, only about 7% of cutaneous melanomas harbor mutations in LIG4, according to TCGA database, however, further analysis suggests that greater number of patients could benefit from PARP targeting due to deficiencies in other factors of cNHEJ pathway factors [203]. LIG4 deficiency has previously been detected also in high-risk neuroblastoma cell lines, thus suggesting that such patients could benefit from this approach [204].

Interestingly, poor prognosis in acute myeloid leukemia (AML) is often associated with internal tandem duplications (ITD) in FLT3 (fms related tyrosine kinase 3). Cells expressing FLT3-ITD demonstrate decreased levels of Ku proteins, crucial for cNHEJ, and elevated level of LIG3α, the component acting in altNHEJ. Such cells are characterized by frequent DNA deletions and DSB repair pathway involving microhomology regions. PARP inhibition can potentially result

in accumulation of lethal DSB in FLT3-ITD expressing leukemia cells [205, 206].

7. PARG INHIBITION

In the early steps of response to a DNA strand interruption, PARP proteins covalently attach multiple subunits of ADP-ribose to themselves and to other acceptor proteins using NAD^+ as a substrate. This process is crucial for repair of SSBs and DSBs as well as for restart of stalled replication forks. Such signal is responsible for chromatin remodeling and recruitment of multiple repair agents. Under pathological conditions, prolonged oxidative, or nitrosative stress caused by increased number of DNA lesions, expression of PARP protein and its activity is extremely elevated resulting in NAD^+ and ATP depletion. Such situation leads to a parthanatos – PARP1-dependent cell death caused by hyper[ADP-ribosyl]ation of target proteins and mitochondrial dysfunction [101, 207]. Modifications induced by PARP are not permanent. It has been suggested that removal of ADP-ribose moieties is crucial for further steps of repair [208]. Poly[ADP-ribose] glycohydrolase (PARG) and ADP-ribosyl hydrolase 3 (ARH3) are the enzymes responsible for removal of ADP-ribose units. PARG exhibits activity of endo- and exoglycohydrolase which allows it to reverse PARP modifications by hydrolysis of ribose-ribose bonds present in poly[ADP-ribose] chains and returning acceptor proteins to their native form [209]. PARG deficiency results in elevated sensitivity to genotoxic stress and genomic instability [210, 211]. Loss of PARG has also been associated with neurodegeneration [207, 212]. PARG targeting generates a new possibility for specific synthetic lethality-based killing of cells deficient in HR repair factors such as BRCA1/2, PALB2, or BARD1. Inhibition of PARG, just as in PARP inhibition, resulted in the death of HR-deficient cells due to inability to resolve arrested replication forks. Interestingly, PARP and PARG do not share all partners in synthetic lethal interaction, which might point to slightly different functions of these proteins. The results obtained varied depending on the cell line used in the analysis and on the factor absent in PARG inhibited cells [213-215]. Targeting PARG in HR-deficient cancer types needs further examination and therefore opens new perspectives in the field of personalized anticancer therapy.

8. RAD52 INHIBITION IN BRCA-DEFICIENT CELLS

PARP1 inhibition results in disruption of a plethora of cellular processes in which this protein is involved, among others, chromatin modeling, telomere maintenance and DNA repair or transcription [216, 217]. Affecting such a wide range of processes may result in induction of side effects in normal cells also. What is more, majority of currently available compounds also show activity toward other proteins from the PARP family, due to their structural similarity, this thereby widens the spectrum of aftereffects [218]. For these reasons, further efforts are focused on identification of novel targets which act the most exclusively in backup pathways. Targeting factors which are not part of a broad spectrum of processes but are specific for a unique pathway could significantly lower the number of side effects. An example of such target can be RAD52 which has been reported to exhibit synthetic lethality interactions with BRCA1/2, PALB2, and RAD51 paralogs [161, 219]. RAD51 binding and filament formation during HR repair is primarily carried out by BRCA protein in mammalian cells, whereas, RAD52 serves as a factor crucial for backup pathway exhibiting activities of binding ssDNA, RAD51, and RPA coated ssDNA overhangs. Previous studies have showed that inhibition of RAD52 in BRCA-proficient cells has no effect, whereas, BRCA-deficient cells react with reduced number of RAD51 binding foci and less effective HR [220]. In cells harboring mutations of BRCA1/2 genes, alternative RAD52-RAD51 pathway could be activated in order to provide cell viability [221]. Therefore, RAD52 protein could become a novel target for synthetic lethality-based personalized anticancer therapy in tumors deficient in RAD51 localizing mediator proteins like BRCA1/2 or PALB2, for example in familial breast or ovarian cancer. Extensive studies have generally confirmed the great potential of this approach on multiple cell lines as well as in patient derived of leukemia cells, resulting from the previously described BRCA1/2 down regulation in BCR-ABL expressing cells [222-224]. Analysis has also shown the ability of RAD52 inhibiting single-agent therapy to kill both progenitor and cancer initiating stem cells by accumulation of toxic DSBs and activation of error-prone repair processes giving rise to genomic instability [225]. Thus, such approach may find a clinical application in tumors displaying BRCA-defective phenotype.

9. SYNTHETIC LETHALITY-BASED ANTICANCER THERAPY IN CLINICAL TRIALS

The initial development stages of therapy based on small molecules inhibiting PARP activity exploited mainly their role as radio- and chemo-sensitizing drugs, however, their utility in a single-agent treatment has not been assessed [226]. Therefore, the ability of PARP

inhibitors to selectively kill BRCA-deficient cells has been extensively described in multiple preclinical *in vitro* and *in vivo* studies, which has contributed to diverse successive clinical tests. To the present moment, a number of PARP inhibitors have been designed and current clinical trials are focused on investigating their efficiency in monotherapy in cancer types expressing changes in DNA repair systems as well as in combinatorial therapy demonstrating synergistic effects of such compounds with alkylating agents or radiation [227]. This group includes orally delivered drugs - olaparib (AZD2281, Lynparza™), niriparib (MK4827, Zejula™), rucaparib (AG14699, Rubraca®), veliparib (ABT888), and talazoparib (BMN673) (Table **1**). Majority of PARP inhibitors are nicotinamide analogues able to reduce NAD-binding catalytic activity of PARP proteins, however, they differ in bioavailability, ability to trap PARP on the DNA strand, and efficiency of inhibiting target protein [172, 226]. Due to the high similarity of the catalytic domain of PARP proteins, many inhibitors are not able to distinguish between different PARP isoforms. PARP1 and PARP2 share over 68% homology in NAD^+ binding domain [228]. The first PARP inhibitors were developed in 1980s and were aimed at enhancing the effect of chemo- and radiotherapy. Studies on agents like 3-aminobenzamide (3-AB), which sensitized cancer cells to the methylating factors contributed towards better understanding of PARP activity [229]. On the other hand, the first generation of inhibitors never made it to the clinical trials due to the high concentrations which had to be used to achieve the desired effect. The second generation of PARP inhibiting agents developed in early 1990s were based on the quinazoline structure. Compounds like PD 128763 or NU1025 have been characterized by higher effectiveness and specificity [174, 230, 231]. Research over modifications of these inhibitors have led to discovery of PARP catalytic domain residues which when bound may contribute to enhanced inhibiting effect of the drugs. For instance inhibitors with carboxamide group in their second aromatic ring have been shown to be more potent due to their ability to create additional hydrogen bounds with Glu988 residue which is responsible for the major catalytic activity of PARP proteins and takes part in the elongation of ADP-ribose chains [174, 232]. The third generation of PARP inhibitors consists of new and more effective agents including a few benzimidazole-based compounds. Novel PARP inhibitors such as talazoparib demonstrate an elevated metabolic stability, a more potent action, a favorable bioavailability and pharmacokinetics. Such increased profile makes it possible to achieve cancer cell response using much lower doses as compared to other PARP inhibitors [233]. On 19th December 2014 the first PARP1 inhibitor – olaparib (Lynparza™) was approved by FDA and European Medicines Agency (EMA) for single-agent use in synthetic lethality-based anticancer therapy of BRCA-deficient ovarian cancer patients [26]. Similarly, two years later, FDA approved rucaparib (Rubraca®) for the treatment of BRCA-mutated ovarian cancer patients [234]. On March 27th 2017 another PARP inhibitor niraparib (Zejula™) was approved as a therapy for patients with recurrent EOC as well as fallopian tube cancer and peritoneal cancer who are responsive to the platinum-based chemotherapy. In clinical trials treatment showed the best results in the cohort of patients with germline BRCA mutations in comparison to the cohort without mutations in BRCA and to the cohorts receiving placebo [235]. Thus, these three milestones point to a promising new direction of personalized medicine, they are of clinical significance and offer many possibilities in the treatment of repair-deficient cancer types.

10. IDENTIFICATION OF POTENTIAL THERAPY RECIPIENTS

Today, the efforts of pharmaceutical companies are focused not only on drug designing, but also on development of a method which would allow identification of patients that would be responsive to PARP inhibitors and who would benefit from that approach. One of such tests is FDA approved BRACAnalysis CDx® (Myriad Genetics) which identifies potential olaparib and niraparib responsive ovarian cancer patients by assessing data on alterations in BRCA1/2 sequence *via* Sanger sequencing performed in order to identify single nucleotide polymorphism (SNP), small insertions and deletions. The method also consists of multiplex PCR BART® acquiring data about extensive deletions or insertions. Genomic DNA isolated from peripheral blood or buccal mouthwash samples constitute the material to be used in method. Easily accessible samples are also a limitation of this method to detection of only germline mutations in BRCA genes. For this reason, company is currently developing equivalent method of TumorBRACAnalysis CDx® that analyzes samples derived from preserved tumor tissue [236-238]. Improved modification of BRACAnalysis CDx® - MyChoice HRD® (Myriad Genetics) is another diagnostic test currently under clinical trials. It is performed on genomic DNA acquired from paraffin or formalin preserved tissue samples. In addition the tests included in BRACAnalysis CDx®, myChoice HRD® assess additional information on genomic instability status NGS-

Table 1. Clinical trials evaluating effectiveness of PARP inhibitors in single-agent therapy.

ClinicalTrials.gov identifier/ Trial name	Phase	Study design	Study population	Dose	Primary outcome
OLAPARIB					
NCT00516373	I	Single arm, open label	Malignant advanced solid tumor refractory to standard therapy	DDS, 10 mg QD = 400 mg BID	DLT, MTD
NCT01844986 (SOLO1)	III	Randomized, double blind, placebo controlled	BRCA-mutated OC following first line platinum-based chemotherapy	300 mg tablets, BID	PFS
NCT01874353 (SOLO2)	III	Randomized, double blind, placebo controlled	Platinum sensitive relapsed BRCA-mutated OC following CR or PR to platinum-based chemotherapy	300 mg tablets, BID	PFS
NCT02282020 (SOLO3)	III	Randomized, open label	Platinum sensitive relapsed BRCA-mutated OC	300 mg tablets, BID	PFS
NCT01078662	II	Non-randomized, single arm, open label	Advanced BRCA-mutated OC, BC, PC, PaC	400 mg, BID	TRR
NCT00494442 (ICE-BERG2)	II	Non-randomized, open label	Advanced BRCA1 or BRCA2 associated OC	100 mg/ 400 mg, BID	OTR
NCT00628251 (ICE-BERG3)	II	Randomized, open label	Advanced BRCA1 or BRCA2 associated OC who have failed previous platinum-based chemotherapy	200 mg/ 400 mg, BID	PFS
RUCAPARIB					
NCT01482715 (Study 10)	I/II	Non-randomized, single arm, open label	BRCA-mutated OC or other solid tumor	DDS	DLT, PK, ORR
NCT 01891344 (ARIEL2)	II	Single arm, open label	Platinum sensitive relapsed high-grade EOC, FTC, PPC	600 mg, BID	DP, ORR
NCT01968213 (ARIEL3)	III	Randomized, double blind, placebo controlled	Platinum sensitive relapsed HGSOC, EOC, FTC, PPC following CR or PR to platinum-based chemotherapy	600 mg, BID	DP, PFS
NCT02855944 (ARIEL4)	III	Randomized, open label	Platinum sensitive relapsed BRCA-mutated high-grade EOC, FTC, PPC	600 mg, BID	PFS
NCT02952534 (TRITON2)	II	Single arm, open label	Metastatic castration-resistant PC with homologous recombination gene deficiency	600 mg, BID	ORR, PSA
NCT02975934 (TRITON3)	III	Randomized, open label	Metastatic castration-resistant PC with homologous recombination gene deficiency	600 mg, BID	PFS
NIRAPARIB					
NCT00749502	I	Non-randomized, open label	Advanced solid tumors and hematologic malignancies	DDS, 30 mg = 400 mg, QD	DLT, inhibition of PARP activity

(Table 1). Contd…..

ClinicalTrials.gov identifier/ Trial name	Phase	Study design	Study population	Dose	Primary outcome
NCT02354586 (QUADRA)	II	Single arm, open label	Advanced relapsed HGSOC, FTC, PPC who have received 3-4 previous chemotherapy regimens	300 mg, QD	Antitumor activity
NCT01847274 (NOVA)	III	Randomized, double blind, placebo controlled	Platinum sensitive OC, FTC, PPC who have responded to the previous platinum-containing therapy	300 mg, QD	PFS
NCT02655016 (PRIMA)	III	Randomized, double blind, placebo controlled	Advanced OC following CR or PR on front-line platinum-based chemotherapy	300 mg, QD	PFS
VELIPARIB					
NCT00387608	0	Single arm, open label	Advanced solid tumors and hematologic malignancies	10 mg/ 25 mg/ 50 mg, single dose	PK, change in tumor PAR level
NCT01540565	II	Single arm, open label	Persistent or recurrent EOC, FTC, PPC with a germline BRCA-mutations	400 mg, BID	AEs, OTR
NCT00892736	I	Single arm, open label	Refractory BRCA-mutated solid cancer, platinum-refractory OC, FTC, PPC or basal-like BC that do not respond to previous therapy	400 mg, BID	MTD, DLT
NCT01472783	I/II	Single arm, open label	BRCA-mutated and platinum-resistant or partially platinum-sensitive relapsed EOC	300 mg, BID	MTD, DLT, ORR
TALAZOPARIB					
NCT01286987	I	Single arm, open label	Advanced or recurrent solid tumors with DNA-repair pathway deficiencies	DDS, 0.025 = 1.1 mg, QD	MTD
NCT02286687	II	Non-randomized, open label	Advanced cancer with alterations in BRCA or other genes involved in BRCA pathways, homologous recombination defects or PTEN alterations	1 mg, QD	CB
NCT01945775 (EMBRACA)	III	Randomized, open label	Advanced and/or metastatic BRCA-mutated BC	1 mg, QD	PFS
CEP9722					
NCT00920595	I	Single arm, open label	Advanced solid tumors	DDS, 150 mg, QD	MTD, DLT

(AEs – adverse events, BC – breast cancer, BID – twice daily, CB – clinical benefit, CR – complete response, DDS – dose determining study, DLT – dose-limiting toxicities, DP – disease progression, EOC – epithelial ovarian cancer, FTC – fallopian tube cancer, HRD – homologous recombination deficiency, MTD – maximum tolerated dose, OC – ovarian cancer, ORR – objective tumor response, OTR – overall response rate, QD – once daily, PaC – pancreatic cancer, PC – prostate cancer, PFS – progression-free survival, PK – pharmacokinetics, PPC – primary peritoneal cancer, PR – partial response, PSA – prostate specific antigen, TRR – tumor response rate).

based identifying three components: LOH, TAI and large-scale state transitions. This method is dedicated to potential niraparib-responsive ovarian cancer, metastatic, and primary breast cancer [237, 239]. Similarly, Foundation Medicine Inc. has developed FoundationFocus CDxBRCA® - a companion diagnostic test which uses NGS for detection of BRCA1/2 mutations in tumor samples collected from women with ovarian cancer which could be responsive to rucaparib treatment. The company has also designed a massive comprehensive genome profiling diagnostic test - FoundationOne® which using NGS-based analysis screens simultaneously coding regions of over 300 tumor related genes and intron sequences of 28 genes whose changes have often been associated with cancer. The method allows for detection of all kinds of genomic alterations, including insertions, deletions, base substitutions, or copy-number alterations (CNAs). Such massive diag-

nostic examination is performed from a small amount of tissue acquired from needle biopsies fixed in formalin or paraffin [237, 240, 241]. Foundation Medicine Inc. is currently conducting clinical trials of an ARIEL2 companion diagnostic test which will be able to not only detect BRCA-mutated patients, but also non-BRCA-mutated ones expressing "BRCAness" phenotype. Moreover, novel comprehensive methods identifying HR-deficient patients are at an early preclinical stages of development. A good example is Gene Expression and Mutation Analysis (GEMA) which is potentially able to identify recipients of PARP inhibitor-based therapy by detecting BRCA and DNA-PK-deficiencies using Reverse Transcription-quantitive PCR (RT-qPCR), flow cytometry, and microarrays or by detecting oncogenes like BCR-ABL1 and AML1-ETO [24, 242]. Some authors have successfully used PARP inhibitors to target GEMA-selected BRCA/DNA-PK-deficient proliferating and DNA-PK-deficient quiescent leukemia steam and progenitor cells triggering cell death due to the dual synthetic lethality [24, 242]. The described diagnostic tests offer genetic vulnerability-screening methods, consequently making it possible to exploit the concept of synthetic lethality-based personalized strategy in patients with defects in repair systems.

11. OLAPARIB

Olaparib (Lynparza™) ($C_{24}H_{23}FN_4O_3$, 4-[3-(4-cyclo propanecarbonylpiperazine-1-carbonyl)-4-fluoro benzyl]-2H-phthalazin-1-one) is a small molecule inhibitor of PARP1/2/3 proteins, named KU-0059436, that was initially developed by Kudos (Fig **3**). The name was changed to AZD2281 after the company was taken over by AstraZeneca. Olaparib is one of PARP-inhibiting compounds belonging to the bicyclic lactams [243, 244]. Olaparib is the first PARP inhibitor to find application in clinical practice. It is absorbed fast after oral intake and the highest plasma concentration is usually achieved after 1-3 hours [26]. Currently approved olaparib dose is 400 mg received twice a day in a capsule formation with or without food. This dose, after to the initial phase I trials in ovarian cancer patients, has been defined as a maximum tolerated dose (NCT00516373)[245]. Because of low solubility of the inhibitor, each capsule delivers 50 mg of olaparib, which means that a patient has to take 16 capsules a day to reach the desired daily dose. Between 2008 and 2012 the phase I clinical trials compared the bioavailability, efficiency, and safety of 300 mg dose delivered in two 150 mg tablet formulation twice a day (2 x 150 mg twice a day) in comparison to the current dose. Due to the promising results, tested dose was recommended for further analysis in phase III clinical trials [246]. SOLO1 (NCT01844986) and SOLO2 (NCT01874353) are undergoing phase III, double-blind, placebo-controlled clinical trials of described 300 mg twice a day dosage in platinum-sensitive patients with BRCA1/2 defective high-grade serous ovarian cancer (HGSOC), peritoneal cancer, fallopian tube cancer, or high grade endometrioid cancer [247, 248].

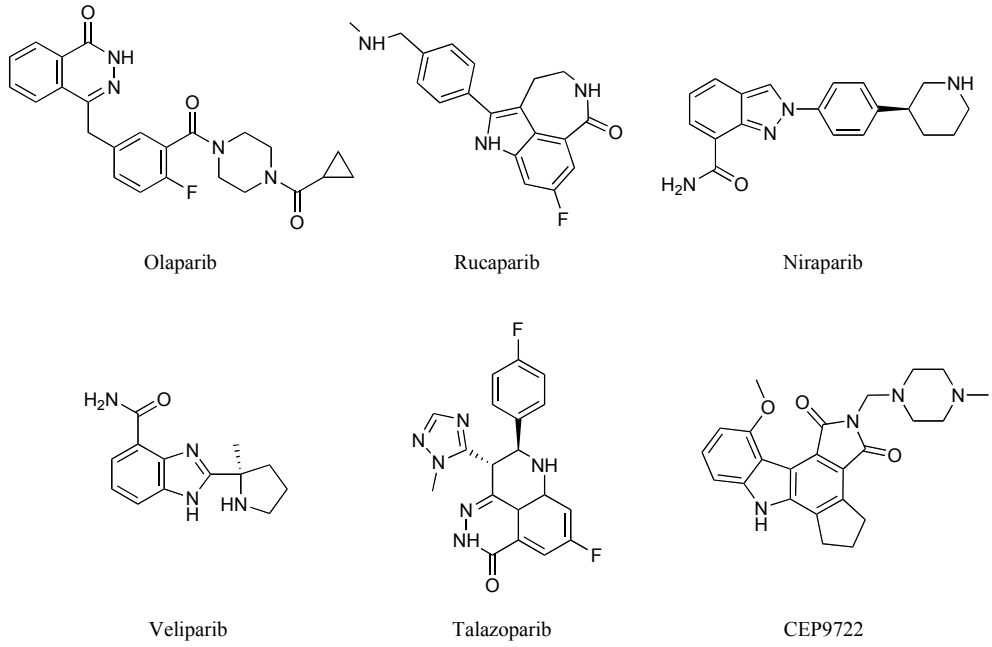

Fig. (3). Chemical structures of PARP inhibitors in clinical trials**.**

Olaparib was the first PARP inhibitor to obtain approval of EMA and accelerated approval of US FDA for treatment of BRCA1/2 mutated ovarian cancer that have been treated with three or more lines of chemotherapy. Decision was based on the phase II open label, non-randomized, non-comparative clinical trial (NCT01078662) which analyzed response of 137 patients with ovarian cancer with detected mutations in BRCA genes to olaparib administered orally in dose of 400 mg twice a day (50 mg x 8 twice daily). Over a third of patients (46/137) demonstrated complete or partial response (objective response rate ORR equal 34%)[249]. Also many other clinical trials at different stages have supported the results. Phase I initial trials assessed data considering safety, tolerability, toxicity, and pharmacokinetics of olaparib [245]. Expanded program of these clinical studies focused on response of patients with BRCA1/2 mutated ovarian, fallopian tube, or primary peritoneal cancer to treatment with 200 mg olaparib twice a day showed that 40% (20/50) achieved partial or complete response, according to Response Evaluation Criteria in Solid Tumors (RECIST) and/or marker analysis. What is more, the study showed a significant correlation between clinical response and platinum sensitivity of tumor [250].

Phase II non-randomized trials confirmed the safety and efficacy of olaparib treatment in advanced ovarian cancer with mutated BRCA1 or BRCA2 gene. Response of patients given either maximum tolerated dose (MTD) or smaller dose of 100 mg twice a day was assessed. In the group of patients receiving 400 mg olaparib twice a day, the ORR reached 33% (11/33), whereas in the second group treated with lower doses, only 13% showed some response (NCT00494442) [169]. A phase II open label, randomized studies compared the response of BRCA-defective ovarian cancer patients prior to platinum-based chemotherapy to either different doses of olaparib (200 or 400 mg twice daily) or pegylated liposomal doxorubicin (PLD) (50 mg/m^2every 28 days) which represented an approved approach for treatment of patients with post-platinum treatment recurrent or progressed ovarian cancer. Interestingly, no statistically significant differences were found between olaparib and PLD group in ORRs or progression-free survival (PFS) length (NCT00628251) [251]. Yet another phase II single-arm study for the first time reported effectiveness of olaparib in treatment of sporadic ovarian cancer without mutations in BRCA1/2 genes (ORR = 24%, 11/46 patients). Study has also analyzed response of triple-negative breast cancer (TNBC) patients but no confirmed ORRs have been reported [252]. Extensive phase II placebo-controlled trials achieved significant improvement of PFS in the maintenance through olaparib treatment (400 mg twice a day) of platinum-sensitive HGSOC patients who had previously received platinum-based treatments. Both BRCA-mutated and "BRCAness" expressing patients reached longer median PFS than placebo treated ones, however, the difference was more significant for patients with defective BRCA1/2, who benefited mostly from olaparib treatment (NCT00753545) [253, 254]. Olaparib treatment response rate of recurrent ovarian, breast, pancreatic or prostate cancer patients with detected BRCA1/2 germline mutation was evaluated in other large scale single-arm, non-randomized phase II studies. All 298 patients assigned to the trial were refractory to conventionally used therapy. Overall tumor response after a 400 mg administration twice daily reached 26.2%. The best response was noticed in platinum-resistant ovarian cancer carriers (31.1% ORR, 60/193). It is interesting to point that patients with metastatic pancreatic cancer who received previously two chemotherapy regimens in the trial reached 21.7% response rate (NCT01078662) [255]. These clinical researches also defined the occurrence of adverse events (AEs) during olaparib treatment, most of which were mild-to-moderate, these effects included fatigue, headache, gastrointestinal symptoms, anorexia, and anemia [253-255]. Although a great majority of AEs were not severe, trials reported also a small number of events which could be potentially threatening to patient's life. This includes myelodysplastic syndrome or AML (MDS/AML) which were reported in less than 1% of olaparib treated patients and were fatal in the majority of reports. Lynparza™ treatment also reports less than 1% of cases of pneumonitis [248, 256].

Current findings of olaparib-based single-agent treatment indicated confirmed durable effects in recurrent ovarian cancer patients with germline mutations in BRCA genes who received at least three regimens of chemotherapy. This promising data translated into a tremendous number of clinical trials over olaparib in single-agent and combination therapy (over 100 studies at different stages due to the clinicaltrials.gov database) [257]. Although current olaparib treatment is focused only on FDA approved monotherapy, possibly it will be utilized in the future in a greater spectrum of cancer patients, either alone or supporting other therapeutic compounds.

12. RUCAPARIB

Rucaparib (Rubraca®) is the second FDA approved PARP1/2/3 small molecule inhibitor [258]. It was ini-

tially developed by Pfizer (named PF-1367338) and later, in 2011, transferred to Clovis Oncology Inc. (CO-338) [243]. Its full chemical name is 6H-pyrrolo[4,3,2-ef][2]benzazepin-6-one,8-fluoro-1,3,4,5-tetrahydro-2-[4-[(methylamino)methyl]phenyl] ($C_{19}H_{18}FN_3O$) (Fig. **3**). Cytotoxicity induced by this compound results in elevated number of PARP/DNA complexes which may result in unsuccessful DNA repair, apoptosis, and cell death [259]. The efficacy of rucaparib in treatment of orthothopic glioblastoma xenografts was shown to be low due to its limited delivery to brain [260]. In 2016, rucaparib was granted the FDA approval for the single-agent treatment of patients with advanced ovarian cancer with both germline and somatic deletions in BRCA genes. Patients legible to be enrolled in the treatment must have been previously treated with two or more regimens of chemotherapy [259]. In preclinical *in vitro* and *in vivo* trials, rupacarib demonstrated promising results in cancer cells lines and xenografts with identified mutations and epigenetic silencing of BRCA genes. Positive results were associated with mutations in other HR-related gene – XRCC3 [261, 262].

A phase I/II open label, dose-escalation studies evaluated MTD, safety, pharmacokinetics, and preliminary efficacy of rucaparib treatment in patients with solid tumors whose disease continued to deteriorate after treatment with standard therapy. Analyzed doses spectrum ranged from 40 mg administered once daily to 840 mg taken twice a day. Basing on antitumor effectiveness and manageable toxicity, research determined the optimal, recommended administration to be 600 mg of rucaparib twice a day. What is more, rucaparib demonstrated promising anticancer activity in both platinum-sensitive and resistant group of ovarian cancer patients (Study10, NCT01482715) [263-265]. This recommended dose was later used in further clinical trials and is currently used in the clinical treatment administered in two 300 mg tablets twice a day [259].

Currently further development of rucaparib treatment is focused on three main trials - ARIEL2, ARIEL3 and ARIEL4 trials (Assessment of Rucaparib in Ovarian Cancer Trial) which are based on cooperation between Clovis Oncology Inc. and Foundation Medicine Inc. Programs are aimed at identifying a molecular signature of ovarian cancer cases which could benefit the most from rucaparib-based therapy. ARIEL2 is a phase II open label trial conducted on patients with relapsed or progressed high grade endometrioid epithelial ovarian, fallopian tube or primary peritoneal cancer who had previously received three or more regimens of chemotherapy and had been categorized as platinum-sensitive. Primary results from ARIEL2 show response according to RECIST and/or marker analysis in both BRCA-mutated patients and BRCA-wild type patients who registered high level of LOH in their tumor tissue (NCT 01891344) [266]. Further extended analysis has confirmed previous trend and showed response in 69% patients with detected mutations in BRCA genes. Interestingly, response occurred in both germline and somatic mutated tumors. Research has also confirmed response in group of patients with wild type of BRCA but high level of LOH (39%). Only 11% of patients with no detected BRCA-mutated or BRCA-like variant responded to the rucaparib treatment [267]. In a group of women with platinum-sensitive deleterious mutation in BRCA genes who had already received two to four chemotherapy regimens and experienced progression-free interval (PFI) above 6 months rupacarib demonstrated a high antitumor activity with ORR reaching 81% (21/26) [268]. ARIEL3 is a phase III randomized, double-blind trial indicating the effectiveness of rucaparib maintenance treatment of platinum-sensitive HGSOC, endometrioid epithelial ovarian, fallopian tube, and primary peritoneal cancer in comparison to placebo group (NCT01968213) [257, 269]. ARIEL4 is a randomized, open label phase III study comparing rucaparib treatment with standard chemotherapy (NCT02855944) [257, 269].

Effectiveness of rucaparib treatment is also being evaluated in prostate cancer in phase II and phase III studies. They aim to identify patients with metastatic, castration-resistant tumors with evidence of HR-deficiencies who may benefit from rucaparib-based treatment. TRITON2 (Trial of RucaparIb In prosTate indicatiONs) is an open label phase II trial on the response of the described patients to rucaparib monotherapy (NCT02952534). TRITON3 (phase III open label trial), on the other hand, compares response to rucaparib with response to standard chemotherapy (NCT02975934) [257].

The most common treatment-related AEs observed during clinical trials included gastrointestinal symptoms, fatigue, and increase in AST/ALT. MDS/AML has been reported in 0.5% of patients (2/377) treated with rupacarib [259, 268].

13. NIRAPARIB

Niraparib (Zejula™, MK-4827) is a recently FDA approved highly selective PARP1/2 small molecule inhibitor developed by Tesaro Inc. [243]. Its full chemical name is 2-{4-[(3S)-piperidin-3-yl] phenyl}-

2H-indazole-7-carboxamide ($C_{26}H_{30}N_4O_5S$) (Fig. **3**). Niraparib exhibits 100-fold higher selectivity for PARP1 and PARP2 than for other members of PARP protein family [270]. It has demonstrated very attractive pharmacokinetics when administered orally once a day in doses of 300 mg in three 100 mg capsules independently from food intake. Just like olaparib, this inhibitor is rapidly absorbed, reaching its peak plasma concentration within 3 hours after an oral administration. Niraparib is able to cross the blood-brain barrier. It has been approved in the USA for the maintenance therapy of recurrent epithelial ovarian, fallopian tube, and primary peritoneal cancer patients who had previously received platinum-based therapy and had showed complete or partial response [271]. Also EMA has currently approved niraparib for review of Marketing Authorization Application (MAA) as a single-agent treatment of platinum-sensitive recurrent ovarian cancer [272].

In preclinical *in vitro* tests, niraparib has demonstrated selective antiproliferative activity in BRCA1/2-mutated tumor cells in comparison to normal BRCA-proficient cells. Cytotoxic activity of the compound resulted in cell death due to induction of cell cycle arrest, apoptosis and mitotic catastrophe [273]. Additionally, *in vivo* studies over response to different niraparib doses have showed not only tumor regression, but also excellent tolerability (no mortality and less than 10% of body weight loss) [270]. A number of studies have also demonstrated ability of niraparib to radio sensitize human lung, breast, and neuroblastoma cancer cell lines as well as xenograft mice. Combinatory treatment with PARP inhibitor and radiation significantly increased the number of DSBs expressed in the elevated level of phosphorylated histone γH2A.X [274-276]. In addition, other *in vivo* studies have evaluated the response of five models of patient-derived HGSOC xenografts to niraparib treatment. Tumor regression was only achieved in model with hypermethylated promoter of RAD51C gene and in one out of two models with deletion detected in BRCA2 gene. Maintenance therapy with niraparib was generally able to delay progression of disease in BRCA2-deficient mice [277].

Initial phase I/Ib dose-escalation trials aimed to investigate maximum tolerated dose, pharmacokinetics, and pharmacodynamics of niraparib monotherapy in 100 patients with solid tumors. The compound was well tolerated up to the dose of 300 mg administered once daily which was recommended for further phase II tests. Preliminary antitumor activity analysis suggested that carriers of BRCA1/2-defective ovarian (40%, 8/20) and breast cancer (50%, 2/4) could potentially benefit from niraparib. A better response was also correlated with platinum-sensitivity (NCT00749502) [278].

There is presently an ongoing phase II trial QUADRA single-arm test of niraparib treatment in relapsed HGSOC, fallopian tube cancer or primary peritoneal cancer patients who had received previously 3 or 4 regimens of chemotherapy. Patients enrolled in the trial must have experienced a platinum-based first-line therapy response lasting over 6 months (NCT02354586) [257, 279]. The development process of niraparib bases also on extensive phase III double-blind, placebo-controlled trial NOVA which has been recently completed. The study evaluated efficacy of niraparib single-agent treatment in platinum-sensitive recurrent ovarian cancer patients in comparison to placebo treatment. During the examination two groups were created, one with germline mutation of BRCA1/2, the other without detected alterations. Beneficial effect of niraparib therapy was most pronounced in the group of patients with platinum-sensitive ovarian cancer with detected mutations in BRCA genes. In this group PFS reached 21 months, compared to 5.5 months in placebo group and 12.9 months in group of niraparib treated patients without detected mutations in BRCA genes but categorized as homologous recombination deficient patients by myChoice HRD® (Myriad) (NCT01847274) [280]. The most commonly reported AEs after niraparib treatment included mild-to-moderate incidents such as nausea, anemia, thrombocytopenia, and fatigue. NOVA trial reported also 5 cases (1.4%) of MDS/AML in a group of 372 patients treated with niraparib [278, 280].

PRIMA, which is a phase III randomized, double-blinded, placebo-controlled trial, evaluates efficacy of niraparib maintenance treatment in stage III or IV ovarian cancer patients who had responded to first-line platinum-based therapy. Patients undergoing the clinical trial are tested for HR deficiencies (NCT02655016) [257]. Currently, possible beneficial outcome of niraparib-based treatment is also being analyzed in other solid tumors. This includes phase III open label trial BRAVO in patients with advanced or metastatic HER2-negative breast cancer with detected germline abnormalities in BRCA genes. The trial compares response to niraparib with the treatment chosen by physicians (NCT 01905592) [257].

14. VELIPARIB

Veliparib (ABT-888, NSC-737664) is a potent PARP1/2 small molecule inhibitor developed by Abbott [243]. It is a bezimidazole compound which has

demonstrated promising efficacy and tolerability in preclinical trials. Its full chemical name is 2-[(2R)-2-Methylpyrrolidin-2-yl]-1H-benzimidazole-4-carboxamide ($C_{13}H_{16}N_4O$) (Fig. **3**). Veliparib exhibits good oral bioavailability and is able to cross blood-brain barrier. In a single-agent therapy, veliparib doesn't demonstrate any antiproliferative effects; thereby it inhibits PARP1/2 and exhibits very promising chemo- and radio sensitizing activities [281-283]. Nowadays veliparib is mainly examined in combinatory therapy with different cytotoxic agents or ionizing radiation.

Phase 0 trial evaluated pharmacokinetics and pharmacodynamics of veliparib in recurrent solid tumors and lymphoid malignancies. Thirteen patients were divided into three groups and administered a single dose of either, 10 mg, 25 mg, or 50 mg of veliparib. Drug was well tolerated and demonstrated a good oral bioavailability (NCT00387608) [284]. Phase II open label trial on BRCA-mutated recurrent ovarian cancer patients analyzes the benefit of veliparib as a single-agent therapy. The compound is being administered twice daily in a dose of 400 mg. Results have shown 26% (13/50) of response in analyzed group of patients. The response reached 35% and 20% in the groups of platinum-sensitive and platinum-resistant patient respectively. The most common AEs reported in the group of patients treated with veliparib were fatigue, nausea, vomiting, and anemia (NCT01540565) [285]. Effectiveness of veliparib monotherapy is being currently evaluated in phase I clinical trials on groups of patients with malignant solid tumors that had not been responsive to previous treatment regimens (NCT 00892736). Phase I/II open label trial is investigating veliparib-based monotherapy in patients with BRCA-mutated relapsed ovarian cancer (NCT01472783) [257].

Veliparib has exhibited previously ability to sensitize tumor cells to chemo- and radiotherapy, therefore, this ability is widely utilized in multiple clinical trial of this compound in combination with other agents inducing DNA lesions [286-288].

15. TALAZOPARIB

Talazoparib is an oral PARP1/2 inhibitor initially developed by Lead Therapeutics (named LT673) but acquired first by BioMarin (named BMN673) and then by Medivation Inc. (now a part of Pfizer Inc.) [243]. Its chemical name is (8S,9R)-5-fluoro-8-(4-fluorophenyl)-9-(1-methyl-1H-1,2,4-triazol-5-yl)-8,9-dihydro-2H-pyrido[4,3,2-de]phthalazin-3(7H)-one ($C_{19}H_{14}F_2N_6O$) (Fig. **3**). This small molecule inhibitor exhibits unusually high potency and selectivity of its action. Talazoparib is able to trap PARP/DNA complexes approximately 100-fold more effectively than previously described inhibitors, namely olaparib and rucaparib, however, their ability to inhibit catalytic activity of PARP is similar [233, 289]. The novel structure of this compound provides for unique, extensive binding with PARP1/2 [290]. In preclinical *in vitro* and *in vivo* tests, talazoparib has demonstrated promising antitumor activity, particularly in BRCA-deficient cells and xenografts [291, 292]. Moreover, there is a correlation between molecular signatures of BRCAness with response to talazoparib [293].

Effectiveness and safety of talazoparib treatment is currently being tested in a number of clinical trials. A two-part, phase I dose-escalation trial has evaluated MTD to be 1 mg a day. Preliminary antitumor activity analysis in patients with advanced or recurrent solid tumors has confirmed response in 50% (7/14) of BRCA-deficient breast and ovarian cancer carriers. Response reached 42% (5/12) in group of patients with pancreatic and lung cancer. Most common talazoparib-related AEs included fatigue and anemia, gastrointestinal issues, and thrombocytopenia (NCT01286987) [294, 295]. Phase II non-randomized study is currently testing response of patients with advanced solid tumors with BRCAness or BRCA-mutated genes to talazoparib administered at 1 mg a day (NCT02286687) [296]. Efficacy of talazoparib in comparison to standard chemotherapy is currently being tested in EMBRACA phase III randomized, open label trial in advanced or metastatic breast cancer patients with detected germline mutations in BRCA1/2 genes (NCT01945775) [257].

16. CEP9722

CEP9722 is a novel small molecule oral PARP1/2 inhibitor currently being developed by Cephalon (part of Teva Pharmaceutical Industries Ltd.) as both single-agent therapy and in combination with other different drugs. Its chemical name is 11-methoxy-2-((4-methylpiperazin-1-yl)methyl)-4,5,6,7-tetrahydro-1H-cyclopenta[a]pyrrolo[3,4-c]carbazole-1,3(2H)-dione ($C_{24}H_{26}N_4O_3$) (Fig. **3**). Preliminary study over CEP9722 has shown, *in vitro* and *in vivo,* antitumor activity when administered either alone or in combination with other anticancer drugs. However, what is even more important about this is that it did not induce myelotoxicity [297, 298]. Phase I dose-escalation trial has evaluated MTD to be 750 mg of CEP9722 per day. The results have also shown this compound to exhibit only modest myelosuppresion (NCT00920595) [299].

CEP9722 is currently under further development either alone or in combination with other anticancer agents (NCT00920595), (NCT01345357).

17. PARP INHIBITORS IN COMBINATION WITH CHEMO- AND RADIOTHERAPY

PARP inhibitors have shown an ability to selectively eliminate cells with defective repair pathways. However, their original aim was to sensitize cells towards other anticancer agents and their utility as single-agent therapy had not been expected. Multiple *in vitro* and *in vivo* studies over utility of PARP inhibitors in antitumor treatment have proven their ability to elicit a profound radio- and chemosensitizing activity and currently a broad spectrum of agents is being tested on their potential clinical use in combination with different PARP inhibitors (Table **2**) [276, 281, 283, 300]. A combination of three factors - reduced HR functionality in cancer cells, PARP inhibition, and use of DNA damaging agent could bring promising therapeutic results. For instance, many patients receive radiotherapy as part of their anticancer therapy. Exposure to IR causes accumulation of DNA lesions the repair of which involves recruitment of PARP1, which then creates a complex with DNA [301]. Studies indicate that 1 Gy of radiation induces average of 35 DSBs per cell [302]. For these reasons, PARP1–deficient cell lines and xenografts are found to be sensitive to IR [168, 303]. This potential treatment is nowadays being tested in a number of clinical trials including a study over combination of veliparib with radiotherapy in patients with solid tumors (NCT01264432) or a phase I trial over olaparib and radiation therapy in TNBC (RadioPARP, NCT03109080) [288]. A combination of radiation and PARP inhibitors has been predicted to improve its efficacy in tumor cells and has elicited specific ability to eliminate cancer cells.

The main purpose of chemotherapeutic agents currently in use is to either introduce DNA damage or exploit abnormalities occurring in the repair pathways of cancer cells. PARP inhibitors, which also cause destabilization of repair processes, have demonstrated extensive chemopotentiating effects. Temozolomide (TMZ) and dacarbazine (DTIC) are compounds of cytotoxic therapy being used in brain and melanoma cancer treatment, respectively. They both are cytotoxic agents that introduce alkyl groups to guanine bases [304, 305]. Out of wide spectrum of their methylation sites, the most frequent ones are N7-methylguanine (N7MeG) and N3-methylguanine (N3MeG). The most toxic of lesions induced by alkylating agents is O6-methylguanine (O6MeG) that represents 0.3 to 8% of all adducts created by alkylating agents in cells. O6-methylguanine-DNA methyltransferaze (MGMT) is a major alkyltransferaze responsible for acquired resistance to cytotoxic agents such as TMZ or DTIC due to its activity in removing alkyl group from oxygen by transferring it to the cysteine in its catalytic domain, resulting in MGMT protein deactivation. If O6MeG is not repaired before a replication process, which may be a result of MGMT depletion or natural low level of MGMT in the tissue, faulty mismatch repair (MMR) mispairs O6MeG with thymine, later leading to generation of secondary lesions and subsequently to creation of DSBs [306]. Therefore, it has already been demonstrated that MGMT-deficient cells exhibit elevated level of γH2A.X, suggesting that the fallout of impaired MGMT dependent repair is formation of DSBs. Further effects of impaired O6MeG repair include SCEs, chromosomal aberrations, point mutations, cell death, and also tumor initiation and/or progression [307]. Unsuccessful repair of O6MeG leads to replication forks stalling, therefore, a repair pathway which appears to protect cells from O6MeG-triggered cell death is HR. Cells with impaired HR are more sensitive to alkylating agents than HR-proficient cells. This explains the reason for effectiveness of alkylating agents in combination with synthetic lethality-based anticancer therapy [308]. Likewise, synergism of PARP inhibitors and alkylating agents appears to be the stronger, the better inhibitor traps PARP protein on DNA. Alkylating agents are potent at enhancing sensitivity of multiple tumor types to PARP inhibitors and a number of clinical trials are currently testing the efficacy of their use in combination with inhibitors like olaparib, niraparib, talazoparib, or veliparib (NCT01858168, NCT00516 802, NCT01294735, NCT02116777, NCT01009788) [257, 309, 310].

Platinum salts are other DNA-damaging chemotherapeutic used in anticancer therapy in combination with PARP inhibitors. Highly reactive platinum analogs are able to covalently interact with nucleophilic residues in DNA and generate interstrand, and more frequently intrastrand crosslinks, therefore, arresting transcripting or replicating machinery progression. Such arrest could lead to miscoding and collapse of replication forks, SSBs and DSBs [311]. Combination of PARP inhibitors with compounds like cisplatin or carboplatin has demonstrated promising results in preclinical trials and is currently further being tested in several clinical studies (NCT00782574,NCT01445418) [281, 312, 313].

Table 2. Clinical trials evaluating effectiveness of PARP inhibitors in combination with different anticancer agents.

Treatment	NCT no./Trial Name	Phase	Study Design	Study Population	Primary Outcome
RADIATION					
Veliparib, Radiation	NCT01264432	I	Single arm, open label	Advanced solid malignancies with peritoneal carcinomatosis, EOC, FTC, PPC	MTD
Olaparib, Radiation	NCT03109080 (RadioPARP)	I	Single arm, open label	Advanced or metastatic TNBC	MTD
ALKYLATING AGENTS					
Olaparib, Temozolomide	NCT01858168	I	Non-randomized, open label	Recurrent or metastatic Ewing's Sarcoma following failure of prior chemotherapy	MTD
Niraparib, Temozolomide	NCT01294735	I	Non-randomized, open label	Recurrent solid tumors, advanced glioblastoma multiforme, advanced melanoma	MTD, DLT
Talazoparib, Temozolomide	NCT02116777	I/II	Single arm, open label	Refractory or recurrent malignancies	MTD, DLT, PK
Veliparib, Temozolomide	NCT01009788	II	Single arm, open label	Metastatic BC and BRCA-mutated BC	ORR
Olaparib, Dacarbazine	NCT00516802	I	Non-randomized, single arm, open label	Advanced melanoma	MTD, DLT
PLATINUM SALTS					
Olaparib, Cisplatin	NCT00782574	I	Single arm, open label	Advanced solid tumors	MTD
Olaparib, Carboplatin	NCT01445418	I	Non-randomized, open label	BRCA-mutated BC and OC, sporadic TNBC and OC	MTD
TAXANES					
Olaparib, Paclitaxel	NCT01924533	III	Randomized, double blind, placebo controlled	Advanced gastric cancer which have progressed after first-line therapy	OS
GEMCITABINE					
Olaparib, Gemcitabine	NCT00515866	I	Randomized, open label	Advanced solid tumors	MTD
TOPOISOMERASE INHIBITORS/POISONS					
Olaparib, Topotecan	NCT00516438	I	Non-randomized, single arm, open label	Advanced solid tumors	MTD
Veliparib, Topotecan	NCT00553189	I	Non-randomized, open label	Refractory solid tumors and lymphomas	MTD
Olaparib, Liposomal doxorubicin	NCT00819221	I	Single arm, open label	Advanced solid tumors	MTD
Veliparib, Liposomal doxorubicin	NCT01145430	I	Single arm, open label	Recurrent OC, FTC, PPC or metastatic BC	MTD, DLT

(BC – breast cancer, DLT – dose-limiting toxicities, EOC – epithelial ovarian cancer, FTC – fallopian tube cancer, MTD – maximum tolerated dose, OC – ovarian cancer, ORR – objective tumor response, OS – overall survival, PK – pharmacokinetics, PPC – primary peritoneal cancer, TNBC – triple-negative breast cancer)

PARP inhibitors are also expected to enhance the effect of taxanes (paclitaxel or docetaxol) which are suppressors of microtubule dynamics that induce their polymerization properties. As a result they block mito-

sis and cause cell death [314]. These agents are being tested in gastric cancer, which often carries mutations in the ataxia telangiectasia mutated (ATM) gene [315]. Deficiency of ATM was previously correlated with sensitivity to PARP inhibition, therefore, agents like olaparib in combination with paclitaxel are currently under development (NCT01924533) [197, 300, 316].

Gemcitabine is an antitumor agent that increases replication stress by inhibiting DNA chain elongation and blocking processivity of replication forks. It is generally used either alone or in combination with other compounds like taxanes or platinum salts. Preclinical studies have demonstrated synergistic action of PARP inhibitors and gemcitabine [317]. These studies are currently being expanded in clinical trials that analyze the safety and tolerability of PARP inhibitors like olaparib or veliparib administered in combination with gemcitabine (NCT00515866, NCT02860819).

Additional chemotherapeutics under evaluation for therapy in combination with PARP inhibitors are topoisomerase inhibitors and poisons. This group of compounds includes camptothecin, irinotecan or topotecan which are topoisomerase 1 poisons/inhibitors and mitoxantrone, etoposide or doxorubicin which interact with topoisomerase 2 [300]. Topoisomerase 1 is able to induce breaks in one strand of DNA giving rise to SSBs, whereas type 2 topoisomerase induces DSBs by cutting both strands of DNA simultaneously. This process is required for DNA relaxation and torsions removal during processes like replication or transcription. Inhibition of these enzymes results in creation of stabilized complex of enzyme fixed covalently with DNA during replication process, which subsequently prevents strand ligation and gives rise to SSBs and DSBs due to a collision of replication forks with a topoisomerase/DNA fixed complex [318, 319]. PARP1 activity is crucial for repair of topoisomerase 1 inhibition effects by tyrosyl-DNA phosphodiesterase 1 (TDP1) [320]. For this reason, synergism of PARP inhibitors and compounds like camptothecin or topotecan are currently undergoing clinical trials (NCT00516438, NCT00553189). Topoisomerase type 2 inhibitors can either fix an enzyme on the DNA (mitoxantrone or doxorubicin) or weaken its ATP catalytic activity (bisdioxopiperazines or novobiocin) [321]. Their synergistic effect with PARP inhibitors is under tests in clinical studies (NCT00819221, NCT01145430).

In addition to chemotherapeutic compounds, PARP inhibitors can be combined with targeted agents utilizing cancer cells vulnerabilities. This group includes drugs like erlotinib, an epidermal growth factor receptor (EGFR) inhibitor approved for treatment of EGFR-mutated non-small cell lung cancer (NSCLC) which in combination with olaparib had a significant antitumor activity in EGFR-overexpressing BRCA1/2-wild type ovarian cancer cell lines and xenograft mice [322]. Anti-angiogenetic agents like vascular endothelial growth factor receptor (VEGFR) inhibitors constitute yet another group of compounds. Interestingly, VEGFR3 inhibition in ovarian cancer CSCs has been associated with decreased expression of BRCA1/2 which can potentially sensitize cells that exhibit primary or secondary resistance to PARP inhibitors [323].

In addition, inhibition of histone deacetylase (HDAC) could promote sensitivity of cancer cell to PARP inhibitors. Activity of HDAC is responsible for removing acetyl groups from histones and other non-histone proteins. Eleven HDAC proteins grouped in three classes are crucial for maintaining the structure of chromatin during such processes as transcription or DNA repair. Multiple tumor types have been reported to overexpress class I HDACs which has been associated with decreased survival. These data points to the role of HDACs in tumor cell maintenance [324]. HDAC inhibition results in decreased expression of DSB repair proteins including RAD51, RAD51D, FANCD (Fanconi anemia complementation group D) and Ku70. Class I HDAC inhibition in melanoma cells resulted in abolition of their resistance to alkylating agents by impairing HR repair pathway responsible for removal of O6MeG adducts from DNA under conditions of low MGMT expression [325]. HDAC inhibition-triggered HR-deficiency could potentially sensitize cells to PARP inhibitors. These synergistic interaction have been confirmed in TNBC cell lines and in mice xenografts [326]. However, this model has not yet been analyzed in clinical trials.

18. NOVEL POTENTIAL RAD52 INHIBITORS

The concept of RAD52 targeted therapy in BRCA1/2-deficient cells is relatively new, therefore, it is currently focused on further interactions evaluation and development of novel potential RAD52-inhibitors in preclinical *in vitro* and *in vivo* trials. 6-hydroxy-DOPA - a precursor of 6-hydroxydopamine - is a small molecule RAD52 inhibitor. It has been shown to selectively reduce the proliferative potential of BRCA1/2-deficient cancer cells and to lead to cell death [222]. Huang *et al*. have reported development of two potent RAD52 inhibitors - D-G09 and D-I03 which were able to inhibit RAD52 activity and suppress growth of BRCA1/2-deficient cells [223]. Sullivan *et al*. have

aimed Phe79 residue of DNA binding domain I and designed aptamer F79 to disrupt interactions between RAD52 and DNA. F79 induced cell death in BRCA1/2-deficient cancer cells and did not affect normal cells [225]. In addition, adenosine 5'-monophosphate (A5MP), and also its mimic 5-aminoimidazole-4-carboxamide ribonucleotide 5' phosphate inhibited RAD52 activity *in vivo* and exerted synthetic lethality against BRCA1 and BRCA2-mutated carcinomas [327]. Moreover, small-molecule inhibitors identify the RAD52-ssDNA interaction as critical for recovery from replication stress and for survival of BRCA2-deficient cells [328]. Targeting RAD52 in tumors depleted in BRCA1/2 activity offers a novel therapeutic approach and drugs currently in use in preclinical trials need further extensive investigation into their safety and application effectiveness.

19. RESISTANCE TO SYNTHETIC LETHALITY

The growing interest in synthetic lethal interactions has led to appearance of numerous clinical trials on therapy based on PARP inhibitors used either alone or in combination with other compounds. Deregulation in HR repair system expressed in BRCA mutations has been associated with improved survival and prolonged progression-free period [329]. However, patients often develop resistance to treatment with platinum compounds or PARP inhibitor. This has become a vexing challenge to clinical utilization of synthetic lethality-based therapies. Taking into consideration underlying mechanisms, resistance could also arise after treatment with potential RAD52 inhibitors, for they utilize the same cancer cell vulnerabilities as PARP inhibitors.

BRCA mutations, for example hypomorphic BRCA mutations, differ in effectiveness of HR impairment. Mice carrying a missense mutation in $BRCA1^{C61G}$ RING domain appear to be much more resistant to olaparib treatment than mice with knock-out of BRCA1. The relatively higher level of RAD51 foci and lower level of DSBs induced by olaparib in tumors with mutated $BRCA1^{C61G}$ RING as compared to BRCA-deficient tumors suggests that resistance may arise due to residual activity of mutated protein [330]. The majority of mutations, both somatic and germline, occurring in BRCA1/2 genes lead to the frame shift or premature termination of gene transcription, giving rise to shorter, dysfunctional proteins [331]. Treatment utilizing cytotoxic compounds that damage DNA and inhibitors that activate error-prone repair pathways very often backfires due to induction of secondary mutations restoring function of BRCA1/2 proteins and HR system, therefore, granting tumor resistance to PARP inhibitors and platinum salts based therapy [332-334]. Detection of secondary mutations in BRCA1/2 has appeared to predict platinum resistance better than detection based on progression-free interval from last platinum-based treatment. BRCA1/2 reverse mutations were more common in patients that had experienced recurrent tumors prior one or more therapy regimens [335].

Although secondary mutations that restore function of BRCA1/2 have been demonstrated to be highly associated with tumor resistance to PARP inhibition, not all therapy refractory tumors carry them, suggesting presence of other mechanisms facilitating tumor resistance to the treatment [335]. Mutation in previously described antagonist of BRCA1 – 53BP1 is another mechanism of resistance to synthetic lethality-based therapy with PARP inhibitors. The loss of this protein along with BRCA1/2 deficiency results in repair rewiring, leading to partial restoration of HR pathway [336, 337]. Lastly, overexpression of p-glycoprotein efflux pump (PgP), a multidrug-resistance protein, results in acquired resistance to PARP inhibitors. Activity of this transmembrane protein is responsible for transporting drugs from inside the cell to its outer environment, which results in reduction of intracellular concentration of drug and its limited delivery to the target. Acquired resistance to olaparib has been observed in mice overexpressing Abcb1a/b genes coding murine PgP efflux pump. Later treatment with PgP inhibitor – tariquidar managed to overcome the resistance and resulted in sensitization of recurrent tumors to olaparib [338, 339].

However, the issue of drug resistance is a challenging problem that requires further research. For instance, it is currently unclear whether resistance will also arise in response to combination therapy or maybe it would be possible to delay resistance acquisition or even prevent it. Another issue is how factors like drug level, its scheduling and sequence of administration in combination with other agents lead to acquisition of resistance. Finding solutions to these key issues is critical not only for the development of PARP inhibitors based treatment, but also appears to affect all synthetic lethality-based therapies.

CONCLUSION

For the past few decades, development of specific and effective anticancer therapy has proven to be a challenging and often futile effort. Synthetic lethality represents a new approach for targeted therapy against unique molecular signature of different tumors. Utiliza-

tion of genomic instability and vulnerabilities arising specifically in cancer cells appears to be an elegant method of tumor elimination, without harm to normal cells and tissues. FDA approval granted in the recent years to three PARP inhibitors – olaparib, niraparib and rucaparib, pushed the anticancer therapy to a new trajectory of personalized treatment utilizing the unique molecular changes of each patient. Efficacy and tolerability data from currently conducted clinical trials continue to give us encouraging results of application of synthetic lethality in clinical practice. Novel diagnostic comprehensive analyses are able to search for molecular tumor signatures that determine tumor sensitivity to applied therapy. What is more, extensive studies over these interactions have broadened our knowledge on molecular biology of cancer cells and have revealed new therapeutic targets beyond BRCA1/2 defects. Tumor-specific deregulations impairing the DNA repair systems are more common than initially estimated therefore they appear to be a promising target for synthetic lethality-based therapy. Abnormalities in repair systems refer not only to genetic changes in their components, but also to events like hypermethylation of genes promoter sequences that result in their silencing and decreased expression that depleting cells their activity. However, treatment with compounds that lead to induction and accumulation of toxic DNA lesions may seem like skating over thin ice. Activation of error-prone repair systems and treatment based on cytotoxic agents could induce secondary mutations restoring function in genes responsible for primary DNA repair pathway deactivation, subsequently, granting tumor therapy resistance. Therefore, it is crucial to identify mechanisms of tumor resistance and investigate methods of either preventing or overcoming them if they have already developed. Despite the undeniably enormous potential of synthetic lethality in anticancer therapy, this approach is still in its infancy. Further, researches will provide us with broader insight into efficacy and long term effects of synthetic lethality-based therapies. However, the number of interactions currently utilized in synthetic lethality-based therapy appears to have only just begun to scratch the surface in the spectrum of possibilities that this approach creates.

LIST OF ABBREVIATIONS

53BP1	=	p53 binding protein 1
AEs	=	adverse events
altNHEJ	=	alternative non-homologous end joining
AML	=	acute myeloid leukemia
APLF	=	PNKP-like factor
APTX	=	aprataxin
ARH3	=	ADP-ribosyl hydrolase 3
ATM	=	ataxia telangiectasia mutated
BARD1	=	BRCA1 associated RING domain
BER	=	base excision repair
BLM	=	Bloom syndrome RecQ like helicase
bNHEJ	=	back-up non-homologous end joining
CNAs	=	copy-number alterations
cNHEJ	=	classical/canonical non-homologous end joining
CPCs	=	cancer progenitor cells
CRISPR	=	clustered regularly interspaced short palindromic repeats
CSCs	=	cancer stem cells
CtIP	=	CtBP interacting protein
DNA-PKcs	=	catalytic subunit of DNA-dependent protein
DSBR	=	double strand break repair
DSBs	=	double strand breaks
DTIC	=	dacarbazine
EGFR	=	epidermal growth factor receptor
EMA	=	European Medicines Agency
EOC	=	epithelial ovarian cancer
EXO1	=	exonucleases 1
FANCD	=	Fanconi anemia complementation group D
FDA	=	Food and Drug Administration
FEN1	=	flap structure-specific endonuclease 1
GEMA	=	Gene Expression and Mutation Analysis
HDAC	=	histone deacetylase
HGSOC	=	high-grade serous ovarian
HR	=	homologous recombination
IR	=	ionizing radiation
ITD	=	internal tandem duplications
LIG1/3/4	=	ligase I/III/IV
LOH	=	loss of heterozygosity

MAA = Marketing Authorization Application
MDS = myelodysplastic syndrome
MGMT = O6-methylguanine-DNA methyl-transferaze
MiSL = Mining Synthetic Lethals
MMEJ = microhomology-mediated end joining
MMR = mismatch repair
MRE = complex of MRE11
RAD50 =
NBS1 =
MTD = maximum tolerated dose
N3MeG = N3-methylguanine
N7MeG = N7-methylguanine
NGS = next-generation sequencing
NHEJ = non-homologous end joining
NSCLC = non-small cell lung cancer
O6MeG = O6-methylguanine
OGG1 = 8-oxoguanine DNA glycosylase 1
ORR = objective response rate
PALB2 = partner and localizer of BRCA2
PARG = poly[ADP-ribose] glycohydrolase
PARP = poly [ADP-ribose] polymerase
PAXX = paralog of XRCC4 and XLF
PFI = progression-free interval
PFS = progression-free survival
PgP = p-glycoprotein efflux pump
PLD = pegylated liposomal doxorubicin
RECIST = Response Evaluation Criteria in Solid Tumors
ROS = reactive oxygen species
RPA = replication protein A
RT-qPCR = Reverse Transcription-quantitive PCR
SCEs = sister chromatid exchanges
SDSA = synthesis-dependent strand annealing
shRNA = short hairpin RNA
SNP = single nucleotide polymorphism
SSA = single strand annealing
SSBR = single strand break repair
SSBs = single strand breaks
ssDNA = single stranded DNA
TAI = telomeric allelic imbalance
TDP1 = tyrosyl-DNA phosphodiesterase 1
TMBC = triple-negative breast cancer
TMZ = temozolamide
VEGFR = vascular endothelial growth factor receptor
XLF = XRCC4-like factor
XRCC1/2/3/4 = X-ray repair cross-complementing protein 1/2/3/4

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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MDPI

Review

RAD52 as a Potential Target for Synthetic Lethality-Based Anticancer Therapies

Monika Toma [1,2], Katherine Sullivan-Reed [1], Tomasz Śliwiński [2] and Tomasz Skorski [1,*]

[1] Sol Sherry Thrombosis Research Center and Fels Institute for Cancer Research and Molecular Biology Lewis Katz School of Medicine, Temple University, Philadelphia, PA 19140, USA; monikatoma3@gmail.com (M.T.); katherine.s.reed@temple.edu (K.S.-R.)

[2] Laboratory of Medical Genetics Faculty of Biology and Environmental Protection, University of Lodz, 90-236 Lodz, Poland; tomasz.sliwinski@biol.uni.lodz.pl

* Correspondence: tskorski@temple.edu; Tel.: +1-215-707-9157

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Abstract: Alterations in DNA repair systems play a key role in the induction and progression of cancer. Tumor-specific defects in DNA repair mechanisms and activation of alternative repair routes create the opportunity to employ a phenomenon called "synthetic lethality" to eliminate cancer cells. Targeting the backup pathways may amplify endogenous and drug-induced DNA damage and lead to specific eradication of cancer cells. So far, the synthetic lethal interaction between BRCA1/2 and PARP1 has been successfully applied as an anticancer treatment. Although PARP1 constitutes a promising target in the treatment of tumors harboring deficiencies in BRCA1/2—mediated homologous recombination (HR), some tumor cells survive, resulting in disease relapse. It has been suggested that alternative RAD52-mediated HR can protect BRCA1/2-deficient cells from the accumulation of DNA damage and the synthetic lethal effect of PARPi. Thus, simultaneous inhibition of RAD52 and PARP1 might result in a robust dual synthetic lethality, effectively eradicating BRCA1/2-deficient tumor cells. In this review, we will discuss the role of RAD52 and its potential application in synthetic lethality-based anticancer therapies.

Keywords: synthetic lethality; dual synthetic lethality; RAD52; PARP1; DNA repair

1. Introduction

Over the past years, physicians and scientists have been implementing major changes in medical practice, leading to a shift from traditional "trial and error" approach to personalized therapy based on the individual features of each patient. Targeted anticancer therapy is a promising tool of current medicine, which allows us to improve the effectiveness of the treatment and increase the overall survival of patients diagnosed with cancer. Multiple studies and clinical trials conducted nowadays utilize personalized anticancer therapy as an approach to provide a great chance to fulfill the need for rationally designed selective treatment. Carcinogenesis is a complex process involving the development of genetic instability, which is responsible for the accumulation of mutations and tumor progression [1]. Therefore, a personalized approach would aim to identify these specific changes in the DNA of an individual patient's cancer cells, which would allow us to specifically target them, and not harm normal cells and tissues.

Maintenance of genome integrity is critical for cell survival. For that reason, double-strand breaks (DSBs), which disrupt DNA continuity, are among the most toxic lesions, often causing mutations and chromosomal aberrations resulting in neoplastic transformation. In human cells, repair of DSBs is carried out by two major mechanisms—homologous recombination (HR) and non-homologous

end-joining (NHEJ) [2]. NHEJ is the main repair pathway in quiescent cells, whereas HR works primarily by utilizing the short proximity of the homologous sequence in proliferating cells.

Canonical DNA-PK-mediated NHEJ (D-NHEJ) is a predominant repair system in normal quiescent cells, while PARP1-mediated alternative NHEJ (B-NHEJ) serves as backup. In normal proliferating cells, PARP1-mediated base excision repair (BER) is employed to prevent replication fork collapse and eventual DSB formation. BRCA1/2-RAD51-dependent HR (BRCA1/2 HR) serves as a main DSB repair pathway and RAD52-RAD51 HR (RAD52 HR) serves as an alternative pathway (Figure 1) [3].

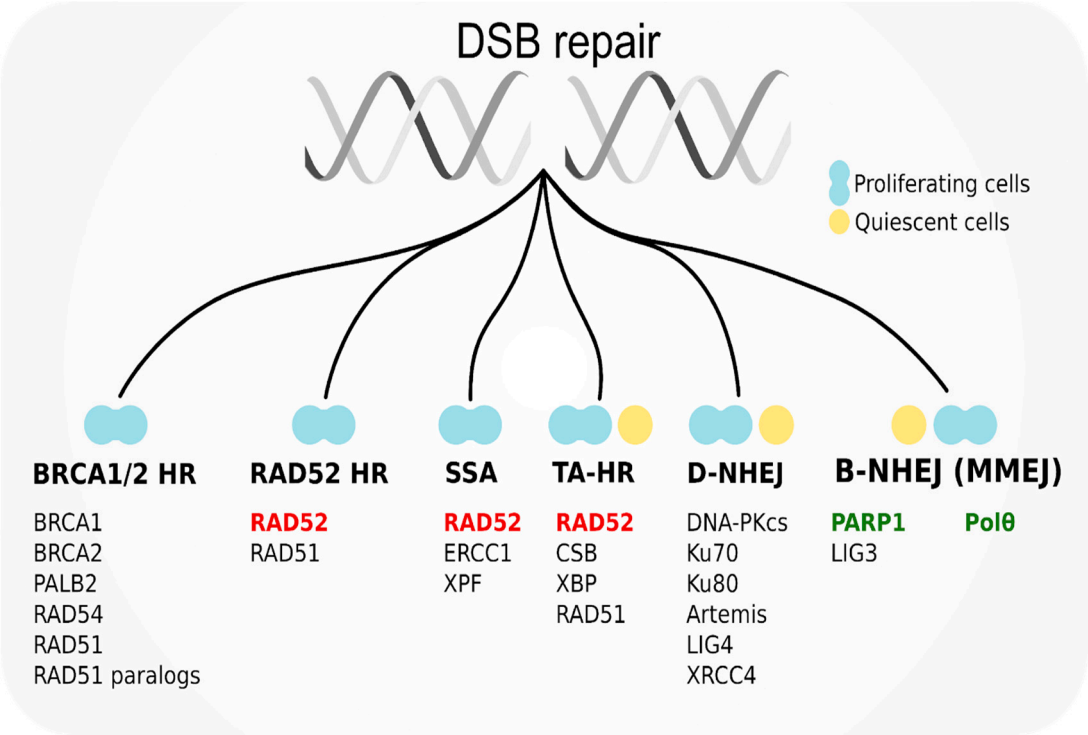

Figure 1. Double-strand break (DSB) repair mechanisms in quiescent and proliferating cells and major proteins participating in them. RAD52 as a potential target for synthetic lethality-based therapy has been marked in red. poly(ADP-ribose) polymerase 1 inhibitors (PARP1) and Polθ—promising partners for dual synthetic lethality have been marked in green.

Tumor-specific alterations in DSB repair mechanisms are currently under broad investigation as a novel target for personalized anticancer therapy. Loss of one of the repair pathways is highly probable in cancer cells due to their genomic instability. Under such conditions, cell survival depends fully on the alternative pathway, which compensates for the deficit. Inactivation of the alternative pathway causes synthetic lethality, where the simultaneous loss of function of two genes/pathways results in cell death; however, the individual inactivation of either of these genes/pathways does not affect cell viability [4]. In the context of anticancer treatment, targeting the alternative pathway in tumors that are deficient in the primary repair system will lead to an accumulation of toxic lesions and specific eradication of cancer cells, with low risk for normal cells. Synthetic lethality was first mentioned as a potential tool for anticancer therapy in 1997 by a Nobel Prize winner, Leland H. Hartwell, who understood the great therapeutic potential of this approach as well as the possibilities of using synthetic lethality to identify interaction networks underlying a malignant phenotype [5]. Almost 20 years later, the poly (ADP-ribose) polymerase 1 (PARP1) inhibitor (PARPi) olaparib became the first synthetic lethality-based treatment approved by the Food and Drug Administration (FDA) as a single-agent therapy for BRCA-deficient ovarian and breast cancer patients [6]. While normal cells treated with PARPi are able to efficiently repair DSBs using BRCA1/2-mediated HR, cancer cells carrying defects in HR accumulate high levels of toxic lesions that leads to synthetic lethality and apoptosis [7]. Recently,

three other PARPi: rucaparib, niraparib, and talazoparib, obtained FDA approval, and numerous compounds are in various stages of clinical trials as single-agent therapies and in combination with other cytotoxic compounds or radiotherapy [8].

Although currently the synthetic lethal interaction between BRCA1/2 inactivation and PARP1 inhibition is the only one which successfully found application in medicine, the sensitivity of tumors carrying other DNA damage response (DDR) defects to PARPi and to novel inhibitors against new targets for synthetic lethality-based therapy are under investigation [9]. One of the reasons for searching for novel synthetic lethal avenues is that the effect of PARPi is usually short-lived. In many cases, tumor cells become resistant to treatment due to a variety of mechanisms, including secondary mutations restoring function of BRCA1/2, or overexpression of the P-glycoprotein efflux pump, which limits the delivery of the drug into the cells [10]. Additionally, loss of function mutations in 53BP1 or E2F7 may lead to partial restoration of HR and subsequently, the emergence of PARPi resistance [11,12]. Therefore, there is a need to develop a strategy, which would allow for an increase in the effectiveness of PARPi, and the more rapid and robust eradication of tumor cells to eliminate the emergence of PARPi-resistant clones.

It has been suggested that the alternative RAD52-RAD51-dependent HR pathway remains active in BRCA-deficient cells, protecting them from the synthetic lethal effect of PARPi [13]. Since the role of RAD52 appears to be important in BRCA-deficient cells in particular, targeting RAD52 could constitute an attractive anticancer therapeutic approach [14,15].

2. RAD52 Protein: Structure and Functional Modifications

During the IVth International Yeast Genetics Conference in 1970, it was proposed that all genetic loci, which mutations confer to X-ray sensitivity, would be given the name "*rad*" followed by an identification number. *Rad52* was first established four years later in *S. cerevisiae,* where its mutation resulted in the abolishment of all recovery processes after irradiation with X-rays [16].

The crystal structure of purified human RAD52 has suggested it forms a ring-shaped undecamer; however, biophysical studies show that RAD52 in vitro could exist as a heptameric ring with a positively charged ssDNA-binding groove running around the structure (Figure 2) [17,18]. DNA-binding domains have, however, been found on both C- and N-terminal parts of the molecule, therefore supporting speculation of another binding-region outside the first groove of the protein oligomer [18]. RAD52 also contains a residue responsible for its import into the nucleus (nuclear localization signal = NLS) which, in human RAD52, is located at the C-terminal end of the protein [19]. The RAD52 NLS is weak when the protein is in monomeric form and allows for only slow migration into the nucleus; however, in the oligomeric ring structure, the additive effect of seven NLS would allow for more efficient transport to the nucleus. For this reason, the most likely formation of RAD52 heptamer is occurring in the cytoplasm [20]. The N-terminal domain of RAD52 allows for its heptamerization, and it possesses the ability to interact with RAD59. The RAD52/RAD59-dependent recombination pathway appears to be important for the processing of faulty Okazaki fragments [21]. The C-terminal and the central domains of RAD52 facilitate recombination "mediatory function" of the protein.

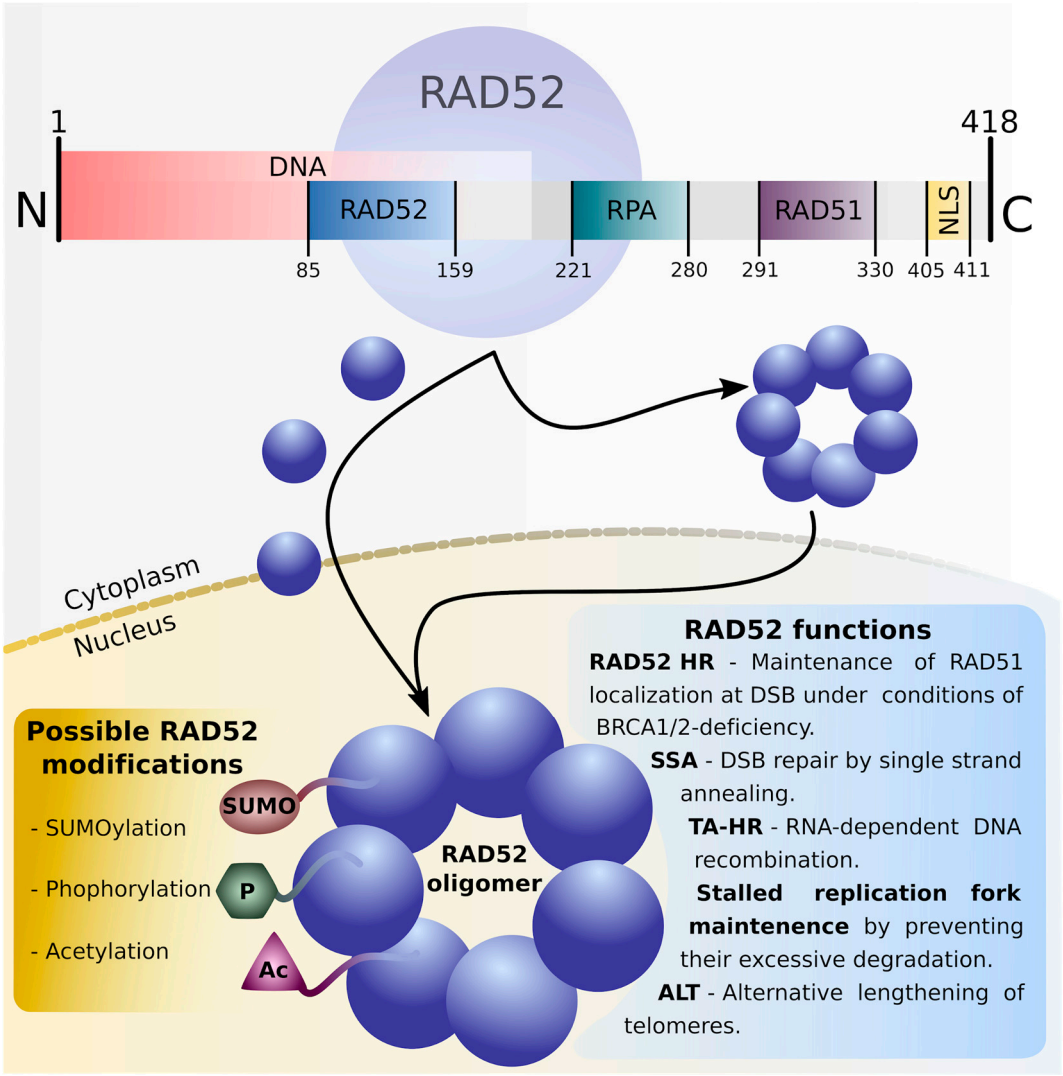

Figure 2. Human RAD52 structure, post-translational modifications, and functions. N-terminal fragment of RAD52 contains a region responsible for its oligomerization and binding with DNA molecule. C-terminal area includes domains interacting with replication protein A (RPA) and RAD51 recombinase, as well as nuclear localization signal (NLS) region responsible for RAD52 transportation to the nucleus. According to the "nuclear retention model", RAD52 monomer possesses a weak NLS signal allowing only slow transport to the nucleus where RAD52 undergoes oligomerization. The "additive NLS model" suggests formation of RAD52 ring in the cytoplasm, resulting in an additive NLS effect and more robust RAD52 ring transportation to the nucleus. Activity of RAD52 and its participation in different recombination processes can be modulated by post-translational modifications including SUMOylation, phosphorylation, and acetylation.

A variety of post-translational modifications including acetylation, phosphorylation, or (SUMO)ylation modulates the function of numerous proteins. The involvement of human RAD52 in HR repair depends on its acetylation by histone acetyltransferases (HATs) p300/CBP [22]. Unacetylated RAD52 dissociates from DSB along with RAD51 recombinase. The acetylation status of RAD52 is maintained by continuous cooperation between HATs and the histone deacetylases (HDACs) sirtuin2 (SIRT2) and SIRT3 [22]. RAD52 can also undergo (SUMO)ylation which does not influence its protein–protein interactions, although it delays recombination by inhibition of DNA-binding and strand annealing activities [23]. SUMO modification also sustains the activity of yeast Rad52 and protects it from degradation [24]. Phosphorylation of RAD52 by c-ABL1 kinase at tyrosine 104 seems to

enhance ssDNA annealing activity and inhibit dsDNA binding abilities of RAD52 [25]. Constitutively active oncogenic BCR-ABL1 kinase facilitates nuclear localization of RAD52 and stimulates SSA repair in leukemia cells [26,27].

3. Role of RAD52 in DNA Repair

RAD52 is able to bind ssDNA, facilitating a major role in single strand annealing (SSA) and HR repair of DSBs based on the homologous strand. RAD52 can also operate on single-ended DSBs, preventing excessive degradation of stalled replication fork by converting them into a compact conformation that is less available for reversal enzymes [28]. In checkpoint-deficient cells, RAD52 reverses stalled replication forks to the form in which they can be cleaved by the MUS81/EME1 complex during the process of break-induced replication (BIR) [29]. In fact, RAD52, through its ssDNA annealing activity, is suspected to assemble a displacement loop (D-loop) which invades the homologous chromosome and allows for BIR progression on the template of the homologous sequence [30]. RAD52 can also prevent chromosome end exposure by copying telomere caps from other chromosomes in a subtype of HR—alternative lengthening of telomeres (ALT) [31].

3.1. Homologous Recombination (HR)

Although in *S. cerevisae* RAD52 is a predominant recombination protein acting alone in facilitating RAD51 loading onto ssDNA, in mammals its role seems to be diminished by other proteins, namely BRCA1/2. *Rad52-/-* mice are viable, fertile, and show only a slight decrease in HR activity [32]. However, overexpression of RAD52 in mammalian cells enhanced their resistance to ionizing radiation, indicating the importance of RAD52 in the DNA damage response [33]. It has been demonstrated that in the absence of the BRCA1/2-dependent HR pathway, cell viability may be dependent on RAD52-RAD51, indicating that in mammalian cells, HR operates with at least two alternative sub-pathways: BRCA1/2-dependent canonical mechanism and RAD52-dependent alternative repair [34–36]. In the latter, RAD52 interacts with RAD51 and places it on RPA-coated ssDNA overhangs, which is possible thanks to the strong inhibitory effect that RAD52 exerts on the RPA-ssDNA complex. It has been suggested that once RAD51 is localized at the DSB, most of RPA and RAD52 are displaced from the DNA; however, some persist surrounded by recombinase filaments, possibly stabilizing further steps of HR [37,38].

3.2. Single Strand Annealing (SSA)

In general, DSB end resection and the creation of single-stranded overhangs is a pivotal moment of DNA repair which allows for the cell to choose between not only NHEJ and HR, but also between HR and SSA depending on how far resection has proceeded [39,40]. SSA events require sufficient resection to have direct sequence repeats presented in the form of ssDNA. 53BP1 is a factor that is responsible for the suppression of BRCA1-mediated end resection and the promotion of D-NHEJ. It has been suggested that the absence of 53BP1 leads to hyper-resection of DSBs in G2/S phase. This stage leads to the switch from error-free HR to mutagenic RAD52-mediated SSA. Therefore, cells lacking BRCA1 and 53BP1 require RAD52 for the maintenance of DSBs [41]. In addition, other factors—BRCA1, RNF168, RIF1, histone H2A.X—which inhibit end resection, were shown to suppress SSA [42–44].

In SSA, 5′ to 3′ end resection within tandem repeats exposes about 25 nt ssDNA overhangs. RAD52 interacts with RPA-coated overhangs and aligns the complementary regions. It was suggested that after finding initial homology, a further search for stronger interactions and more extensive homology continues without complex dissociation. The alignment occurs due to the overlapping of nucleoproteins present on the opposite sites of DSB [45]. After final homology is achieved, the endonucleolytic complex ERCC1/XPF, in cooperation with RAD52, trims 3′ overhangs. Final gap filling and strand ligation follow this step. SSA often results in the generation of deletions during the step where 3′ ssDNA overhangs are trimmed. Additionally, since SSA uses as templates repetitive elements that are present in multiple other genetic loci, SSA may also lead to translocations [46].

3.3. RNA-Dependent DNA Recombination

Although HR is mostly active during G2/S phase due to the short proximity to homologous sequence of sister chromatid or homologous chromosome, it appears that a HR sub-pathway that uses RNA transcripts as a template is active at transcriptionally active regions during G1/G0 phase of the cell cycle. RNA polymerase II can bypass different base modifications, however single strand breaks (SSBs) and DSBs result in permanent blockage of the enzyme. Such damage in transcriptionally active regions is expected to be more toxic than in any other genome area [47].

Under conditions of low abundance of BRCA1/2 during G0 and early G1 phase, its task of RAD51 recruitment to a DSB is fulfilled by RAD52 [48–50]. It appears that RAD52 may not only show affinity to ssDNA but also to RNA, and it might be active in repair mechanisms in differentiated, non-dividing cells [49]. Cocaine syndrome B protein (CSB) is expected to be the key protein in transcription-coupled homologous recombination (TC-HR). It detects stalled RNA polymerase and interacts directly with HR proteins RAD51C and RAD52, directing them to DNA damage in coding regions [51]. RAD52 binds to R-loops, which are three stranded DNA-RNA hybrids that allow for repair on the template of RNA transcript. In transcription-associated homologous recombination repair (TA-HR**)** RAD52 is recruited to the RNA-DNA hybrid at the DSB and promotes ERCC excision repair 5 (XPG)-mediated processing, leading to HR-based repair [52].

Two models indicate how RAD52 might promote RNA-mediated repair. In the first, RAD52 directs RNA to the DSB, where it finds homologous sequences with both its termini, creating a synapse that conjoins the ends. In the second model, RAD52 creates an RNA-DNA hybrid at 3′ ssDNA overhang. The overhang created by RNA is then used as a template for reverse transcription, before finally being degraded by RNase H. In the final steps, homology between the created ssDNA and the other end of the DSB allows for end joining and RAD52-promoted SSA [53].

It has been established that not only yeast, but also human RAD52 promotes RNA-templated DNA repair. RNA could constitute a stable template for DSB repair in differentiated cells that do not undergo divisions thus do not have sister chromatid as a template [50,51,54].

4. Synthetic Lethality Targeting RAD52

BRCA1 and *BRCA2* are tumor suppressor genes in which mutations have been widely correlated with hereditary and sporadic breast and ovarian cancer [55]. BRCA1 is responsible for directing DSB repair pathway choice towards recombination-based mechanisms and interacting with proteins participating in end resection. The recombination mediator BRCA2 contains ssDNA, dsDNA, and RAD51-binding domains, which facilitate the formation of recombinase RAD51-ssDNA filament during HR repair [56].

In contrast to the severe effects of RAD52 depletion in yeasts, only a mild effect on recombination was observed in RAD52-deficient vertebrate cells, and *Rad52-/-* mice are viable and fertile [32,57]. However, cancer cells that are deficient in BRCA substitute its activity with RAD52, which, thanks to its ssDNA and RAD51-binding sites, is able to manage HR in a BRCA-independent manner [34]. The fact that RAD52 is essential in human cells only under conditions of BRCA-deficiency makes it an attractive target for synthetic lethality-based anticancer therapy. Such approach is based on natural genetic interactions between DNA repair mechanisms and it utilizes cancer-specific defects. Targeting RAD52 in BRCA-depleted cancer cells will sensitize them to the toxic effect of DSBs, while normal cells and tissues with intact BRCA1/2-dependent HR should not be influenced.

It has been reported that one probable mechanism of synthetic lethality in RAD52/BRCA1/2-depleted cells is the activity of the endonuclease/exonuclease/phosphatase family domain containing protein 1 (EEPD1) [58]. In cells depleted in BRCA, 5′ endonuclease EEPD1 can nick stalled replication forks independently of BRCA to initiate end resection by EXO1, creating the ssDNA 3′ overhangs that are required for HR [59]. Downregulation of EEPD1 results in the suppression of synthetic lethality in RAD52/BRCA1/2-deficient cells. EEPD1-mediated cleavage of stressed replication forks creates a toxic intermediate, which under conditions of impaired BRCA- and RAD52-dependent

HR, is dependent fully on error-prone systems for repair, resulting in accumulation of lethal damage in the cell's genome [58]. Additionally, other evidence confirms that the malfunction of factors like BRCA2 or RAD52, which are responsible for the prevention of excessive degradation of stalled replication forks, might also be responsible for the development of toxic intermediates that lead to cell death [28,60,61].

Several laboratories have focused on the development of a small-molecule inhibitor of RAD52 (RAD52i), which could be utilized to trigger synthetic lethality under conditions of depleted BRCA1/2.

4.1. F79

In 2013, the first paper was published that confirmed the successful inhibition of RAD52 in human BRCA1/2-deficient leukemia cells derived from patients [14]. The goal was achieved by the utilization of synthetic peptide aptamer F79 that probably interferes with DNA binding by RAD52.

The authors proposed to use the F79 aptamer to treat leukemias displaying low levels of BRCA1/2. To test this hypothesis, they conducted an analysis of the response of different leukemia types displaying a variety of mutations that cause a low level of one of the members of BRCA-HR pathway, which includes: BCR-ABL1-positive chronic myeloid leukemia (CML) cells in which BRCA1 is downregulated [62], PML-RAR-positive acute promyelocytic leukemia (APL) cells with downregulation of RAD51C (RAD51 paralog), and samples from leukemias which express low levels of BRCA1/2 due to unknown mechanisms. Therefore, another goal of the research was to identify a cohort of patients who could potentially benefit from targeting RAD52.

F79 was able to selectively eliminate BRCA-deficient leukemia cells, with low risk for normal cells. It resulted in synthetic lethality in leukemias carrying BCR-ABL1 and PML-RAR oncogenes as well as epigenetic modifications resulting in BRCA-ness. In vivo tests showed significantly extended life spans of F79 treated SCID mice carrying BCR-ABL1—positive leukemia. F79 treatment resulted in synthetic lethality in *BRCA1/2*-mutated breast, pancreatic, and ovarian cancer cells and displayed synergistic effect with approved drugs such as imatinib (approved for BCR-ABL1-positive leukemia) and ATRA (for PML-RAR-positive leukemia) [14].

4.2. 6-OH-dopa

Another small-molecule RAD52i is 6-hydroxy-dopa (6-OH-dopa) [63]. 6-OH-dopa disrupts formation of the RAD52 heptameter superstructure and its dissociation, leading to abolished recruitment of RAD52 to DNA damage sites. 6-OH-dopa was reported to specifically inhibit SSA but had little to no effect on HR or D-NHEJ in BRCA-proficient cells. It selectively halted the proliferation of BRCA1-depleted triple negative breast cancer (TNBC) cells. Selective growth blockage after treatment with 6-OH-dopa was also observed in BRCA-deficient AML and CML cells derived from patients. BRCA-deficient cells treated with the inhibitor demonstrated increased level of DNA damage, thus resulting in increased apoptosis [63].

6-OH-dopa is a dopaminergic toxin derivative and has been reported to contribute to Parkinson disease and degeneration of mitral neurons [64]. Therefore, it is unlikely that 6-OH-dopa finds application in anticancer therapy.

4.3. A5MP and AICAR/ZMP

Another screen of libraries of drug-like compounds and FDA-approved drugs identified two substances, which were able to inhibit ssDNA binding by human RAD52. One of the leading compounds—adenosine 5′-monophosphate (A5MP) was able to halt the proliferation of BRCA1-deficient HCC1937 breast cancer cells and did not influence cells with restored BRCA1 expression [65]. The second identified compound—5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) 5′monophosphate = ZMP is known to imitate A5MP, and similarly to A5MP it disrupts ssDNA-RAD52 binding. Although ZMP is not able to penetrate the cell membrane, its membrane-permeable precursor AICAR is spontaneously phosphorylated intracellularly, generating high levels of ZMP within the cell [66]. AICAR treatment was able to disrupt SSA repair and

cisplatin-induced formation of RAD52-ssDNA foci in BRCA1-deficient cells. Both A5MP and ZMP localize at the intersection between two molecules of RAD52, interacting with its DNA-binding domain. Similarly to A5MP, AICAR was able to eliminate BRCA1-deficient HCC1937 cells and BRCA2-deficient Capan1 pancreatic adenocarcinoma cells. BRCA1/2-reconstitution resulted in complete abrogation of the sensitivity to the compound. Ectopic expression of BRCA1 resulted in reversion of sensitivity to AICAR in Capan1 and BCR-ABL1 leukemia cells implicating the synthetic lethal interactions between BRCA-deficiency and AICAR [65].

4.4. D-I03

Huang et al. conducted a high throughput screen and identified 17 compounds able to almost completely prevent RAD52-mediated D-Loop formation in vitro tests [67]. The compound D-I03 exhibited the strongest inhibitory effect and preferentially suppressed the proliferation of BRCA-deficient cells in all experimental setups, with no effect in BRCA-proficient counterparts. In BCR-ABL1-positive CML cells which express low levels of BRCA1, treatment led to selective growth inhibition in comparison to BRCA1-proficient control cells [67]. D-I03 led to inhibition of RAD52 but not RAD51 foci formation after cisplatin treatment, and significantly reduced level of SSA repair with no influence on HR. It has been reported that D-I03 binds directly to RAD52 and impairs its DNA-annealing activity [67].

4.5. '1', '6' and NP-004255

Compounds '1' ((-)-Epigallocatechin) and '6' (Epigallocatechin-3-monogallate) are RAD52i identified in Hengel at al. [68]. They interact directly with the ssDNA binding groove running around the RAD52 oligomer, and disrupt DNA wrapping by RAD52. However, unlike previous inhibitors '1' and '6' do not disrupt the RAD52 ring assembling process. NP-004255 (Corilagin) was identified in the screen of natural products library. It is a macrocyclic ester, which similarly to '1'and '6,' binds the RAD52 oligomer in the ssDNA interacting groove, abolishing the activity of this region. Importantly, '1' and '6' compounds were able to inhibit RAD52 binding to RPA-coated ssDNA and its ability to anneal ssDNA. Both compounds were also able to decrease RAD52-dependent BIR in hydroxyurea-treated, checkpoint-depleted cells [29,68]. '1' was able to significantly reduce the viability of BRCA2 or MUS81-depleted cells under conditions of replication stress [68].

4.6. F779-0434

Compound F779-0434 exhibits high affinity for RAD52 and stably binds to the protein. F779-0434 interacts with RAD52 residue Lys152, which plays a major role in ssDNA binding. The compound disrupts RAD52-ssDNA interactions and selectively eliminated BRCA2-mutated pancreatic adenocarcinoma Capan1 cells at the concentration range 10–40 μM, whereas the BRCA2-proficient BxPC3 cell line viability was not significantly influenced. The compound could be further investigated as a promising tool for targeted therapy of cancer cells with deficiencies in BRCA1/2-based HR-pathway [69].

5. Dual Synthetic Lethality as an Aggressive Anticancer Strategy

Most recently, a new strategy named "dual synthetic lethality" emerged from the idea of simultaneous aiming at two targets: (A) Two different types of cancer cells (D-NHEJ—deficient quiescent cells and BRCA1/2 HR—deficient proliferating cells) = "dual cellular synthetic lethality" (Figure 3A) [70], (B) two different repair pathways within the cell (PARP1 and RAD52) = "dual pathways synthetic lethality" (Figure 3B) [71] and (C) two different functions of the same protein (the NAD binding niche of PARP1 and histone 4-mediated activation of PARP1)—"dual molecular synthetic lethality" (Figure 3C) [72]. "Dual synthetic lethality" is an aggressive anticancer approach, which has a chance to increase the effectiveness of cancer cell elimination, thus preventing the emergence of drug-resistant cells [71].

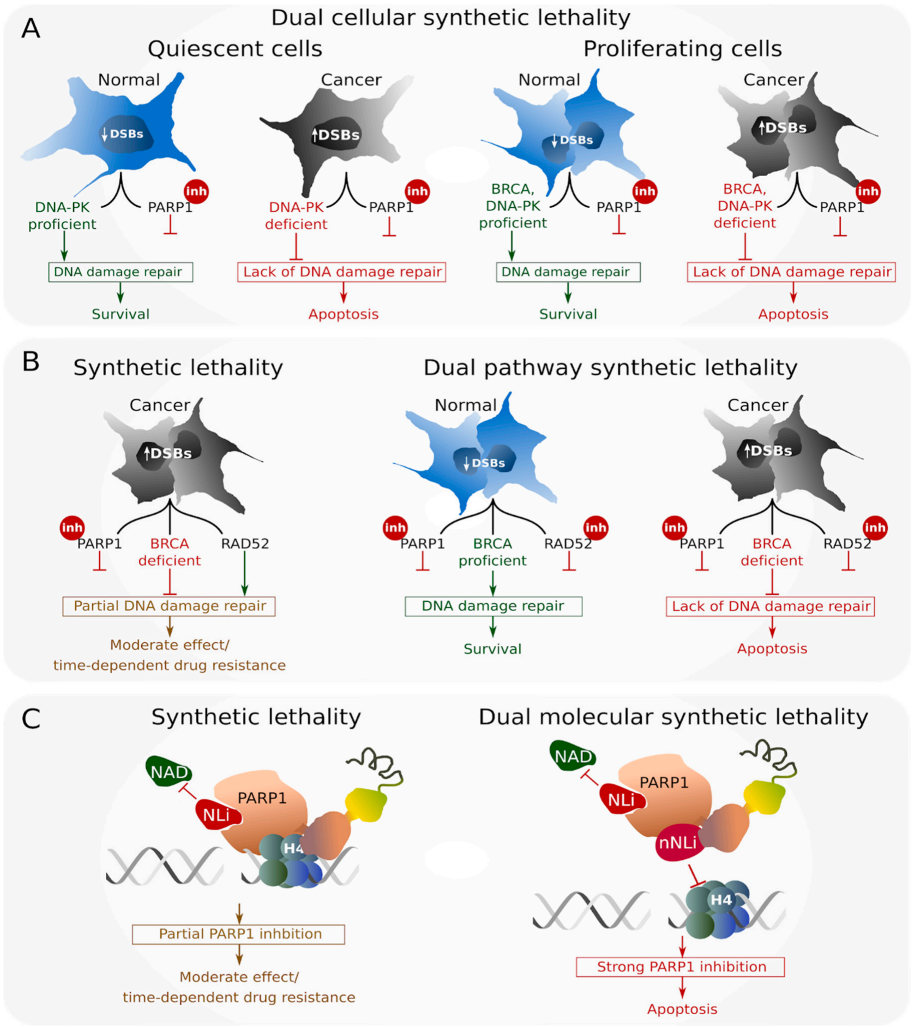

Figure 3. Dual synthetic lethality strategies: (**A**) Dual cellular synthetic lethality—inhibition (inh) of PARP1 gives a chance to simultaneously eliminate DNA-PK (DNA-dependent protein kinase)—deficient quiescent and BRCA (breast cancer susceptibility protein)-deficient proliferating cancer cells accumulating high numbers of DSBs, with no harm for BRCA-proficient normal cells; (**B**) dual pathway synthetic lethality—simultaneous inhibition of RAD52 and PARP1 exerts synergistic synthetic lethality effect against BRCA-deficient cancer cells with no toxicity to BRCA-proficient cells; (**C**) dual molecular synthetic lethality—anti-PARP1 activity of the combination of NAD-like inhibitor (NLi) reducing the catalytic activity of PARP and non-NAD-like inhibitor (nNLi), which abolishes activation of PARP1 by histone H4, resulting in synergistic effect eliminating BRCA-deficient cancer cells.

5.1. Dual Cellular Synthetic Lethality

Tumor bulk is a heterogeneous agglomeration of cells, containing a small cohort of cancer stem cells (CSCs) which display tumor-initiating properties, and give rise to cancer progenitor cells (CPCs) which constitute the majority of cancer's mass. CSCs consist of proliferating and quiescent cells. Most of the currently available drugs fail to eradicate CSCs due to their chemo- and radiotherapy-resistance associated with quiescence, and their ability to reproduce tumor mass from even a small number of tumor-initiating cells [73]. Dual cellular synthetic lethality offers the possibility to eradicate quiescent and proliferating CSCs and proliferating CPCs (Figure 3A). PARPi was able to cause extensive "dual cellular synthetic lethality," simultaneously eliminating DNA-PK-deficient quiescent leukemia CSCs and BRCA1/2-deficient proliferating CSCs and CPCs [70]. Thus, "dual cellular synthetic lethality" is a strategy of great potential allowing for the eradication of therapy-refractory cancer cells utilizing specific vulnerabilities to DNA repair inhibitors.

5.2. Dual Pathway Synthetic Lethality

At this time, only PARPi are being applied in personalized anticancer therapy of BRCA-deficient ovarian and breast cancer patients. However, the effect of PARPi is usually temporary and the majority of patients develop therapy resistance. "Dual pathway synthetic lethality" expands the synthetic lethal approach to simultaneous targeting of two repair mechanisms. RAD52 maintains residual HR in BRCA-deficient PARPi-treated cancer cells, so simultaneous targeting PARP1 and RAD52 represents an attractive therapeutic approach [71].

Rad52-/-Parp1-/- mice are normal but show delay in the appearance of BRCA1-deficient leukemia when compared to single knockout mice [71]. Simultaneous inhibition of PARP1 and RAD52 by small molecule inhibitors resulted in the synergistic accumulation of lethal DSBs and complete elimination of BRCA1/2-deficient cell lines in comparison to individual agent treatment (Figure 3B). Combination of PARPi and RAD52i also effectively eliminated primary leukemia cells displaying "BRCA1/2-ness", while individual compounds generated only a partial effect. "Dual pathway synthetic lethality" could be even enhanced by addition standard therapeutic drugs (e.g., imatinib or daunorubicin). Additionally, combination of PARPi and RAD52i inhibitors exerted a synergistic effect against BRCA-deficient tumors in immunodeficient mice with low toxicity to normal cells and tissues [71]. Therefore, "dual pathway synthetic lethality" simultaneously targeting PARP1 and RAD52 offers a promising and very aggressive therapeutic approach against HR-compromised tumors, allowing for more robust elimination of cancer cells and preventing the emergence of drug resistance.

PARPi could potentially be used in combination with inhibitors of other pathways. Polθ (encoded by *PolQ*), is a unique DNA polymerase that contains a helicase-like domain at its N-terminal end. Polθ plays an essential role in B-NHEJ, particularly in microhomology-mediated end-joining (MMEJ) [74]. However, it has been recently suggested that Polθ might also interact with RAD51 and thus may regulate HR repair [75]. The expression of Polθ is relatively low in normal human cells; however, its elevated expression is often associated with poor prognosis in breast cancer [76,77]. Simultaneous inactivation of HR factor *Fancd2* and *Polq* in mice resulted in embryonic lethality, whereas knockdown of Polθ in HR-deficient epithelial ovarian cancer (EOC) and breast cancer cells caused increased cell death, therefore suggesting synthetic lethal interactions between HR and Polθ-mediated repair [75,78]. Polθ depletion resulted in increased sensitivity of HR-deficient cells to PARPi, with no effect on HR-proficient counterparts. *Fancd2-/-Polq-/-* mouse embryonic fibroblasts (MEFs) exhibited high sensitivity even to low doses of PARPi [75]. Therefore, the simultaneous targeting of PARP1 and Polθ might constitute an interesting strategy for treatment of HR-depleted tumors; however, this strategy needs further investigation.

5.3. Dual Molecular Synthetic Lethality

"Dual molecular synthetic lethality" simultaneously targets two functions within one molecular target (Figure 3C). Although this approach has not been expanded in the context of RAD52, double inhibition of PARP1 has recently been investigated. All currently clinically utilized PARPi are designed to target the nicotinamide adenine dinucleotide (NAD)-binding site on the PARP1 protein [79,80]. This domain is present not only among the PARP protein family but also in many other enzymes utilizing NAD as a cofactor, which means that NAD-like PARP inhibitors can affect the activity of much broader group of proteins, leading to toxic effects. Recently discovered non-NAD-like PARPi seems to be as effective as NAD-like inhibitors, but less toxic [72,81].

5F02 is a non-NAD-like compound which interferes with PARP1 interactions with histone H4, resulting in the inhibition of PARP1 enzymatic activity [81,82]. 5F02 has been proven effective and selective against breast, prostate, and kidney cancer cells; however, the combination of 5F02 with NAD-like inhibitor generated synergistic anti-tumor effect in comparison to each compound administered individually [81]. Combination of NAD-like and non-NAD-like inhibitors was exceptionally effective in vitro and in vivo against BCR-ABL1-positive BRCA1-deficient CML in chronic phase, at the same time causing little or no toxicity to normal cells and tissues [72]. While

NAD-like PARPi usually lead to the accumulation of DSBs, non-NAD-like 5F02 did not seem to induce DSBs. Therefore, the course of action of this inhibitor needs further investigation [72].

6. Conclusions

The success of synthetic lethality with PARP1 gave hope for the development of highly personalized therapies that take into consideration the molecular uniqueness of each cancer case. Although PARP1 constitutes a promising target in the treatment of tumors harboring deficiencies in BRCA-mediated HR, some tumor cells acquire therapy-resistance and survive, resulting in disease relapse. Targeting RAD52 represents a next step in synthetic lethality-based anticancer therapy. The fact that its activity is limited exclusively to DNA repair and its absence is only lethal under conditions of HR-deficiency makes RAD52 an attractive target for personalized, highly specific anticancer therapy of HR-compromised tumors. Currently available RAD52 inhibitors prevent the creation of the RAD52-DNA complex, which is crucial in all RAD52-mediated processes. Additionally, the recently developed "dual synthetic lethality" strategy might increase the effectiveness and specificity of cancer cell elimination via targeted approach and prevent the emergence of drug-resistant cells. In the context of this strategy, the combination of RAD52i with PARPi has been reported to exert synergistic lethal effect in HR-deprived cells.

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Abbreviations

ALT	**alternative lengthening of telomeres**
APL	acute promyelocytic leukemia
B-NHEJ	backup non-homologous end-joining
BER	base excision repair
BIR	break-induced replication
BRCA1/2 HR	BRCA1/2-mediated homologous recombination
CML	chronic myeloid leukemia
CPC	cancer progenitor cell
D-NHEJ	DNA-PK-mediated non-homologous end-joining
DDR	DNA damage response
D-Loop	displacement loop
DSB	DNA double strand break
EOC	epithelial ovarian cancer
HR	homologous recombination
MMEJ	microhomology-mediated end-joining
NHEJ	non-homologous end-joining
PARP1i	poly(ADP-ribose) polymerase 1 inhibitor
RAD52 HR	RAD52-mediated homologous recombination
RAD52i	RAD52 inhibitor
SSA	single strand annealing
SSB	DNA single strand break
ssDNA	single stranded DNA
TA-HR	transcription-associated homologous recombination
TC-HR	transcription-coupled homologous recombination
TNBC	triple negative breast cancer

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Research Paper

PARP1 inhibitor olaparib (Lynparza) exerts synthetic lethal effect against ligase 4-deficient melanomas

Małgorzata Czyż[1], Monika Toma[2], Anna Gajos-Michniewicz[1], Kinga Majchrzak[1], Grazyna Hoser[3], Janusz Szemraj[4], Margaret Nieborowska-Skorska[5], Phil Cheng[6], Daniel Gritsyuk[5], Mitchell Levesque[6], Reinhard Dummer[6], Tomasz Sliwinski[2], Tomasz Skorski[5]

[1]Department of Molecular Biology of Cancer, Medical University of Lodz, 92-215 Lodz, Poland

[2]Department of Molecular Genetics, University of Lodz, 90-236 Lodz, Poland

[3]Department of Flow Cytometry, Medical Center for Postgraduate Education, 01-813 Warsaw, Poland

[4]Department of Medical Biochemistry, Medical University of Lodz, 92-215 Lodz, Poland

[5]Department of Microbiology and Immunology, Temple University Lewis Katz School of Medicine, Philadelphia, PA 19140, USA

[6]Department of Dermatology, Faculty of Medicine, University Hospital Zürich, and University of Zürich, CH-8952, Zürich, Switzerland

Correspondence to: *Tomasz Skorski,* ***email:*** *tskorski@temple.edu*
Tomasz Sliwinski, ***email:*** *tomsliw@biol.uni.lodz.pl*

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ABSTRACT

Cancer including melanoma may be ''addicted" to double strand break (DSB) repair and targeting this process could sensitize them to the lethal effect of DNA damage. PARP1 exerts an important impact on DSB repair as it binds to both single- and double- strand breaks. PARP1 inhibitors might be highly effective drugs triggering synthetic lethality in patients whose tumors have germline or somatic defects in DNA repair genes. We hypothesized that PARP1-dependent synthetic lethality could be induced in melanoma cells displaying downregulation of DSB repair genes. We observed that PARP1 inhibitor olaparib sensitized melanomas with reduced expression of DNA ligase 4 (LIG4) to an alkylatimg agent dacarbazine (DTIC) treatment *in vitro*, while normal melanocytes remained intact. PARP1 inhibition caused accumulation of DSBs, which was associated with apoptosis in LIG4 deficient melanoma cells. Our hypothesis that olaparib is synthetic lethal with LIG4 deficiency in melanoma cells was supported by selective anti-tumor effects of olaparib used either alone or in combination with dacarbazine (DTIC) in LIG4 deficient, but not LIG4 proficient cells. In addition, olaparib combined with DTIC inhibited the growth of LIG4 deficient human melanoma xenografts. This work for the first time demonstrates the effectiveness of a combination of PARP1 inhibitor olaparib and alkylating agent DTIC for treating LIG4 deficient melanomas. In addition, analysis of the TCGA and transcriptome microarray databases revealed numerous individual melanoma samples potentially displaying specific defects in DSB repair pathways, which may predispose them to synthetic lethality triggered by PARP1 inhibitor combined with a cytotoxic drug.

INTRODUCTION

While melanomas can be successfully treated in the early stages, the appearance of metastasis in distant organs worsens prognosis and drops median survival below nine months [1]. Despite of the recent advances in melanoma treatment, including immunotherapies and targeted therapies, a resistance is developed in the majority of patients [2] indicating that genotoxic therapies might still be needed. It has been suggested

that cancer cells survive genotoxic stress due to acquired abnormalities in DNA repair system [3]. The 'addiction' of cancer cells to compensatory DNA repair mechanisms, especially double strand break (DSB) repair, may create an opportunity to target these pathways to eliminate malignant cells [3, 4].

DSBs are highly cytotoxic DNA lesions caused by reactive oxygen species (ROS), ionizing radiation and genotoxic drugs [4]. In proliferating cells DSBs are usually repaired by two major mechanisms, BRCA1/BRCA2-dependent homologous recombination (HR) and DNA-PKcs-mediated non-homologous end-joining (D-NHEJ), whereas PARP1-dependent back-up NHEJ (B-NHEJ) serves as an alternate mechanism [5–7]. In addition, PARP1 may decrease the number of potentially lethal DSBs, either by stimulation of base excision repair (BER) and single-strand break (SSB) repair and/or by facilitation of MRE11-mediated recruitment of RAD51, as well as, by involvement in relocation of XRCC1, an essential protein for an effective DSB repair and restart of stalled replication forks [8, 9].

It was reported that cells deficient in BRCA1/BRCA2-mediated HR are sensitive to PARP1 inhibitors, such as the recently FDA approved olaparib (Lynparza, Astra-Zeneca) due to induction of synthetic lethality [10]. Since TCGA database analysis revealed that melanoma samples display deregulated expression and/or mutations of the genes encoding DSB repair proteins (Figure 1), we hypothesize that DSB repair deficiencies could sensitize individual melanomas to PARP1 inhibitor administered either alone or in combination with DSB-inducing genotoxic agents, such as dacarbazine (DTIC) [11].

RESULTS

Genes involved in the DSB repair pathway are differentially expressed in patient-derived melanoma cells and in normal melanocytes

To test the potential anti-melanoma effect of PARP1 inhibitors we established six patient-derived melanoma cell lines. Real-time PCR was used to determine the gene expression profile in melanoma cells and in normal human melanocytes. Eight genes were examined, whose products are essential for DSB repair pathways (BRCA1, PALB2, and RAD51 in HR; PRKDC, XRCC6, and LIG4 in D-NHEJ; PARP1 and LIG3 in B-NHEJ). Significant differences were found in the gene expression profiles between melanoma cells and melanocytes. In particular, all melanoma lines showed a decreased level of DNA ligase 4 (*LIG4*) (Figure 2A).

Protein expression status of LIG4, RAD51, PARP1, Ku70 was determined by Western blot analysis in normal melanocytes and melanoma cell lines (DMBC11, DMBC12) (Figure 2B). Both DMBC11 and DMBC12 cell lines displayed elevated expression of RAD51, PARP1 and Ku70 proteins, whereas expression of LIG4 was downregulated.

Olaparib used either alone or in combination with DTIC induced cytotoxic effects in patient-derived LIG4-deficient melanoma cells

To determine the influence of tested compounds on viable cell number, plasma membrane integrity was measured by cytometric analysis (Figure 3A). After

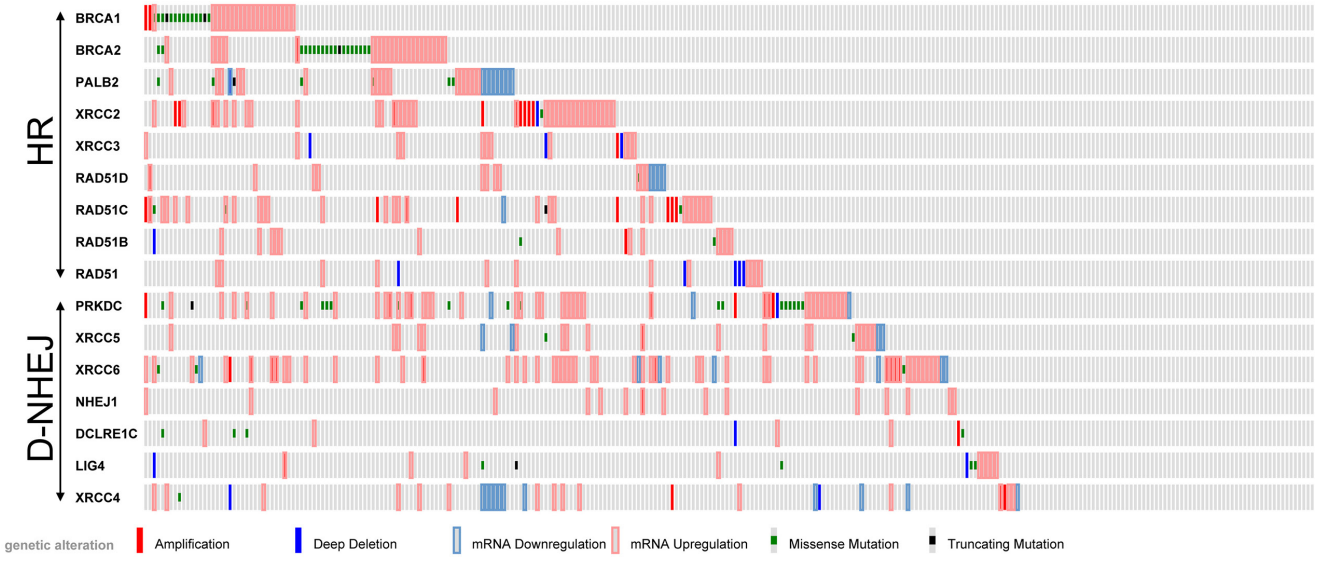

Figure 1: Analysis of TCGA database of 287 individual skin cutaneous melanomas. Deregulated expression (Z-score >2.0) and/or mutations of the genes in DSB repair pathways, HR and D-NHEJ, are shown.

the first 48 hours of treatment, only the combination of olaparib and DTIC markedly reduced viability reaching about 54% of control. The second dose and additional incubation for 72 hours induced cell response to drugs, used either alone or in combination. Normal melanocytes were not affected by the treatments.

Cell death was assessed by the appearance of sub-diploid fraction (subG1, Figure 3B). Sub-diploid DNA content was found in about 55% in DMBC11 cells and 34% in DMBC12 cells after combined treatment with olaparib and DTIC for 48 hours, and this effect was further increased with the next dose and prolonged treatment. This might indicate that these compounds were more likely to induce cell death than cytostatic effects in melanoma cells, which was further confirmed by cell cycle analysis. Cell cycle arrest was not clearly visible in olaparib or DTIC treated melanoma cells, and only a modest fraction of cells treated with olaparib + DTIC accumulated in G2/M (Figure 3C).

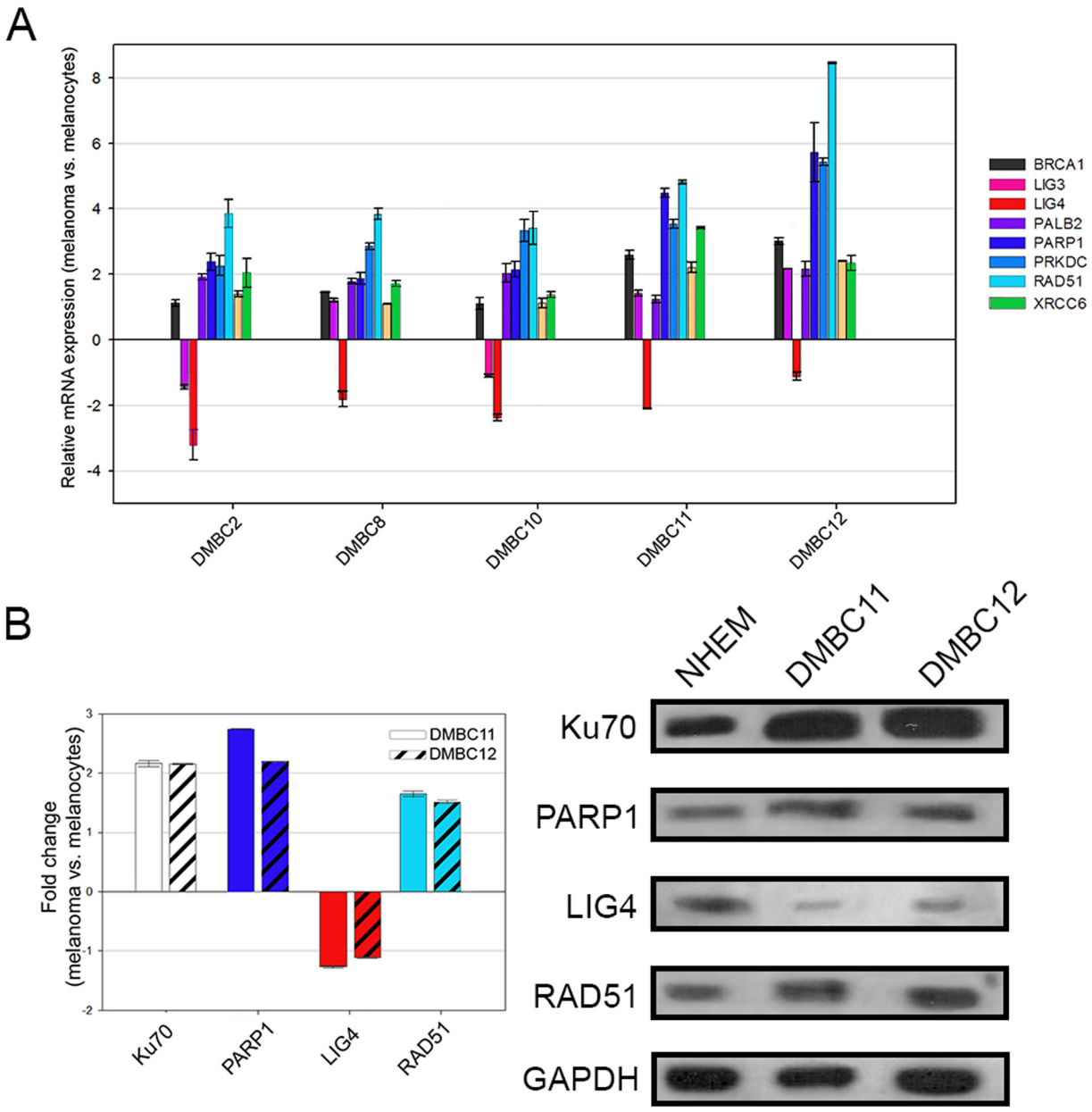

Figure 2: Expression profiles of DNA double-strand break repair genes in melanoma cells compared to melanocytes. **A.** The transcript level of each gene was normalized to the expression of a reference gene (18S RNA). Data is presented as fold change in melanoma cells versus melanocytes, in which expression levels of the genes were set as 1. The mean values ± SD were calculated from 3 experiments performed in triplicates. **B.** The protein level was normalized to the expression of a reference protein, GAPDH. Data is presented as fold change in melanoma cells versus melanocytes, in which the expression levels of the proteins were set as 1. The means ± SD were calculated from 3 experiments. Representative Western blot results are included.

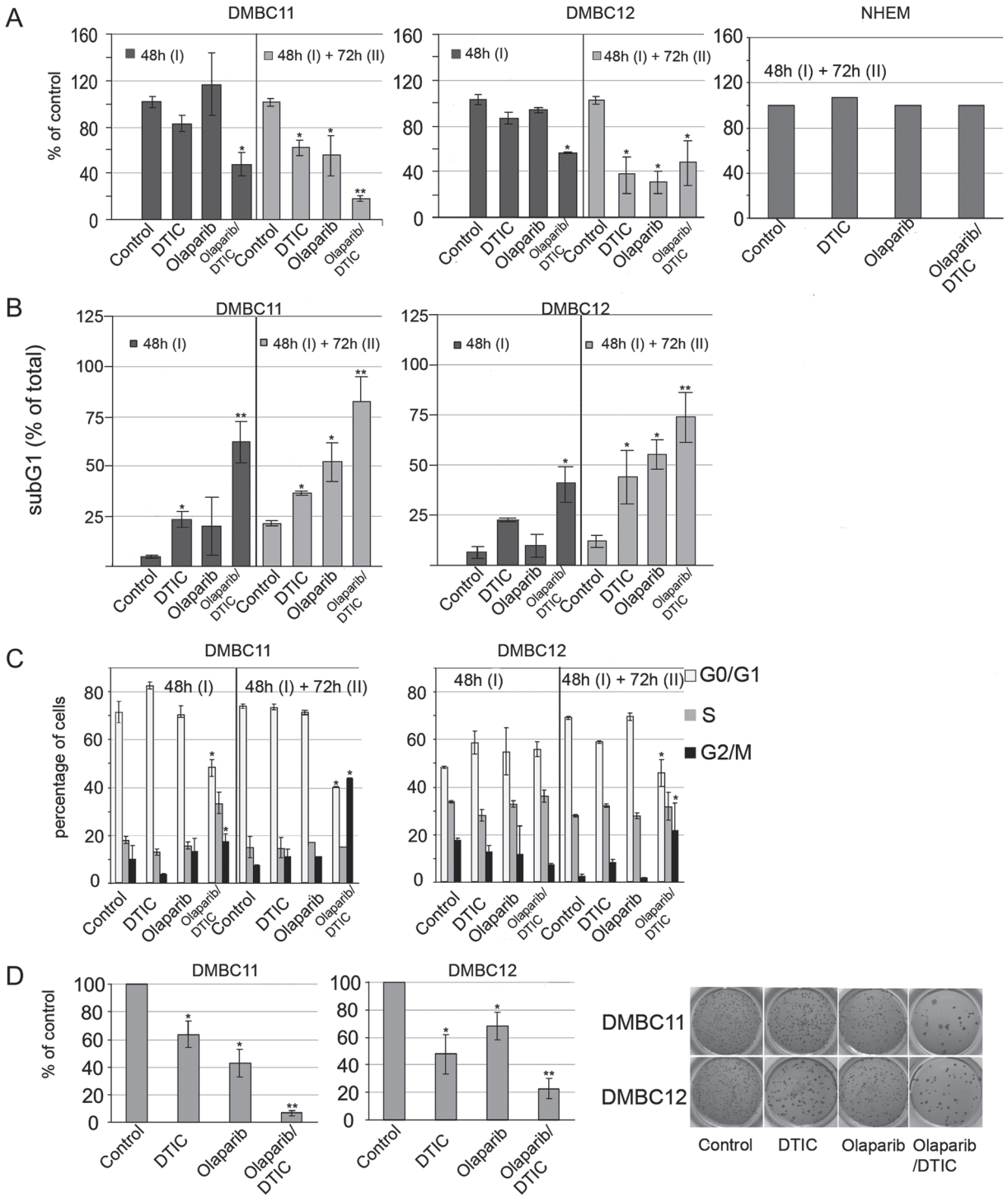

Figure 3: Effects of olaparib and DTIC, used alone or in combination, on viability, distribution in cell cycle and clonogenicity of melanoma cells. A. Viability was measured using PI staining and flow cytometry, and it is shown as % of vehicle control. Means ± SD of 2 independent experiments performed in triplicates are shown. **B.** Cell death was measured by accumulation of melanoma cells in the sub-G1 fraction; mean ± SD of 2 independent experiments. **C.** Distribution of melanoma cells through the cell-cycle phases was analyzed by flow cytometry. Left panel, bars represent cell distribution after 48 hours and after additional 72 hour treatments of DMBC11 and DMBC12 populations with DTIC and olaparib, used alone or in combination. ModFit LT 3.0 software was used to calculate the percentages of cells in each fraction; means ± SD of two independent experiments are shown. Right panel, representative histograms of DMBC11 cells treated with two doses of indicated drugs (48 hours followed by 72 hours). **D.** Clonogenic assay showing the long-term effects olaparib and/or DTIC on melanoma cell lines DMBC11 and DMBC12. Left panel, bars represent clonogenic efficiency in drug-treated melanoma cell populations, expressed as percentages of clonogenic efficiency in vehicle-treated control; mean ± SD of 2 independent experiments. Right panel, photographs of a representative experiment are shown.

Soft agar was used as a semisolid support to obtain spatially distinct colonies. When used alone, DTIC and olaparib reduced the number of colonies (Figure 3D). When drugs were used in combination, the clonogenic efficiency was further reduced.

To validate the importance of reduced level of LIG4 on the susceptibility of melanoma cells to olaparib, LIG4 was ectopically expressed in DMBC11 cell line (Figure 4A). Elevated expression of LIG4 reduced the sensitivity of DMB11 cells to olaparib (Figure 4B). Moreover, *LIG4-/-* pre-B cells were more sensitive to olaparib treatment than parental cells expressing endogenous LIG4 (Figure 4C).

Olaparib and DTIC, used alone or in combination, increase the number of DSBs in patient-derived LIG4 deficient melanoma cells

In normal melanocytes the level of phosphorylated γ-H2AX, which marks DSBs [12], remained unchanged after the treatment. However, DMBC11 and DMBC12 cell lines showed increased levels (5- or 2-fold, respectively) of phosphorylated γ-H2AX in comparison to melanocytes (Figure 5A). Moreover, combined treatment approximately doubled the level of phosphorylated γ-H2AX in both melanoma cell lines in comparison to cells treated with either drug alone.

The neutral comet assay was also used to measure the ability of olaparib and/or DTIC to induce DSBs as described before [13]. DMBC11 and DMBC12 cell lines treated with individual drugs showed increased intensity of DNA tail in comparison to melanocytes indicating accumulation of DSBs (Figure 5B). Moreover, combination of olaparib and DTIC caused more DSBs that individual drug.

Olaparib and DTIC combination reduces melanoma growth in NSG mice

Sub-optimal doses of olaparib or DTIC did not reduce the growth of DMBC11 cells in NSG mice (Figure 6). Interestingly, the combination of olaparib and DTIC exerted modest, but statistically significant anti-melanoma effect. Stronger effect would probably require optimization of the treatment protocol.

DISCUSSION

Synthetic lethality is a phenomenon occurring when simultaneous depletion of a pair of genes or gene products is required for cell death to occur. For example, cells harboring BRCA1/2 inactivating mutations are sensitive to PARP1 inhibitors [14, 15]. Therefore, PARP1 inhibitors may be highly effective drugs in variety of tumors with germline or somatic defects in DNA damage repair genes. In the present study we showed that PARP1 inhibitor olaparib applied alone and in combination with DTIC (a drug used in melanoma treatment) was effective against melanoma cells displaying downregulation of LIG4 without affecting normal melanocytes. This effect was associated with accumulation of toxic DSBs, implicating olaparib-mediated synthetic lethal effect in LIG4 deficient melanoma cells. Downregulated LIG4 and/or Artemis were

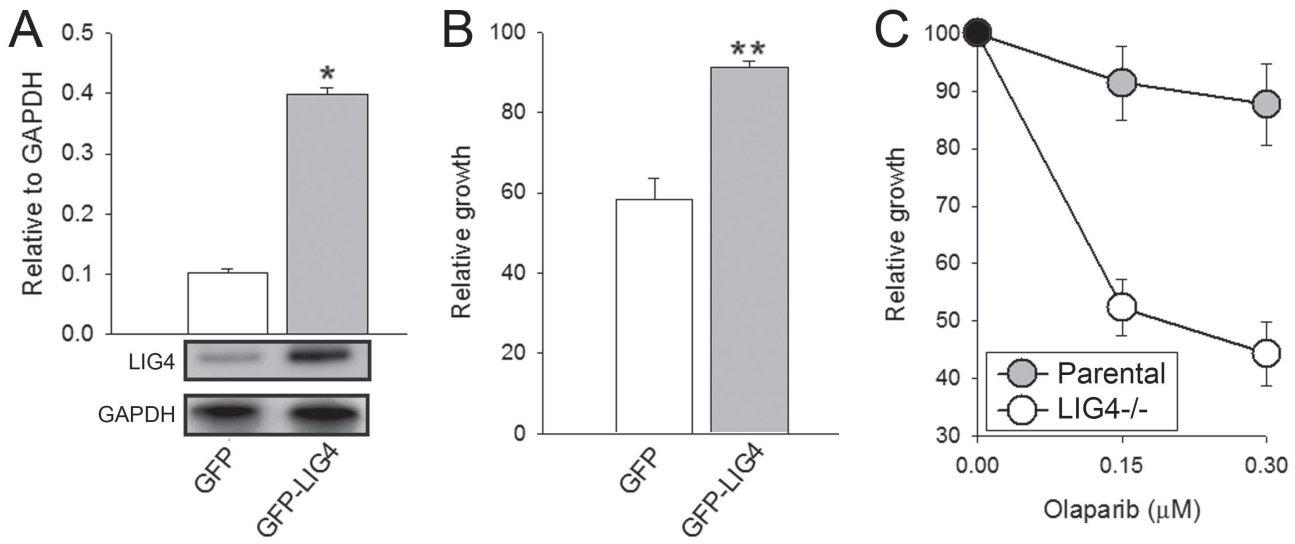

Figure 4: Sensitivity to olaparib depended on LIG4 expression levels. A. Quantification of normalized LIG4 levels to GAPDH in total cell lysates obtained from GFP+ DBM11 cells transfected with expression plasmids encoding GFP or GFP and LIG4. Bars represent mean percentage volume intensity ± SD from 3 experiments; *$p < 0.001$ in comparison with GFP. Representative Western blots of the expression of LIG4 and GAPDH (loading control) are shown. **B.** The effect of olaparib on viability of DMBC11 cells transfected with GFP or GFP + LIG4. Results represent mean ± SD from 3 independent experiments; **$p < 0.05$ in comparison with GFP. **C.** The effect of olaparib on viability of Nalm6 parental and Nalm6 *LIG4-/-* pre-B cells. Results represent mean ± SD from 3 independent experiments.

detected before in cell lines established from high-risk neuroblastomas and therapy-resistant breast carcinomas [16, 17]. However, these studies did not establish that sensitivity to PARP1 inhibitors depended on inhibition of LIG4. Our work for the first time demonstrates that downregulation of LIG4 in melanoma cells is directly responsible for enhanced sensitivity to olaparib.

Our results suggest the new therapeutic approach against melanomas based on synthetic lethality which exploits the reduced levels of LIG4, an essential component of D-NHEJ that performs the final 'end processing' step of DSB repair [18]. When LIG4 expression is reduced, D-NHEJ repair is performed inefficiently, and additional inhibition of PARP1-dependent B-NHEJ, BER and/or replication fork restart by olaparib could result in accumulation of toxic DSBs [5, 7–9]. Altogether, we postulate that D-NHEJ deficiency caused by downregulation of LIG4 could be synthetically lethal with B-NHEJ deficiency induced by PARP1 inhibitor. This hypothesis is supported by the results showing that PARP inhibitors were selectively toxic to LIG4-deficient melanoma and leukemia cells (this work) and that they

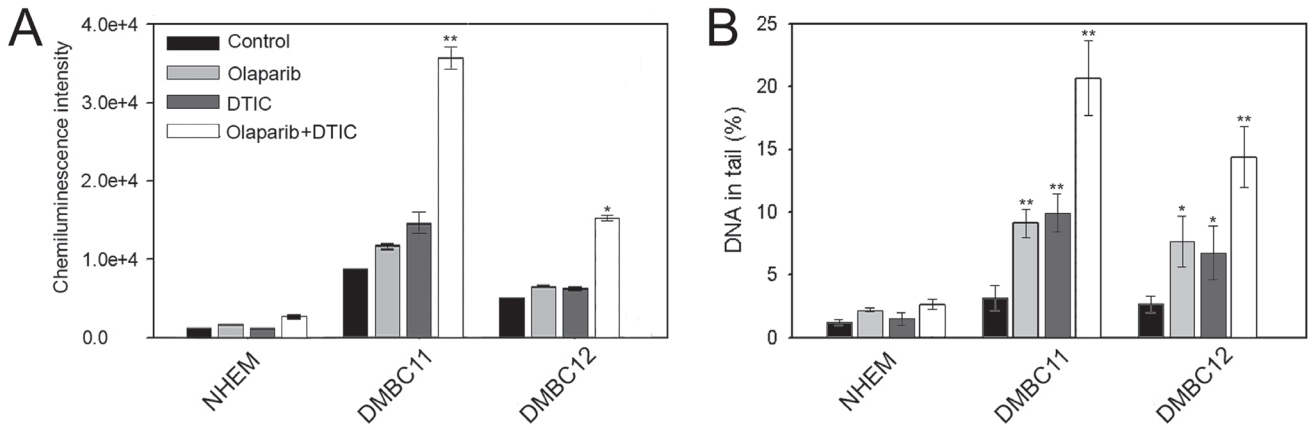

Figure 5: Olaparib and/or DTIC induced DSBs in melanoma cell lines (DMBC11, DMBC12). Cells were treated with 5 μM olaparib and/or 2 mM DTIC for 48 hrs (comet assay) and 120 hrs (γ-H2AX). **A.** The mean values ± SD of γ-H2AX were calculated from 3 ELISA experiments performed in triplicates. **B.** The mean percentage ± SD of DNA in the tails of comets in neutral conditions acquired from one hundred cells/group from 3 experiments. *p<0.05 and **p<0.001 in comparison with control.

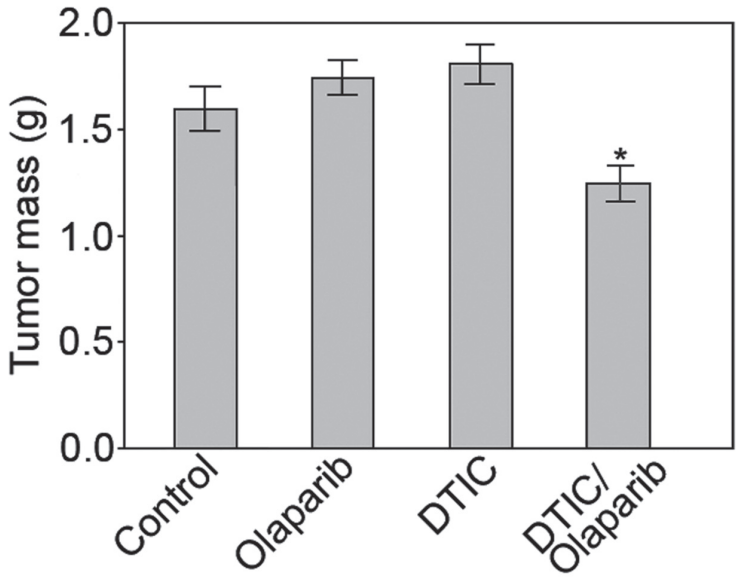

Figure 6: Combination of olaparib and DTIC reduced the growth of human melanoma in immunodeficient mice. NAG mice were injected s.c. with DMBC11 melanoma cells followed by the treatment with olaparib (35 mg/kg twice a day), DTIC (8 mg/kg every second day), or olaparib + DTIC. Data represent mean ± SD of tumor mass from 2 independent experiments, *p<0.05 in comparison with untreated mice.

increased DNA damage induced by radiation exposure in *LIG4-/-* HCT116 colon carcinoma cell line [19].

Although downregulation/mutation of LIG4 (and its partner XRCC4) was detected only in approximately 7% of cutaneous melanomas in TCGA database (Figure 1), inhibition/inactivating mutation of other members of D-NHEJ potentially impairing DSB repair activity by the pathway were detected, too [20]. Moreover, transcriptome analysis by microarrays of 229 melanoma cell lines detected downregulation of at least one member of D-NHEJ pathway (including LIG4) in numerous samples established from patients manifesting different stages of malignancy (Figure 7A). The 229 melanoma cells were grouped by their molecular phenotype, proliferative, intermediate and invasive. The proliferative phenotype is defined by high expression of MITF and low expression of WNT5A, the invasive phenotype is defined by low expression of MITF and high expression of WNT5A, and intermediate phenotype have approximately equal expression of MITF and WNT5A. From the analysis, it seems that the invasive phenotype has greater downregulation in the D-NHEJ genes than proliferative,

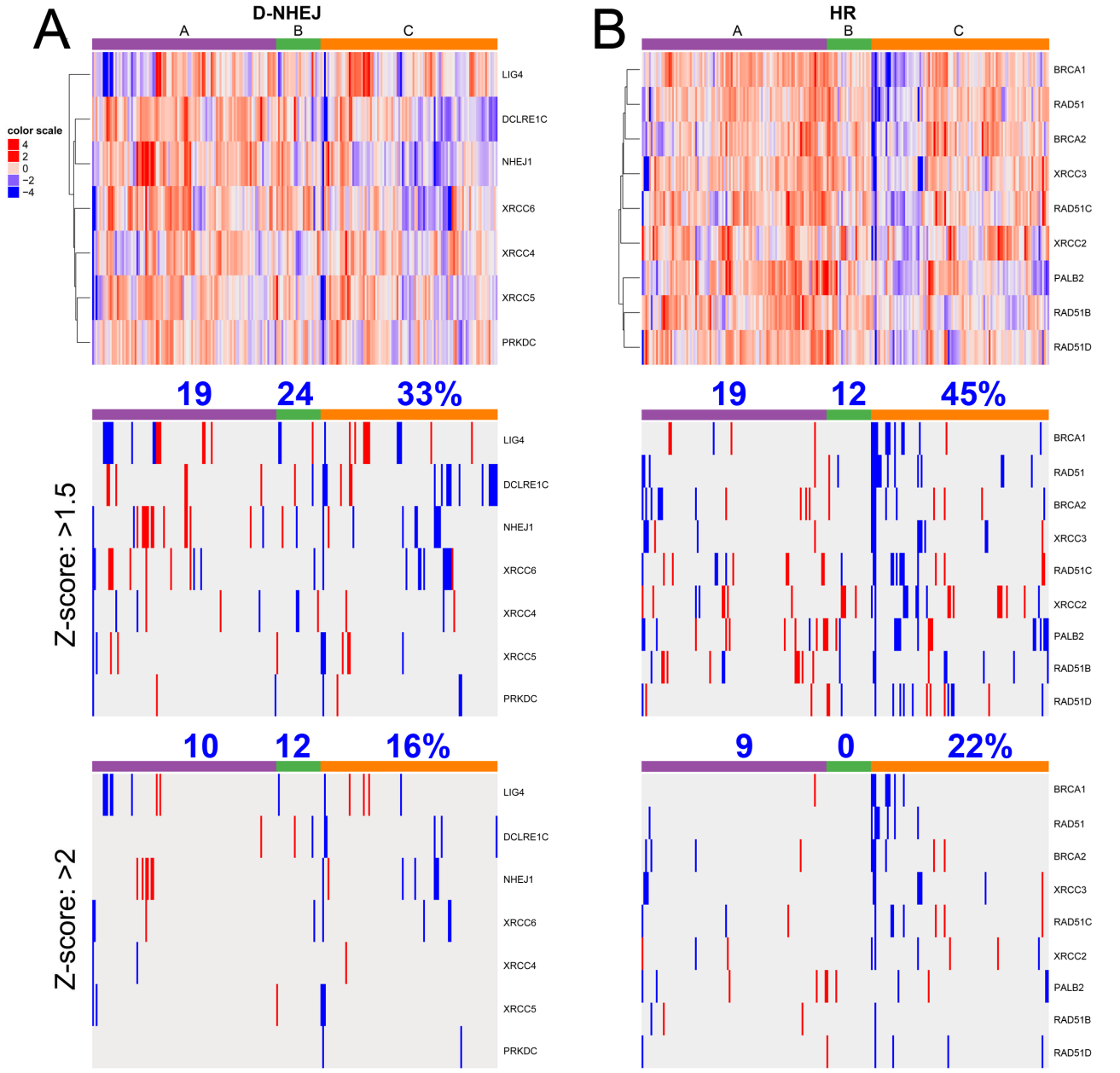

Figure 7: Transcriptome microarrays analysis of expression of the genes in A. D-NHEJ pathway, and B. HR pathway from 229 melanoma cell lines established from patients manifesting the following phenotypes: A- proliferative, C- invasive, and B- intermediate. Percent above column color bar represents number of samples with at least one downregulated gene within the phenotype group.

therefore selected melanomas with an invasive phenotype should display enhanced sensitivity to PARP1 inhibitors.

In addition, multiple melanoma samples displayed downregulation of at least one gene in HR pathway (Figure 7B) with higher frequency in the invasive phenotype suggesting their sensitivity to synthetic lethality triggered by PARP1 inhibitors [21]. In concordance, inhibition of histone deacetylases class I resulted in suppression of HR due to down-regulation of RAD51 and FANCD2 and sensitized malignant melanoma cells to a synthetic lethal effect of olaparib combined with alkylating drug temozolomide [22].

Despite downregulation/mutations of DSB repair genes detected in numerous samples in TCGA and transcriptome microarray databases, melanomas typically do not respond well to DNA damaging agents. Perhaps the degree of downregulation of DNA repair genes is not strong enough to increase the sensitivity to chemotherapeutics in clinical settings. However, as suggested by this work, the effect may become clinically relevant in repair-deficient cells when a genotoxic drug is combined with PARP1 inhibitor, which further enhances DNA damage beyond a reparable threshold.

In summary, PARP1 inhibitor seems to offer additional treatment opportunity to pre-selected melanomas displaying LIG4 (and/or XRCC4) deficiency. In addition, analyses of the already existing databases strongly suggest that numerous melanomas could be sensitive to personalized medicine-guided PARP1 inhibitor-mediated synthetic lethality due to their putative deficiencies in DNA repair pathways. This speculation is supported by phase II study showing almost doubled (although not statistically significant) progression-free survival of the patients with metastatic melanoma treated with veliparib + temozolomide compared with placebo + temozolomide. Perhaps personalized medicine approach is necessary to pre-select patients with melanomas predisposed to synthetic lethality mediated by PARP1 inhibitor.

MATERIALS AND METHODS

In vitro cell cultures

Melanoma cell lines derived from surgical specimens of nodular (DMBC2, DMBC8, DMBC9, DMBC10, DMBC12) and superficial spreading melanoma (DMBC11) were established in the Department of Molecular Biology of Cancer. 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 Stem Cell Medium (SCM) as described elsewhere [23, 24]. Normal Human Melanocytes (NHEMs – Ad, Lonza) were cultured in Melanocyte Cell Basal Medium (MBM) (CC-3250, Lonza) supplemented with growth supplements according to the manufacturer's protocol. Nalm6 parental and Nalm6 LIG4-/- pre-B cells were purchased from HORIZON (www.horizondiscovery.com) and cultured in RPMI medium with 10% FBS (Lonza) and antibiotics (100 IU/ml penicillin, 100 mg/ml streptomycin (Gibco) at 37°C in a humidified atmosphere containing 5% CO_2.

Drug treatment

Melanoma cells and NHEMs were plated at a density of 1 x 10^5 viable cells per well in a 6-well plates one day before drug treatment. Cells were cultured with 5 μM olaparib (Selleckchem), 2 mM dacarbazine (DTIC) (Sigma Aldrich), olaparib + DTIC, or vehicle. After 48 hours, half the cell suspension from each well was taken to determine cell viability after propidium iodide (PI) staining and cell cycle analysis. Following this, 1 ml of fresh medium containing drugs at appropriate concentrations was added to the remaining cell culture for additional 72 hours of culturing.

Clonogenic assay

Melanoma cells were first incubated with compounds at indicated concentrations for 48 hours and then for 72 hours. Then, 1000 single viable cells were transferred to soft agar and clonogenic assay was performed as previously described [23].

Flow cytometry

Flow cytometry and propidium iodide (PI) staining was used to assess changes in viability and cell distribution in cell cycle phases. Cells were analyzed using a FACSVerse flow cytometer (Becton Dickinson, San Jose, California, USA). ModFit LT 3.3 software (Verity Software, Topsham, Minnesota, USA) was used to calculate the percentage of cells in each cell-cycle phase and FACSuit software (Becton Dickinson) was used to calculate the percentages of dead cells in subG1.

Ectopic expression of LIG4

Melanoma DMBC11 cells were transfected with plasmid pCMV6-AC-GFP with cloned human LIG4 cDNA (OriGene Technologies) using lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. GFP+ cells were sorted after 48 hrs and used for the experiments.

Transcriptome microarrays analysis

Microarray data was obtained from NCBI GEO and analyzed for phenotype classes proliferative, intermediate and invasive as described in Widmer et al [25]. Microarray was subset for D-NHEJ genes and HR genes. Z-score cutoffs were set at 1.5 and 2 to detect upregulated and downregulated genes. Samples with at least one downregulated gene were counted.

RNA isolation, cDNA synthesis and Real-Time PCR

Isolation and purification of RNA was performed using total RNA isolation kit (A&A Biotechnology). Subsequently, RNA was transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, California, USA). qRT-PCR was performed using TaqMan® Real-Time PCR Master Mix (Life Technologies) and Agilent Technologies Stratagene Mx300SP working on MxPro software. TaqMan probes (Life Technologies) were used to analyze 8 genes whose products are essential for DSB repair pathways (*BRCA1*, *LIG3*, *LIG4*, *PALB2*, *PARP1*, *PRKDC*, *RAD51*, *XRCC6*), and *18S RNA* (Life Technologies) was included as the reference gene. The cycling parameters were 95°C for 10 minutes, 30 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Western blot analysis

Cell lysates were obtained by incubating a cell pellet with RIPA buffer for 30 minutes. Lysates were than resolved by SDS-PAGE. The proteins were transferred onto an Immobilon-P PVDF membrane (Millipore), which were blotted overnight with primary antibodies recognizing GAPDH, DNA LIG4 (Santa Cruz Biotechnologies), Ku70, RAD51 or PARP1 (ThermoFisher Scientific). This was followed by 1 h incubation with secondary antibodies conjugated with HRP (Anti-Mouse and Anti-Rabbit antibodies, Cell Signaling).

ELISA measurement of γ-H2AX

Cell lines DMBC11, DMBC12 and NHEMs were cultured with vehicle or with drugs on black 96-well plates with a clear bottom. Analysis of the level of phosphorylated histone γ-H2AX was performed using an H2AX Phosphorylation Assay Kit (Millipore, Billerica, MA, USA according to the protocol. Chemiluminescence detection was performed using attached HRP-substrates using a GloMax-Multi device (Promega). Bleomycin at 35 μM for 30 min was used as a control.

Neutral comet assay measurement of DSBs

Cells were cultured with vehicle or drugs for 48 hours and analyzed by neutral version of comet assay to detect DSBs as described before with modifications [13]. Briefly, cells were suspended in 0.75% LMP agarose and casted onto microscope slides precoated with 0.5% NMP agarose. The cells were then lysed for 1 h at 4 °C in a buffer consisting of 2.5 mM NaOH, 100 mM EDTA, 1% Triton X-100, 10 mM Tris, pH 10. After the lysis the slides were placed in an electrophoresis unit, DNA was allowed to unwind for 20 min in the electrophoresis buffer consisting of 100 mM Tris and 300 mM sodium acetate at a pH adjusted to 9.0 by glacial acetic acid. Electrophoresis was conducted in this electrophoresis buffer at 4 °C for 60 min at an electric field strength of 0.41 V/cm (100 mA). The slides were then washed in water, drained and stained with 2 μg/ml of DAPI and examined at 200× magnification in an Eclipse fluorescence microscope (Nikon, Tokyo, Japan) attached to COHU 4910 video camera (Cohu, San Diego, CA, USA) equipped with a UV-1 filter block consisting an excitation filter (359 nm) and a barrier filter (461 nm) and connected to a personal computer-based image analysis system, Lucia-Comet v. 5.41 (Laboratory Imaging, Praha, Czech Republic). Fifty images were randomly selected from each sample and the percentage of DNA in the tail of comets (% tail DNA) was measured. The mean value of the % tail DNA in a particular sample was taken as an index of DSBs in the sample.

Xenograft experiments

24 NSG mice were injected subcutaneously under the right scapula with $1x10^5$ melanoma cells previously suspended in Matrigel. After 4 days tumor-bearing mice were randomly assigned into four groups; untreated, and treated intraperitoneally either with olaparib (35 mg/kg bodyweight twice a day, diluted in DMSO), DTIC (8 mg/kg bodyweight every second day, diluted in PBS) or olaparib with DTIC (same dosing as in monotherapy) for 24 days. After the end of experiment tumors were collected and weighted. The study was approved by the local Ethical Committee.

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

There is no conflicts of interest.

Authors' contributions

M.T. performed RT-PCR, western blot, comet assay and phosphorylated histone H2A.X experiments, analyzed data; A.G-M. performed cell culturing and drug treatment, clonogenic assay; K.M. performed flow cytrometric analysis of viability and cell cycle; G.H. performed experiments with mice; J.S. performed RT-PCR, western blot experiments, analyzed data; M.N-S. performed experiments with NALM 6 cells, analyzed data; P.F.C., D.G., M.P.L., R.D. acquired bioinformatics data; M.C., T.Sk., T.S. conceived the project, designed experiments, analyzed data, wrote the manuscript.

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Research Paper

Eradication of LIG4-deficient glioblastoma cells by the combination of PARP inhibitor and alkylating agent

Monika Toma[1], Monika Witusik-Perkowska[2], Marzena Szwed[3], Robert Stawski[4], Janusz Szemraj[2], Malgorzata Drzewiecka[1], Margaret Nieborowska-Skorska[5], Maciej Radek[6], Pawel Kolasa[7,8], Ksenia Matlawska-Wasowska[9], Tomasz Sliwinski[1] and Tomasz Skorski[5]

[1]Laboratory of Medical Genetics, Faculty of Biology and Environmental Protection, University of Lodz, Lodz, Poland

[2]Department of Medical Biochemistry, Medical University of Lodz, Lodz, Poland

[3]Department of Medical Biophysics, University of Lodz, Lodz, Poland

[4]Department of Clinical Physiology, Medical University of Lodz, Lodz, Poland

[5]Department of Microbiology and Immunology, Fels Institute for Cancer Research and Molecular Biology, Temple University Lewis Katz School of Medicine, Philadelphia, PA, USA

[6]Department of Neurosurgery, Surgery of Spine and Peripheral Nerves, Medical University of Lodz, University Hospital WAM-CSW, Lodz, Poland

[7]Department of Neurosurgery, Medical University of Lodz, Copernicus Memorial Hospital, Lodz, Poland

[8]Social Sciences Academy in Lodz, Lodz, Poland

[9]Division of Pediatric Research, Department of Pediatrics, University of New Mexico Health Sciences Center, Albuquerque, NM, USA

Correspondence to: *Tomasz Sliwinski,* ***email***: *tomasz.sliwinski@biol.uni.lodz.pl*
Tomasz Skorski, ***email***: *tskorski@temple.edu*

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ABSTRACT

Cancer cells often accumulate spontaneous and treatment-induced DNA damage i.e. potentially lethal DNA double strand breaks (DSBs). Targeting DSB repair mechanisms with specific inhibitors could potentially sensitize cancer cells to the toxic effect of DSBs. Current treatment for glioblastoma includes tumor resection followed by radiotherapy and/or temozolomide (TMZ) – an alkylating agent inducing DNA damage. We hypothesize that combination of PARP inhibitor (PARPi) with TMZ in glioblastoma cells displaying downregulation of DSB repair genes could trigger synthetic lethality. In our study, we observed that PARP inhibitor (BMN673) was able to specifically sensitize DNA ligase 4 (LIG4)-deprived glioblastoma cells to TMZ while normal astrocytes were not affected. LIG4 downregulation resulting in low effectiveness of DNA-PK–mediated non-homologous end-joining (D-NHEJ), which in combination with BMN673 and TMZ resulted in accumulation of lethal DSBs and specific eradication of glioblastoma cells. Restoration of the LIG4 expression caused loss of sensitivity to BMN673+TMZ. In conclusion, PARP inhibitor combined with DNA damage inducing agents can be utilized in patients with glioblastoma displaying defects in D-NHEJ.

INTRODUCTION

Glioblastoma (grade IV in WHO Classification of Tumors of the Central Nervous System) [1] is the most frequent primary brain tumor with very poor survival rate (median of 14.6 months) [2]. Currently, treatment bases on surgical resection if feasible, followed by radiotherapy and/or oral chemotherapy with temozolomide (TMZ) – alkylating agent inducing toxic DNA lesions like O6-methylguanine, N7-methylguanine or N3-methylalanine.

These, become highly lethal for cancer cells when DNA repair systems are disrupted [3, 4].

One of the hallmarks of the cancerous cells is genomic instability responsible for accumulation of further genome rearrangements and tumor progression [5]. Development of such abnormalities in primary DNA repair systems results in activation of compensatory DNA repair mechanism, ipso facto, inducing cell "addiction" to the changes it carries. It has been suggested that cancer-specific abnormalities in the functioning of DNA repair systems and pathway redirection events might be responsible for the resistance and survival of cancer cells after exposure to genotoxic stress [6].

DNA double-strand breaks (DSBs) are the most toxic among DNA lesions and can be responsible for genome rearrangements leading to genomic instability, neoplastic transformation and cell death [7]. DSBs can arise due to the exposure to ionizing radiation (IR), reactive oxygen species (ROS) or genotoxic drugs [8]. Two mechanisms predominantly responsible for repair of DSBs in proliferating cells are BRCA1/2-mediated homologous recombination (HR) and DNA-PK-mediated non-homologous end-joining (D-NHEJ). When proper functioning of one of these pathways is compromised, cells redirect functions to an alternative mechanism – PARP1-dependant backup NHEJ (B-NHEJ) [9–11]. PARP1 is a protein playing a critical role in other processes decreasing the number of lethal DSBs - by activation of base excision repair (BER), single strand break (SSB) repair or HR activation at stalled replication forks [12–13]. PARP inhibitors are currently used in synthetic lethality-based personalized therapy in patients with breast and ovarian cancer deficient in BRCA1/2-mediated HR [14–15]. We hypothesized that deficiencies in DSB repair pathways could sensitize glioblastoma cells to PARP inhibitor (PARPi) BMN673 especially when combined with DSB-inducing drug temozolomide [16–18].

RESULTS

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

In order to utilize personalized synthetic lethality approach we determined the expression profile for 3 patient-derived glioblastoma primary cell lines and compared it to the profile of normal human astrocytes (NHA). The subject of our interest were 15 genes involved in DSB repair pathways (BRCA1, BRCA2, PALB2, RAD51B, RAD51C, RAD51D, XRCC2, XRCC3, RAD52 taking part in HR; LIG4, DNA-PKcs, XRCC5, XRCC6 in D-NHEJ; and PARP1, LIG3 in B-NHEJ). Significant changes in the mRNA expression profile of LIG4 was found between glioblastoma cell lines and NHA (Figure 1A). Decreased level of LIG4 at the protein level was then confirmed by Western blot in cancer cell lines in comparison to normal astrocytes (Figure 1B). For further experiments H6 and H7 primary cell lines were chosen due to abundant downregulation of LIG4.

Patient-derived glioblastoma cell lines carry tumor-unique molecular markers

Loss of heterozygosity (LOH) in chromosome loci 10q, 10p and 22q were reported to be one of the most commonly occurring abnormalities during astrocytoma progression [19–20]. Therefore, we analyzed LOH in these locations in order to confirm the presence of glioblastoma cells in tumor samples and established primary cell lines H6 and H7. LOH was examined in two resected primary tumors and two corresponding primary cell lines in comparison to blood samples. LOH in 10q locus were detected in all neoplastic samples (Figure 2) whereas no aberrations were noted in 10p and 22q locus. The presence of the wild-type DNA in the tumor samples have been observed, which may refer to the heterogeneity of cell population in the tumor bulk. Nonetheless control vs corresponding tumor peak height ratio was lower than 50%, whereas in cell lines was more than 65–70%, strongly suggesting that 10q aberration has been propagated in cell culture condition.

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

To analyze the potential anti-glioblastoma effect of PARP inhibitor (BMN673) used either alone or in combination with alkylating agent (TMZ), double staining with propidium iodide (PI) and annexin V was used. Annexin V staining in conjunction with vital dye (PI) distinguishes viable cells from dead, and also early apoptotic cells from necrotic cells. When compared to individual agents, the combination of BMN673 + TMZ exerted significantly stronger anti H6 and H7 glioblastomas effect with only minimal toxicity to normal astrocytes (Figure 3A). The flow cytometry result indicates also that post-treatment cell death occurs majorly via apoptosis as cells were getting accumulated in Q4 quadrant (Annexin V^+, PI^-) and then shifting to Q2 quadrant (Annexin V^+, PI^+) what would characterize slow externalization of phosphatidylserine and prolonged annexin V binding which is typical for apoptosis (Figure 3B) (Supplementary Figure 1). The results were also confirmed with trypan blue staining (Supplementary Figure 2).

Morphological changes induced by BMN673 +/- TMZ were assessed by Calcein AM/PI double staining (Figure 3C). Cells treated with the inhibitors showed the characteristic hallmarks of cellular homeostasis disorders (cellular membrane damage, cell shrinkage and their fragmentation). These morphology changes were much

more noticeable in cancer than in normal cells. These alterations of cellular morphology were in agreement with the increasing number of dead cells stained with PI especially in samples treated with BMN673 + TMZ.

The impact of BMN673 + TMZ on cell cycle phase distribution of glioblastoma cells and normal human astrocytes was analyzed by flow cytometry (Figure 4). The effect of drugs was visible in H6 and H7 glioblastomas as elevation of SubG1 and S phase populations but a picture typical for G2/M arrest was not detected. Interestingly, drug-induced changes in cell cycle phases for NHA cells were slight to none.

Clonogenic assay was used to test the impact of drugs on colony formation ability of cancer cells. When used alone, only TMZ had a significant influence on long-term clonogenic efficiency whereas BMN673 + TMZ were able to almost completely abrogate clonogenic ability of LIG4-deficient glioblastoma cells (Figure 5).

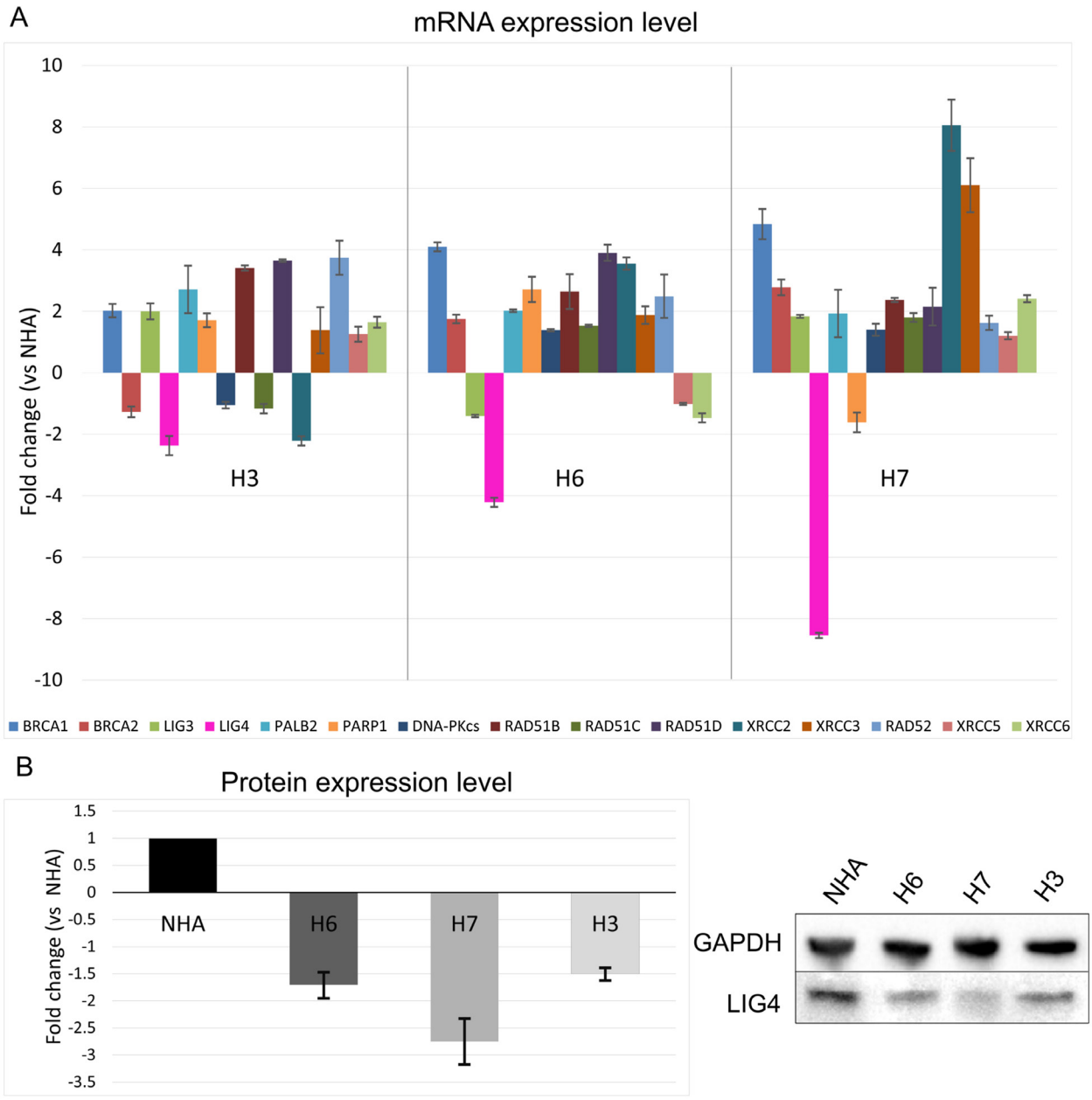

Figure 1: Expression profiles of genes involved in HR, D-NHEJ and B-NHEJ repair systems in glioblastoma cells vs normal human astrocytes. (**A**) mRNA expression level of 15 indicated genes in primary human glioblastoma cell lines (H3, H6 and H7) was normalized to the expression of reference gene – 18S rRNA. Data are presented as a fold change in reference to normal human astrocytes (NHA). Results represent mean value ± SD from 3 independent experiments each performed in triplicates. (**B**) Protein expression level presented as fold change in comparison to NHA where expression was set as 1. The expression level was normalized to the reference protein, GAPDH. Mean ± SD was calculated form 3 independent experiments. Representative Western protein expression analysis of LIG4 and GAPDH (loading control) is shown.

Combination of BMN673 and TMZ induces accumulation of toxic DSBs in patient-derived glioblastoma cells

Phosphorylation of serine 139 on histone 2A.X (γH2A.X) can be used as a marker of DSBs [21]. TMZ treatment increased γH2A.X immunofluorescence in H7 primary cell line (Figure 6A). This effect was remarkably enhanced in both H6 and H7 cell lines when BMN673 and TMZ were used in combination. In NHA cells the level of γH2A.X positive cells stayed at relatively low level regardless from the treatment used.

Neutral comet assay was also employed to detect DSBs after treatment with BMN673 and/or TMZ. After treatment with individual drugs only TMZ enhanced the percentage of DNA in tails of H7 cells in comparison to NHA cells (Figure 6B). Combination of BMN673 and TMZ caused significant increase of DSBs in both glioblastoma cell lines.

Rescue of LIG4 expression caused resistance to BMN673 + TMZ treatment

To determine the role of reduced expression of LIG4 in sensitivity of glioblastoma cells to BMN673+TMZ, H7 cells were transfected with the plasmid carrying LIG4 cDNA followed by treatment with the drugs. Elevated expression of LIG4 resulted in resistance of H7 glioblastoma primary cell line to BMN673 + TMZ (Figure 7).

DISCUSSION

Due to the growing knowledge of genetic and epigenetic changes in tumors the concept of synthetic lethality became lately one of the main areas of searching for new therapy candidates. The phenomenon occurs when simultaneous loss of two genes causes cell death whereas loss of each of these genes individually is not lethal [11]. For instance, BRCA1/2 deficient tumors with impaired homologous recombination repair were reported to be sensitive to PARP inhibition [16–17]. PARPi could be used in combination with the agents inducing DNA damage like doxorubicin, radiation or alkylating drugs [22]. Therefore, we postulated that combination of PARPi may significantly improve the therapeutic outcome of currently used TMZ-based therapy and specifically eradicate glioblastoma cells with disrupted DSB repairing pathways.

LIG4 is a crucial element of D-NHEJ pathway and its low level might result in ineffective functioning of this repair system. Downregulation in LIG4 was previously described in patient-derived high-risk neuroblastomas and correlated with higher stage of disease and lower survival probability [23]. Analysis of available mRNA gene expression databases revealed cohorts of glioblastomas displaying lower expression of LIG4. Mechanisms responsible for reduced expression of LIG4 are not known, but our recent report suggested that inefficient JAK2-STAT5 and/or PI3K-AKT pathways may play a role [24].

To examine the potential therapeutic aspect we generated primary glioblastoma cells with downregulated LIG4 when compared to normal human astrocytes. PARPi BMN673 in combination with alkylating agent TMZ was effective against patient-derived glioblastoma cells displaying downregulation of LIG4 but not against normal human astrocytes. Downregulation of LIG4 in glioblastoma cells was directly responsible for enhanced

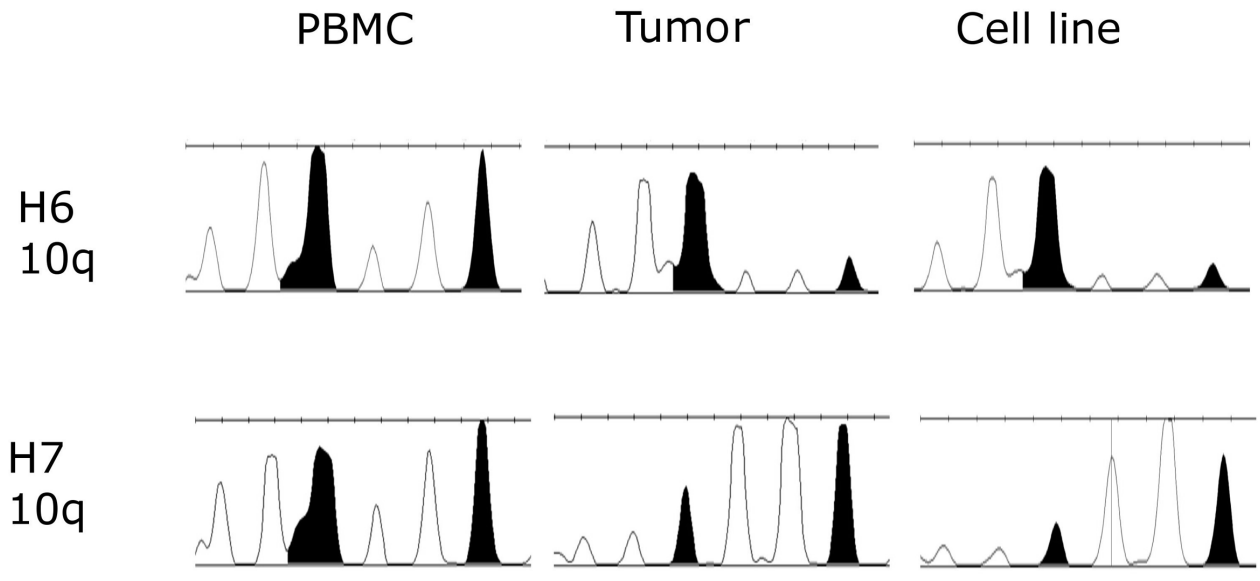

Figure 2: Loss of heterozygosity (LOH) examined in two primary tumor samples and established primary cell lines H6 and H7 in comparison to normal corresponding blood samples. LOH in 10q locus was been observed in all neoplastic samples. As expected, tumor tissue expressed lower level of LOH due to the presence of both normal and cancer cells.

sensitivity to BNM673 as restoration of LIG4 expression resulted in resistance to the treatment.

We postulate that D-NHEJ deficiency resulting from downregulation of LIG4 could be synthetically lethal with B-NHEJ deficiency induced by PARPi in glioblastoma cells exposed to TMZ-induced DNA damage. In concordance, we demonstrated that LIG4 deficient melanoma cells were highly sensitive to the combination of an alkylating agent dacarbazine and PARPi [18]. Morevoer, HCT116 *Lig4-/-* cells were sensitive to the combination of PARPi with radiotherapy [25].

Although downregulation/mutation of LIG4 (and its partner XRCC4) was detected only in approximately 4% of glioblastomas in The Cancer Genome Atlas (TCGA) database [26] inhibition/inactivating mutation of other members of D-NHEJ potentially impairing DSB repair activity were present in up to 20% of the cases. Moreover, transcriptome analysis by microarrays detected downregulation of at least one member of D-NHEJ pathway (including LIG4) in 191 glioblastomas manifesting the proneural, proliferative, proliferative-mesenchymal and mesenchymal phenotypes (Figure 8A) [27]. In addition, multiple glioblastoma samples displayed downregulation of at least one gene in HR pathway (Figure 8B) suggesting their sensitivity to synthetic lethality triggered by PARPi [28].

In summary, this study implicates potential therapeutic effect of PARPi used in combination with DNA-damaging agents in D-NHEJ-deprived glioblastoma cells. Therefore patient pre-selection based on expression of DNA repair genes may be applied for personalized medicine approach to improve the effectiveness of anti-glioblastoma therapy.

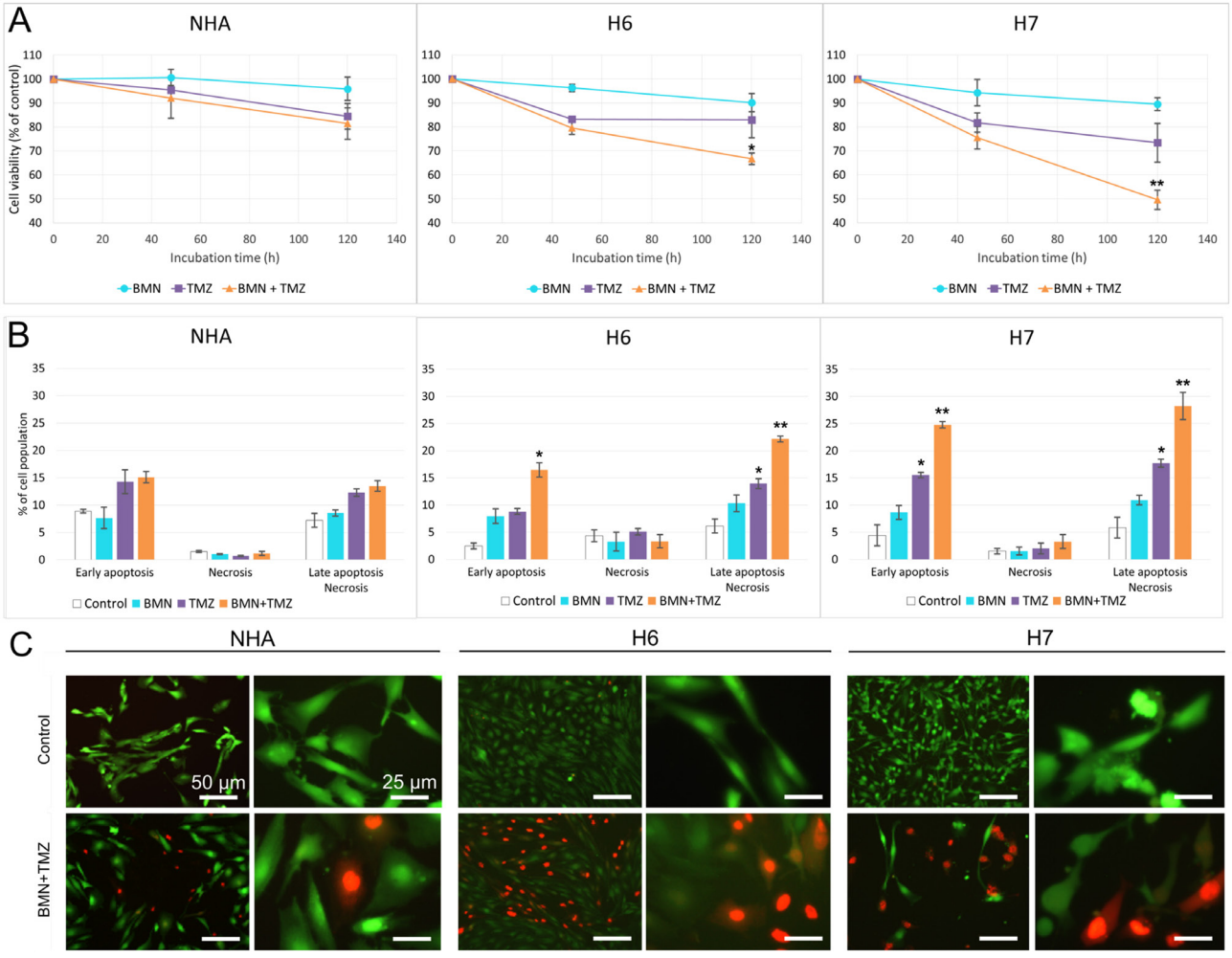

Figure 3: BMN673+TMZ anti-glioblastoma effect. H6, H7 and NHA cells were treated with BMN673 (BMN) and/or TMZ. (**A**) Viability measured as population of Annexin V/PI negative cells in comparison to vehicle-treated control after 48 h and 120 h. Results represent mean % ± SD of 3 independent experiments, $^{*}p < 0.05$, $^{**}p < 0.001$ in comparison with control. (**B**) Quantitative representation of flow cytometry results after 120 h of treatment. Results represent mean value ± SD from 3 independent repeats, $^{*}p < 0.05$, $^{**}p < 0.001$ in comparison with control. (**C**) Morphological changes of normal and cancer cells after 120 h of treatment with BMN673 + TMZ or vehicle (Control). Cells were stained with Calcein AM/ propidium iodide. Note the typical morphological features of cell death: loss of structural framework of nuclei, condensation of chromatin, cell shrinkage and nuclear fragmentation (observed mostly in higher magnification). Cells were analyzed under an inverted fluorescence microscope (Olympus IX70), magnification x100 (scale bar = 50 µm) and x400 (scale bar = 25 µm).

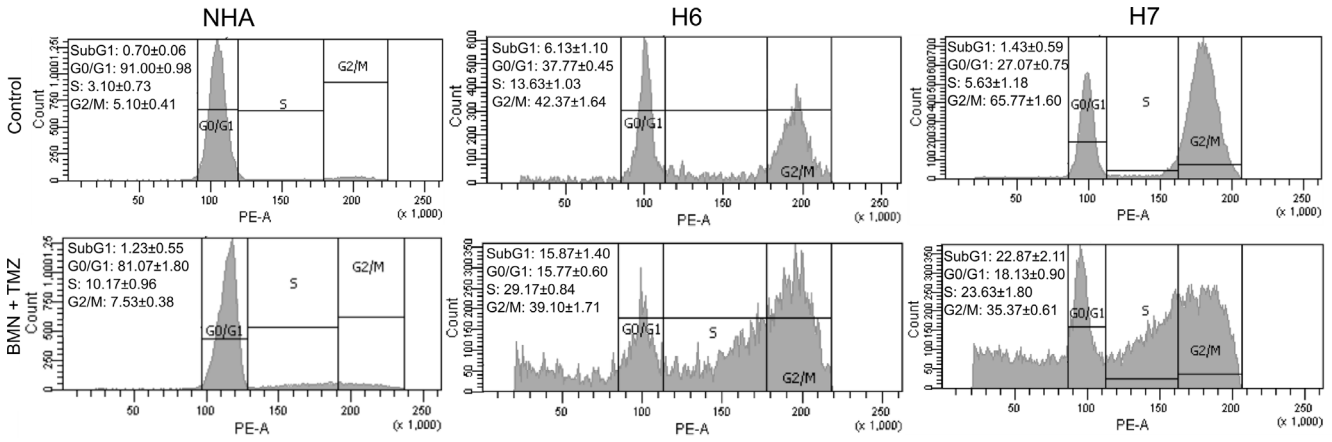

Figure 4: Cell cycle distribution of H6 and H7 glioblastoma cells and NHA cells treated or not with BMN673 + TMZ. Representative graphs of normal human astrocytes (NHA) and H6 and H7 primary cell lines after 120 h incubation with the drugs (BMN + TMZ) or vehicle (Control). Left upper corner of each variant includes quantitative representation of cell population in each cell cycle phase – SubG1, G0/G1, S, G2/M. Values represent mean ± SD from 3 independent experiments.

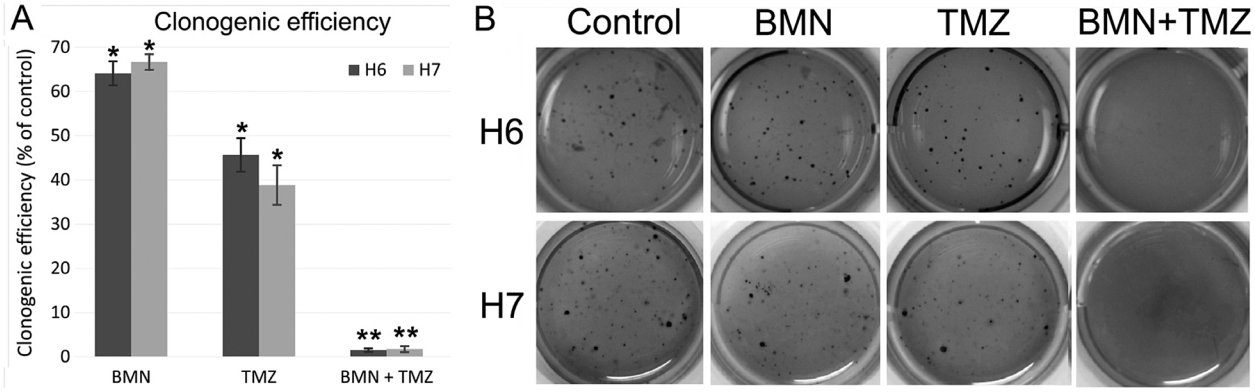

Figure 5: Clonogenic potential of patient-derived glioblastomas after treatment with BMN673 and/or TMZ. (**A**) Cells were treated with either vehicle, BMN673, TMZ and BMN673 + TMZ followed by soft agar culture for 2–3 weeks. Clonogenic efficiency is shown as mean ± SD % of control (cells treated with vehicle) from 3 independent experiments, $^{*}p < 0.05$ and $^{**}p < 0.001$ in comparison to control. (**B**) Photographs of a representative experiment.

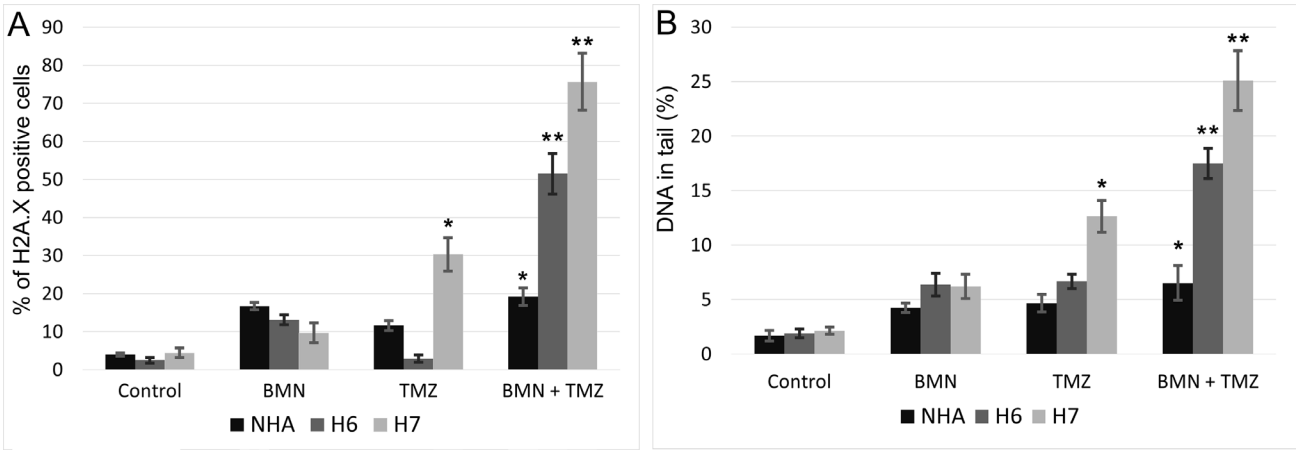

Figure 6: Accumulation of DSB in BMN673+TMZ-treated H6 and H7 glioblastoma cells and in NHA cells. (**A**) DSBs were detected by γH2A.X immunofluorescence. Bars show mean percentage of γH2A.X –positive cells ± SD from 3 independent experiments. (**B**) DSBs were detected by neutral comet assay. Bars show mean percentage of DNA in tail ± SD from 50 randomly selected cells in 3 independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.001$ when compared to control.

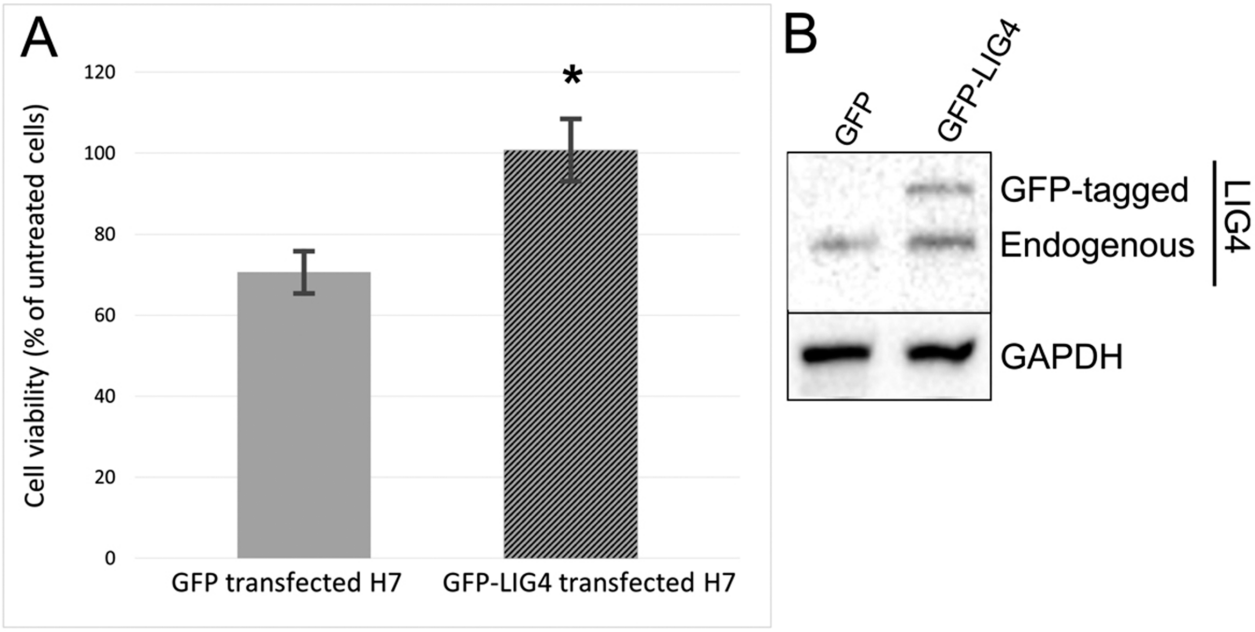

Figure 7: Sensitivity to BMN673+TMZ depends on LIG4 expression. (**A**) The effect of BMN673 in combination with TMZ on H7 cells transfected with GFP (Control) or GFP-LIG4 expression plasmid. Results represent mean % of viable cells ± SD from 3 independent experiments, $^{*}p < 0.05$ comparing with Control. (**B**) Representative Western blot of LIG4 expression in H7 cells transfected with control GFP plasmid and with vector carrying GFP-LIG4. GAPDH is included as a loading control.

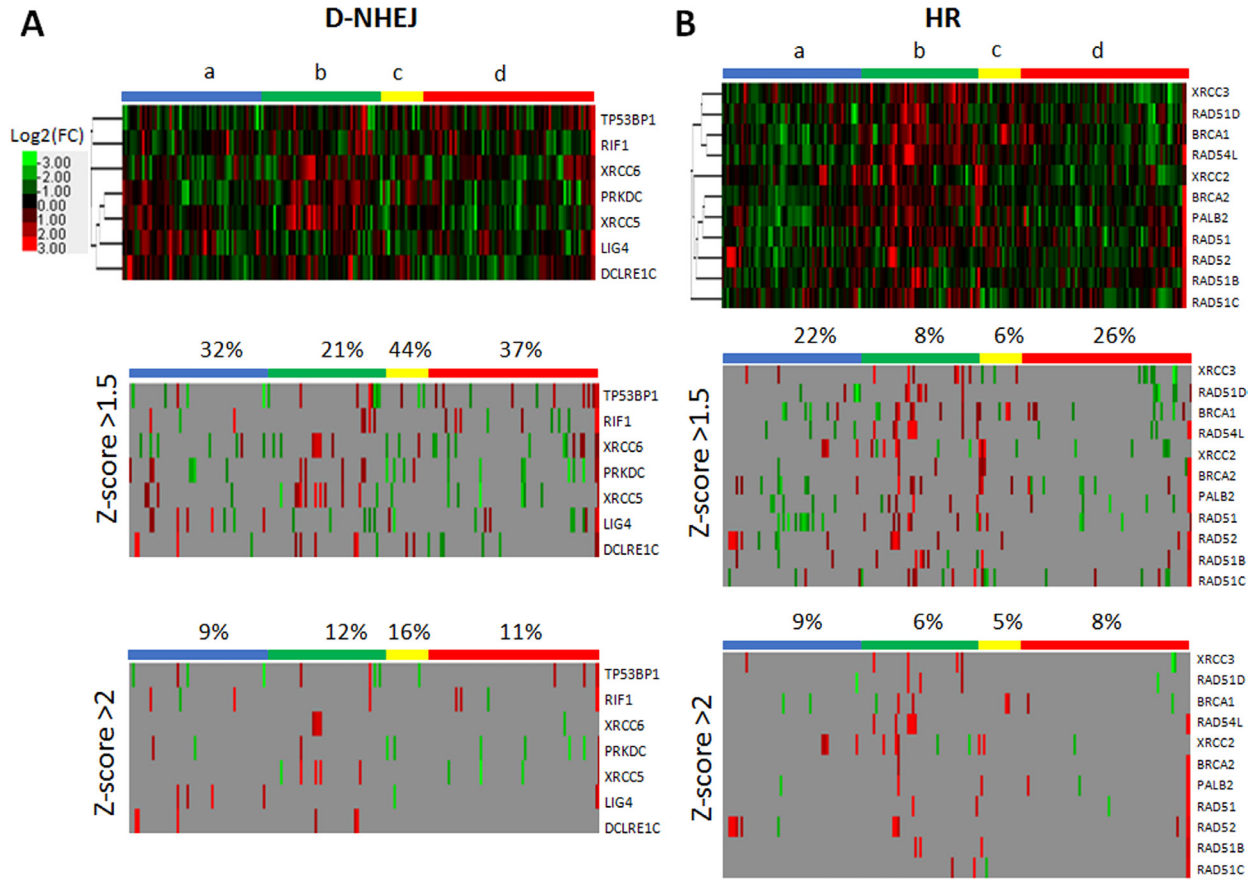

Figure 8: Microarray-based gene expression profiling for the genes in (A) D-NHEJ pathway and (B) HR pathway. Data was obtained for 191 glioblastoma patients manifesting the following phenotypes: a – proneural (n = 56); b – proliferative (n = 48); c- proliferative-mesenchymal (n = 18); d – mesenchymal (n = 69). Percent above column bar represents number of samples with at least one downregulated gene within the phenotype group.

MATERIALS AND METHODS

In vitro cell culture

Glioblastoma specimens, histopathologically classified as clinical stage IV, were obtained from patients of Department of Neurosurgery, Surgery of Spine and Peripheral Nerves, Medical University of Lodz (University Hospital WAM-CSW Łódź) and Department of Neurosurgery, Medical University of Lodz (Copernicus Memorial Hospital, Łódź, Poland). Cell cultures derived from specimens were established in the Laboratory of Molecular Genetics, University of Lodz and named H3, H6 and H7. After several washes tissue fragments were minced with scalpel and cells were filtered through 70 μM pore size cell strainer. Glioblastoma cells were cultured in DMEM medium (Lonza, Basel, Switzerland) supplemented with 10% FBS (Lonza), 100 IU/ml penicillin, 100 μg/ml streptomycin (Lonza) and gentamycin 50 μg/ml (Lonza) in a humidified atmosphere containing 5% CO_2 at 37°C. The normal human astrocytes NHA (Lonza) were grown in ABM Basal Medium supplemented with AGM BulletKit (Lonza) and cultured according to the protocol provided by manufacturer.

Isolation of DNA from tumor, blood and cell culture samples and loss of heterozygosity analysis

Loss of heterozygosity (LOH) analysis was performed using microsatellite markers. The aim of the experiment was to verify presence of genetic aberrations specific for glioblastoma cells in suspected cancerous tissue. The samples were examined for LOH using sets of DNA samples isolated from tumor bulk specimen and corresponding cell culture and peripheral blood samples (PBMC). Isolation and purification of genomic DNA was performed with Genomic Mini and Blood Mini isolation kits (A&A Biotechnology) according to the manufacturer's protocol. The microsatellite markers D10S1709 (10q) (F–GTGAGTCCAGAATCACCCC, R–CAGTGGAAATGGCTCATTTG), D10S1172 (10p) (F–GGATACTACCAAGAGAGAG, R–ATCATCTATCTCTACTATCTG), D22S283 (22q) (F–ACC AACCAGCATCATCAT, R–AGCTCGGGACTTTCTGAG) were selected using the NCBI database [20, 21]. The F primers were 5'-labelled with Fam fluorochrome (Sigma). Each reaction was amplified in volume of 25 μl containing 50 ng of DNA template, dNTP, KAPA Taq DNA Polymerase (Kapa Biosystems) and forward/reverse primers. PCR reaction was carried as follows: 95°C 3 min, (95°C 30s, temperature depending on primer pair 30s, 72°C 45s) x32, 72°C 4 min. PCR products were visualized with a 16-capillary electrophoresis 3130xl Genetic Analyzer (Applied Biosystems). The analysis was performed using Gene mapper 4.1 software and verified manually. Loss of heterozygosity was judged to be present if the allelic signal intensity of the tumor sample was reduced by at least 50% relative to the corresponding allele in the patient's control DNA (PBMC).

RNA isolation, reverse transcription and Real-Time PCR

RNA isolation and purification was performed using RNA isolation kit (A&A Biotechnology). In the next step samples were transcribed into cDNA with SuperScript II Reverse Transcriptase (Invitrogen, Life Technologies, Carlsbad, California, USA) according to the manufacturer's protocol. Real-Time PCR quantitation was carried out using TaqMan Real-Time PCR Master Mix and TaqMan probes (Applied Biosystems, Life Technologies, Carlsbad, California, USA) detecting genes which products are involved in DSB repair pathways (BRCA1, BRCA2, LIG3, LIG4, PALB2, PARP1, PRKDC, RAD51B, RAD51C, RAD51D, XRCC2, XRCC3, RAD52, XRCC6, XRCC7). 18S rRNA TaqMan probe was included as the reference gene. The parameters for Agilent Technologies Stratagene Mx300SP instrument were 95°C for 10 minutes, 30 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Protein isolation and western blot analysis

Protein extraction was performed incubating cell pellet with the mixture of RIPA buffer (Sigma) and protease inhibitor cocktail (Thermo Scientific, Rockford, Illinois, USA). After concentration measurement, 30 μg of cell lysates was resolved on 4–20% ExpressPluS PAGE Gel (GenScript, Piscataway, New Jersey, USA). The proteins were then transferred onto PVDF Transfer Membrane (Thermo Scientific, Rockford, Illinois, USA) using eBlot Protein Transfer device (GenScript, Piscataway, New Jersey, USA). Membranes were blocked and blotted overnight with primary antibodies recognizing LIG4 and GAPDH (Santa Cruz Biotechnology, Dallas, Texas, USA). Membranes were then washed and incubated 1 h with secondary anti-mouse antibody conjugated with HRP (Cell Signaling Technology, Danvers, Massachusetts, USA). The result was visualized using Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, Illinois, USA) and BioRad Universal Hood II with Chemiluminescence System (BioRad, Hercules, California, USA).

Drug treatment

Normal astrocytes and glioblastoma cells were plated in a 6-well plate at a density of 2×10^5 viable cells per well. Cells were cultured with 50 nM BMN673 (Selleckchem), 6.25 μM TMZ (Sigma Aldrich), BMN673

+ temozolomide or vehicle for 48 h followed by the second dose of the compounds and another 72 h of incubation.

Calcein AM/propidium iodide double staining

After the indicated treatments, normal and cancer cells were incubated for 30 min at 37°C with the mixture of 2 mM Calcein AM and propidium iodide 1 mM (Life Technologies, USA) diluted in PBS. Fluorescence emitted by stained cells was then observed in an inverted fluorescence microscope (Olympus IX70, Japan).

Flow cytometry

Flow cytometry and staining with propidium iodide and FITC Annexin V (BD Biosciences) was used to assess changes in viability and to track the mechanism of cell death after treatment. Cells were prepared and analyzed according to the FITC Annexin Apoptosis Detection Kit II (BD Biosciences). To analyze the influence of the compounds on glioblastoma and NHA distribution in cell cycle, cells fixed with 70% cold ethanol were stained with propidium iodide with addition of RNase (BD Biosciences) and analyzed. The extent of DNA DSBs measured by phosphorylation of H2A.X histone was obtained using Alexa Fluor 647 Mouse Anti-H2A.X (pS139) antibody (Becton Dickinson, San Jose, California, USA) after 48 h treatment with the compounds. Fixed cells were washed resuspended in 20 µl BD Perm/Wash™ buffer and stained for 20 min with H2A.X antibody (5 µl/test). All the experiments were performed using a FACS Canto II cytometer (Becton Dickinson, San Jose, California, USA).

Neutral comet assay

Neutral comet assay was performed according to the protocol used in the previous research [19] on cells cultured for 48 h with either drugs or vehicle. 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.

Clonogenic assay

To examine clonogenic activity glioblastoma cells were first cultured with drugs or vehicle for 48 h followed by the second dose of the compounds and another 72 h of incubation. After treatment cell viability was determined by staining with trypan blue and 10^3 cells were resuspended in 700 µl of soft agar (DMEM, 0.4% w/v) and plated over 700 µl of solidified agar underlay (DMEM, 0,5% agar) on a 12-well plate. After solidifying cell layer was covered with medium (changed weekly). After 2–3 weeks colonies were stained with crystal violet (0.5% w/v) and counted under the microscope. Clonogenic efficiency was expressed as percent of untreated control (no. of colonies after treatment vs no. of colonies in control sample × 100%).

Ectopic expression of LIG4

Glioblastoma H6 cells were transfected with pCMV6-AC-GFP plasmid containing human LIG4 cDNA (OriGene Technologies). The method was performed using Lipofectamine 2000 (Invitrogen, Life Technologies, Carlsbad, California, USA). GFP-positive cells were sorted 48 h after transfection.

Microarrays

Microarray data sets were obtained from NCBI GEO (GSE13041). Gene expression profiling was performed as described before [29–30]. Microarray was subset for D-NHEJ genes and HR genes. Z-score cutoffs were set at 1.5 and 2 to detect upregulated and downregulated genes as described before [18].

Statistical analysis

Data was accessed in three independent experiments and presented as mean ± SD. Results were compared using two tailed Student *t* test. *P* values lower than 0.05 were considered significant. The synergistic effect of drugs was studied using response additivity approach.

Study approval

Studies performed on cells derived from surgical specimens were approved by the Ethical Commission of the Medical University of Lodz (no. RNN/194/12/KE) and informed consent was obtained from all patients.

Abbreviations

BER: base excision repair; DSB: DNA double strand break; HR: homologous recombination; IR: ionizing radiation; LOH: loss of heterozygosity; NHA: normal human astrocytes; NHEJ: non-homologous end-joining; PBMC: peripheral blood mononuclear cells; PI: propidium iodide; ROS: reactive oxygen species; SD: standard deviation; SSB: DNA single strand break; TMZ: temozolomide.

Author contributions

M.T. wrote the manuscript, performed cell culturing, LOH sample preparation, drug treatment, RT-PCR, Western blot, flow cytometry, clonogenic assay, ectopic expression of LIG4, analyzed data; M.W-P. performed cell culturing and neutral comet assay; M.S. performed

calcein/propidium iodide double staining; R.S. analyzed LOH results; J.S. performed RT-PCR, analyzed data; M.D. performed cell culturing, DNA and RNA isolation; M.N-S. analyzed data; M.R, P.K. provided with surgical specimens; K.M-W. performed microarray analysis; T.Sk., T.Sl. conceived the project, designed experiments, analyzed data, and wrote the manuscript.

CONFLICTS OF INTEREST

There is no conflicts of interest.

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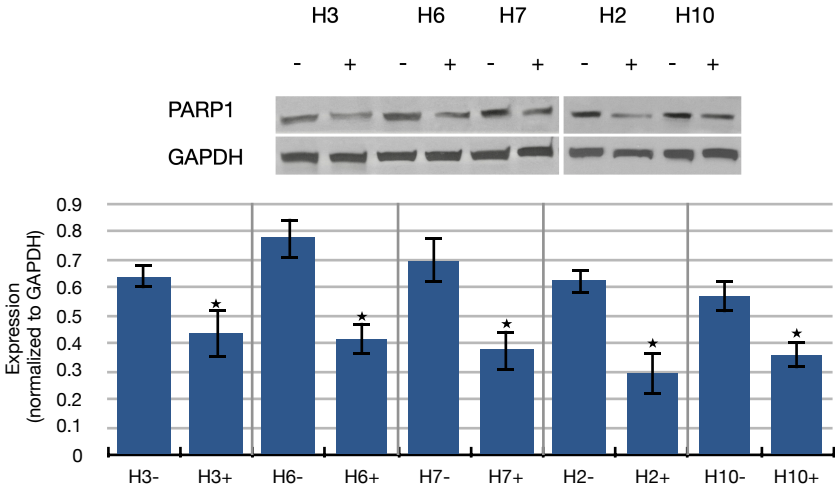
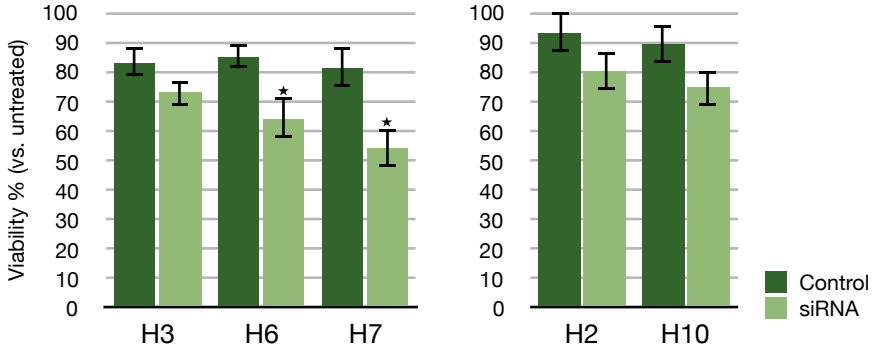
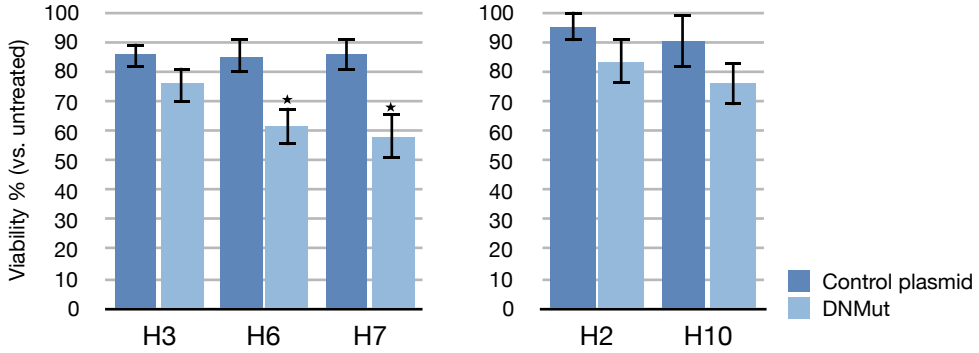
Dane uzupełniające



Wykres 1. Ekspresja PARP1 po zastosowaniu siRNA w celu wyciszenia PARP1. Reprezentatywny wynik eksperymentu Western Blot po wyciszeniu ekspresji PARP1 w badanych liniach (+) i negatywna kontrola (-). Densytometryczna analiza zaprezentowana na wykresie została znormalizowana do ekspresji GAPDH i przedstawiona jako średnia ± SD.



Wykres 2. Żywotność komórek lini H3, H6 i H7 (z obniżonym poziomem LIG4) i linii H2 i H10 (z poziomem LIG4 porównywalnym z komórkami prawidłowymi) po wyciszeniu ekspresji PARP1 i 48h inkubacji ze związkiem alkilującym. Średnia ± SD.



Wykres 3. Żywotność komórek lini glejaka po wywołaniu ekspresji dominującego negatywnego mutanta PARP1(E988K) (DNMut) i po traktowaniu związkiem alkilującym. Średnia ± SD.

Oświadczenia współautorów

mgr Monika Toma
Pracowni Genetyki Medycznej
Uniwersytet Łódzki
ul. Pomorska 141/143
90-236 Łódź

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mgr Monika Toma

mgr Monika Toma

Tomasz Skorski, MD, PhD, DSc
Sol Sherry Thrombosis Research Center and
Fels Institute for Cancer Research and Molecular Biology,
Lewis Katz School of Medicine
Temple University
Philadelphia, PA, USA

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.....

Tomasz Skorski, MD, PhD, DSc

prof. dr hab. Tomasz Śliwiński
Kierownik Pracowni Genetyki Medycznej
Uniwersytet Łódzki
ul. Pomorska 141/143
90-236 Łódź

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KIEROWNIK
PRACOWNI GENETYKI MEDYCZNEJ
Wydział Biologii i Ochrony Środowiska UŁ

prof. dr hab. Tomasz Śliwiński

.....

Prof. dr hab. Tomasz Śliwiński

mgr Monika Toma
Pracowni Genetyki Medycznej
Uniwersytet Łódzki
ul. Pomorska 141/143
90-236 Łódź

16.01.2020 r.

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mgr Monika Toma

mgr Monika Toma

Tomasz Skorski, MD, PhD, DSc
Sol Sherry Thrombosis Research Center and
Fels Institute for Cancer Research and Molecular Biology,
Lewis Katz School of Medicine
Temple University
Philadelphia, PA, USA

16.01.2020 r.

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.....

Tomasz Skorski, MD, PhD, DSc

prof. dr hab. Tomasz Śliwiński
Kierownik Pracowni Genetyki Medycznej
Uniwersytet Łódzki
ul. Pomorska 141/143
90-236 Łódź

20.01.2020 r.

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KIEROWNIK
PRACOWNI GENETYKI MEDYCZNEJ
Wydział Biologii i Ochrony Środowiska UŁ

prof dr hab. Tomasz Śliwiński

.....

Prof. dr hab. Tomasz Śliwiński

mgr Monika Toma
Pracowni Genetyki Medycznej
Uniwersytet Łódzki
ul. Pomorska 141/143
90-236 Łódź

16.01.2020 r.

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mgr Monika Toma

mgr Monika Toma

Katherine Sullivan-Reed, PhD
Sol Sherry Thrombosis Research Center and
Fels Institute for Cancer Research and Molecular Biology,
Lewis Katz School of Medicine
Temple University
Philadelphia, PA, USA

16.01.2020 r.

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Declaration of co-authorship

Regarding the following publication

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I have contributed to the publication by editing the manuscript and I estimate that my overall participation in the article is 3%.

Katherine Sullivan-Reed, PhD

prof. dr hab. Tomasz Śliwiński
Kierownik Pracowni Genetyki Medycznej
Uniwersytet Łódzki
ul. Pomorska 141/143
90-236 Łódź

20.01.2020 r.

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Oświadczam, że w pracy

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KIEROWNIK
PRACOWNI GENETYKI MEDYCZNEJ
Wydział Biologii i Ochrony Środowiska UŁ

prof dr hab Tomasz Śliwiński

.....
Prof. dr hab. Tomasz Śliwiński

Tomasz Skorski, MD, PhD, DSc
Sol Sherry Thrombosis Research Center and
Fels Institute for Cancer Research and Molecular Biology,
Lewis Katz School of Medicine
Temple University
Philadelphia, PA, USA

16.01.2020 r.

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.....

Tomasz Skorski, MD, PhD, DSc

prof. dr hab. n. med. Małgorzata Czyż
Zakład Biologii Molekularnej Nowotworów
Uniwersytet Medyczny w Łodzi
ul. Mazowiecka 6/8
92-215 Łódź

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Oświadczenie o udziale w publikacjach

Oświadczam, że praca

Czyż M*, Toma M*, Gajos-Michniewicz A, Majchrzak K, Hoser G, Szemraj J, Nieborowska-Skorska M, Cheng P, Gritsyuk D, Mitchell Levesque, Dummer R, Śliwiński T, Skorski T. PARP1 inhibitor olaparib (Lynparza) exerts synthetic lethal effect against ligase 4-deficient melanomas. Oncotarget 2016; 7(46): 75551-75560

jest publikacją o równorzędnym pierwszym autorstwie, a mój udział polegał na tworzeniu koncepcji badań, projektowaniu eksperymentów, analizie wyników oraz tworzeniu manuskryptu. Swój udział w artykule oceniam na 35%.

.....

prof. dr hab. n. med. Małgorzata Czyż

mgr Monika Toma
Pracowni Genetyki Medycznej
Uniwersytet Łódzki
ul. Pomorska 141/143
90-236 Łódź

16.01.2020 r.

Oświadczenie o udziale w publikacjach

Oświadczam, że praca

Czyż M*, **Toma M***, Gajos-Michniewicz A, Majchrzak K, Hoser G, Szemraj J, Nieborowska-Skorska M, Cheng P, Gritsyuk D, Mitchell Levesque, Dummer R, Śliwiński T, Skorski T. PARP1 inhibitor olaparib (Lynparza) exerts synthetic lethal effect against ligase 4-deficient melanomas. Oncotarget 2016; 7(46): 75551-75560

jest publikacją o równorzędnym pierwszym autorstwie, a mój udział polegał na wykonaniu analizy RealTime PCR, analizy Western Blot, testu kometowego, badania poziomu ufosforylowanego histonu γH2A.X oraz na analizie wyników. Swój udział w artykule oceniam na 35%.

mgr Monika Toma

mgr Monika Toma

prof. dr hab. n. med. Janusz Szemraj
Zakład Biochemii Medycznej
Uniwersytet Medyczny w Łodzi
ul. Mazowiecka 6/8
92-215 Łódź

16.01.2020 r.

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy

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mój udział polegał na współudziale w wykonaniu analizy RealTime PCR, analizy Western Blot oraz na analizie danych. Swój udział w artykule oceniam na 1%.

KIEROWNIK
Katedry i Zakładu Biochemii Medycznej
Uniwersytetu Medycznego w Łodzi
Prof. dr hab. n. med. Janusz Szemraj

.....
prof. dr hab. n. med. Janusz Szemraj

prof. dr hab. Tomasz Śliwiński
Kierownik Pracowni Genetyki Medycznej
Uniwersytet Łódzki
ul. Pomorska 141/143
90-236 Łódź

20.01.2020 r.

Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy

Czyż M*, Toma M*, Gajos-Michniewicz A, Majchrzak K, Hoser G, Szemraj J, Nieborowska-Skorska M, Cheng P, Gritsyuk D, Mitchell Levesque, Dummer R, **Śliwiński T**, Skorski T. PARP1 inhibitor olaparib (Lynparza) exerts synthetic lethal effect against ligase 4-deficient melanomas. Oncotarget 2016; 7(46): 75551-75560

mój udział polegał na pozyskiwaniu funduszy, tworzeniu koncepcji projektu, projektowaniu eksperymentów, analizie wyników oraz tworzeniu manuskryptu. Swój udział w artykule oceniam na 10%.

KIEROWNIK
PRACOWNI GENETYKI MEDYCZNEJ
Wydział Biologii i Ochrony Środowiska UŁ
prof dr hab. Tomasz Śliwiński

.....
Prof. dr hab. Tomasz Śliwiński

Tomasz Skorski, MD, PhD, DSc
Sol Sherry Thrombosis Research Center and
Fels Institute for Cancer Research and Molecular Biology,
Lewis Katz School of Medicine
Temple University
Philadelphia, PA, USA

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.....

Tomasz Skorski, MD, PhD, DSc

mgr Monika Toma
Pracowni Genetyki Medycznej
Uniwersytet Łódzki
ul. Pomorska 141/143
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Oświadczenie o udziale w publikacjach

Oświadczam, że w pracy

Toma M, Witusik-Perkowska M, Szwed M, Stawski R, Szemraj J, Drzewiecka M, Nieborowska-Skorska M, Radek M, Kolasa M, Matlawska-Wasowska K, Śliwiński T, Skorski T. Eradication of LIG4-deficient glioblastoma cells by the combination of PARP inhibitor and alkylating agent. 2018; 9(96):36867-36877

mój udział polegał na prowadzeniu hodowli komórkowych linii glejaków, badaniu utraty heterozygotyczności (LOH), przeprowadzeniu analizy RealTime PCR oraz Western Blot, analizie danych cytometrycznych, przeprowadzeniu testu klonogenności komórek, transfekcji komórek glejaka wektorem kodującym ligazę IV oraz na analizie otrzymanych danych. Swój udział w artykule oceniam na 66%.

mgr Monika Toma

mgr Monika Toma

Dr n. med. Monika Witusik-Perkowska
Zakład Biochemii Medycznej
Uniwersytet Medyczny w Łodzi
ul. Mazowiecka 6/8
92-215 Łódź

20.01.2020 r.

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mój udział polegał na hodowli linii glejaka i wykonaniu testu kometowego. Swój udział w artykule oceniam na 3%.

Dr n. med. Monika Witusik-Perkowska

prof. dr hab. n. med. Janusz Szemraj
Zakład Biochemii Medycznej
Uniwersytet Medyczny w Łodzi
ul. Mazowiecka 6/8
92-215 Łódź

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Toma M, Witusik-Perkowska M, Szwed M, Stawski R, **Szemraj J**, Drzewiecka M, Nieborowska-Skorska M, Radek M, Kolasa M, Matlawska-Wasowska K, Śliwiński T, Skorski T. Eradication of LIG4-deficient glioblastoma cells by the combination of PARP inhibitor and alkylating agent. 2018; 9(96):36867-36877

mój udział polegał na współudziale w wykonaniu analizy RealTime PCR oraz na analizie danych. Swój udział w artykule oceniam na 2%.

KIEROWNIK
Katedry i [illegible] Medycznej
Uniwer[illegible] w Łodzi
Prof. dr hab. n. med. Janusz Szemraj

.....
prof. dr hab. n. med. Janusz Szemraj

prof. dr hab. Tomasz Śliwiński
Kierownik Pracowni Genetyki Medycznej
Uniwersytet Łódzki
ul. Pomorska 141/143
90-236 Łódź

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mój udział polegał na pozyskiwaniu funduszy, tworzeniu koncepcji projektu, projektowaniu eksperymentów, analizie wyników oraz tworzeniu manuskryptu. Swój udział w artykule oceniam na 10%.

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Tomasz Skorski, MD, PhD, DSc