POMORSKI UNIWERSYTET MEDYCZNY
W SZCZECINIE

Lek. Piotr Kulig

Epigenetyczne aspekty mechanizmu działania bortezomibu, mechanizmów rozwoju lekooporności oraz implikacje kliniczne dla rozwoju potencjalnych terapii adjuwantowych.

Rozprawa doktorska w dziedzinie nauk medycznych i nauk o zdrowiu

Dyscyplina nauki medyczne

Promotor: *dr hab. n. med. Bartłomiej Baumert*

Promotor pomocniczy: *prof. dr hab. n. med. Bogusław Machaliński*

Szczecin 2024

Spis treści

Wykaz skrótów

AZA – azacytydyna

BTZ – bortezomib

DAC – decytabina

DNMT – metylotransferaza DNA

GSEA – gene set enrichment analysis

HDACs – deacetylazy histonowe

IGR – region międzygenowy

IMiDs – leki immunomodulujące

lncRNA – długie niekodujące DNA

MGUS – gammapatia monoklonalna o nieustalonym znaczeniu

miRNA – mikro RNA

MM – szpiczak plazmocytowy

ncRNA – niekodujące RNA

NES – normalized enrichment score

PI – inhibitory proteasomu

piRNA – RNA oddziaływujące na białka piwi

siRNA – małe interferujące RNA

SMM – szpiczak tlący

srRNA – małe regulatorowe RNA

TSS – miejsce inicjacji transkrypcji

VD – 25(OH)D3

VDR – receptor dla witaminy D

VK – witamina K

Wykaz publikacji włączonych do rozprawy doktorskiej

Założenia rozprawy doktorskiej zrealizowano na podstawie cyklu publikacji składającego się z trzech spójnych tematycznie artykułów naukowych, w tym jednej pracy poglądowej (Publikacja 1) oraz dwóch prac oryginalnych (Publikacje 2 i 3). Prace zostały opublikowane w latach 2023-2024. Cykl jest zatytułowany „Epigenetyczne aspekty mechanizmu działania bortezomibu, mechanizmów rozwoju lekooporności oraz implikacje kliniczne dla rozwoju potencjalnych terapii adjuwantowych". W jednej publikacji doktorant jest pierwszym autorem (Publikacja 1), natomiast w dwóch pozostałych publikacjach, równorzędnym, pierwszym autorem (Publikacje 2 i 3).

1. Kulig, P.; Łuczkowska, K.; Bakinowska, E.; Baumert, B.; Machaliński, B. Epigenetic Alterations as Vital Aspects of Bortezomib Molecular Action. *Cancers* 2024, 16, 84. https://doi.org/10.3390/cancers16010084. IF 5.2, MEiN 200 pkt.
2. Łuczkowska, K.*; Kulig, P.*; Rusińska, K.; Baumert, B.; Machaliński, B. 5-Aza-2′-Deoxycytidine Alters the Methylation Profile of Bortezomib-Resistant U266 Multiple Myeloma Cells and Affects Their Proliferative Potential. *Int. J. Mol. Sci.* 2023, 24, 16780. https://doi.org/10.3390/ijms242316780. IF 5.6, MEiN 140 pkt.

3. Łuczkowska, K.*; Kulig, P.*; Baumert, B.; Machaliński, B. Vitamin D and K Supplementation Is Associated with Changes in the Methylation Profile of U266-Multiple Myeloma Cells, Influencing the Proliferative Potential and Resistance to Bortezomib. *Nutrients* 2024, 16, 142. https://doi.org/10.3390/nu16010142. IF 5.9, MEiN 140 pkt.

   * Dwaj pierwsi autorzy mają równorzędny wkład w pracę

   Sumaryczny współczynnik Impact Factor: 16,7

   Sumaryczna liczba punktów Ministerstwa Edukacji i Nauki: 480

Wstęp

Szpiczak plazmocytowy (ang. multiple myeloma, MM) jest nowotworem wywodzącym się z plazmocytów. Choroba najczęściej charakteryzuje się naciekiem szpiku kostnego przez klonalne komórki plazmatyczne, któremu zwykle towarzyszy obecność białka monoklonalnego, tj. immunoglobulin lub ich fragmentów w postaci lekkich lub ciężkich łańcuchów wykrywanych we krwi i/lub w moczu (*1*). Szacuje się, że zaledwie około 3% wszystkich przypadków MM stanowi szpiczak niewydzielający (*2*). Typowo choroba rozwija się ze stanu przednowotworowego, gammapatii monoklonalnej o nieustalonym znaczeniu (ang. monoclonal gammopathy of undetermined significance, MGUS) (*3*), która ewoluuje poprzez szpiczaka tlącego (ang. smoldering multiple myeloma, SMM) do pełnoobjawowego MM. Ryzyko transformacji z MGUS do objawowego, wymagającego leczenia MM wynosi około 1% rocznie (*4*). Choroba jest istotnym problemem klinicznym, gdyż ujmując ją epidemiologicznie stanowi około 10% wszystkich nowotworów hematologicznych oraz 1% wszystkich nowotworów złośliwych (*5*). W Polsce, według danych Narodowego Funduszu Zdrowia, rocznie diagnozuje się około 2600 nowych przypadków MM (*6*). Zapadalność na MM w Europie szacuje się na poziomie 4,5-6/100 000/rok (*7*).

Patogeneza MM jest złożona i wciąż nie do końca poznana. Obecnie postuluje się istnienie wielu mechanizmów przyczyniających się do wystąpienia i rozwoju choroby. Dowiedziono, że translokacje obejmujące *locus* ciężkiego łańcucha immunoglobulin (IgH) oraz dysregulacja genów kodujących cykliny D są kluczowymi zjawiskami w patogenezie MM na poziomie molekularnym. Warto przy tym zauważyć, że zaburzenie sygnalizacji zależnej od cyklin D jest wczesnym zdarzeniem inicjującym w onkogenezie, niezależnie od ploidii klonalnych plazmocytów (*8, 9*).

Symptomatologia choroby jest zróżnicowana. Początkowe objawy mogą być niespecyficzne, w tym należące do kręgu tzw. objawów ogólnych. Wśród nich można wymienić utratę masy ciała, poty nocne, bóle kostne oraz uogólnione osłabienie (*10*). Typową manifestację kliniczną MM dobrze obrazuje akronim CRAB (C - calcium; R – renal/ kidney failure; A – anemia; B – bone lesions). Uszkodzenia narządowe objawiają się hiperkalcemią, niewydolnością nerek, niedokrwistością oraz zmianami kostnymi o charakterze osteolitycznym (*11*). Hiperkalcemia oraz osteoliza wynikają ze zwiększonej resorpcji kostnej na skutek nadmiernej rekrutacji osteoklastów, aktywowanych przez komórki MM, co w konsekwencji często doprowadza do wystąpienia złamań patologicznych (*12*). Patogeneza niewydolności nerek w przebiegu MM jest wieloczynnikowa. Wśród głównych czynników należy wymienić nefrotoksyczny wpływ wapnia oraz białka monoklonalnego, szczególnie łańcuchów lekkich immunoglobulin oraz odkładanie się złogów amyloidu w przebiegu współistniejącej amyloidozy (*13*). Niedokrwistość jest skutkiem nacieku szpiku kostnego przez dyskrastyczne plazmocyty i wyparcia hematopoezy, a także może wynikać z niedoborów erytropoetyny, hemolizy czy przewlekłego stanu zapalnego (*14*).

Początkowo szpiczak plazmocytowy był chorobą o bardzo złym rokowaniu. Jednak rozwój nowoczesnych terapii znacząco zmienił perspektywy w tej grupie chorych i przyczynił się do istotnego polepszenia rokowania (*15*). Pierwszym kamieniem milowym w terapii MM było ponowne odkrycie dla medycyny talidomidu, leku pierwotnie stosowanego przeciwwymiotnie oraz nasennie u kobiet w ciąży, a następnie wycofanego z powodu teratogenności. Talidomid obecnie zaliczany jest do leków immunomodulujących (ang. immunomodulating drugs, IMiDs) I generacji (*16*). Mimo wysokiej skuteczności w leczeniu MM, terapia talidomidem jest związana z szeregiem reakcji niepożądanych. Do najczęstszych powikłań zaliczamy toksyczność hematologiczną, incydenty zakrzepowo-zatorowe oraz rozwój neuropatii obwodowej, które często skutkują koniecznością zaprzestania leczenia (*17*). Obecnie dostępne są IMiDs kolejnych generacji, lenalidomidem oraz pomalidomidem, które znacznie rzadziej przyczyniają się występowania neuropatii obwodowej, przy jednoczesnym zachowaniu wysokiej skuteczności przeciwnowotworowej (*18*, *19*). Obie wspomniane cząsteczki są dziś powszechnie wykorzystywane w wielu złożonych schematach terapeutycznych.

Kolejnym punktem zwrotnym w terapii MM było opracowanie następnej grupy leków, inhibitorów proteasomu (ang. proteasome inhibitors, PIs). Bortezomib (BTZ) to PI I generacji, zmodyfikowana pochodna kwasu borowego, będąca selektywnym

i odwracalnym inhibitorem podjednostki 26S proteasomu, regulująca wewnątrzkomórkową degradację białek (*20*). Precyzyjniej opisując mechanizm działania, BTZ przyłącza się do podjednostki 20S proteasomu, przez co blokuje degradację peptydów znakowanych ubikwityną (*21*). Do kolejnej generacji PIs należą karfilzomib oraz iksazomib. Iksazomib jest jedynym obecnie PI, który może być przyjmowany doustnie (*22*, *23*). Blokada molekularnych szlaków, zaangażowanych w ubikwitynozależną degradację białek, powoduje wewnątrzkomórkową akumulację nieprawidłowo sfałdowanych i defektywnych polipeptydów, co ostatecznie prowadzi do skierowania komórki na drogę zaprogramowanej śmierci, apoptozy (*20*, *24*). BTZ jest najlepiej przebadanym PI, który może być podawany zarówno dożylnie jak i podskórnie. Wykazano, że obie drogi podania leku są równie efektywne. Niemniej jednak, preferowana jest podaż podskórna z powodu większej wygody oraz mniejszej liczby powikłań (*25*). Klirens leku po dostaniu się do krwiobiegu jest bardzo wydajny, a BTZ bardzo szybko przemieszcza się do wnętrza komórek. Należy jednak pamiętać, że eliminacja leku z organizmu jest powolna. Szacuje się, że okres półtrwania cząsteczki przekracza 10 godzin (*20*). BTZ ulega wątrobowemu metabolizmowi przez kompleks enzymatyczny cytochromu P450 do nieaktywnych metabolitów (*26*, *27*), a następnie jest wydalany zarówno drogą nerkową, jak i wraz z żółcią (*28*). Dostosowywanie dawki do stopnia wydolności nerek nie jest potrzebne (*29*). BTZ cechuje się względnie wysoką skutecznością kliniczną, jednak jak każdy lek, nie jest pozbawiony działań niepożądanych. Przeprowadzono wiele badań klinicznych 1/2 fazy, oceniających bezpieczeństwo stosowania BTZ w różnych wskazaniach, ze szczególnym uwzględnieniem MM. Najczęściej raportowane działania niepożądane BTZ to objawy żołądkowo-jelitowe, zmęczenie, neuropatia obwodowa, trombocytopenia oraz neutropenia (*30–36*). Dotychczas nie opisano w literaturze przypadków skumulowanej toksyczności leku (*37–39*). Bezpieczeństwo stosowania BTZ było oceniane zarówno w monoterapii, jak i w schematach wielolekowych, w nowotworach hematologicznych (*40–42*) oraz guzach litych, takich jak rak płuca, piersi i jajnika (*43–46*). Obecnie BTZ ma rejestrację w terapii MM oraz chłoniaka z komórek płaszcza (*47*). Główny mechanizm działania BTZ, tj. hamowanie proteasomu oraz cytotoksyczność spowodowana wewnątrzkomórkową akumulacją białek, są szczegółowo opisane w literaturze. Znacznie mniej wiadomo na temat epigenetycznych aspektów działania leku.

Epigenetyka jest subdyscypliną genetyki, która zajmuje się badaniem mechanizmów regulujących ekspresję informacji genetycznej, niezależnych od zmiany sekwencji nukleotydów. Dotychczas wyróżniono cztery zasadnicze mechanizmy epigenetyczne:

metylację DNA, metylacją i acetylację histonów, remodeling chromatyny oraz niekodujące RNA (*48*). Powyższe zmiany mogą być wywoływane przez bodźce środowiskowe, m.in. poprzez ekspozycję na leki (*49*). Modyfikacje epigenetyczne są potencjalnie odwracalne, co jest szczególnie istotne w kontekście rozwoju nowych strategii terapeutycznych (*50*). Już dziś istnieją zarejestrowane leki, które bezpośrednio interferują z modyfikacjami epigenetycznymi. Taką sztandarową cząsteczką jest azacytyda (AZA), lek hipometylujący, obecnie powszechnie wykorzystywany w leczeniu schorzeń hematologicznych, zwłaszcza ostrej białaczki szpikowej i zespołów mielodysplastycznych (*51*, *52*).

Aktualnie wydaje się, że to immunoterapia odgrywa kluczową rolę w leczeniu MM (*53*). Wiele leków wpływających na funkcję układu odpornościowego jest wykorzystywanych w połączeniu z BTZ, co skutkuje większą skutecznością kliniczną. Poza wspomnianymi już i powszechnie stosowanymi IMiDs, obecnie niezmiernie ważną rolę odgrywają m.in. przeciwciała monoklonalne (*54*), przeciwciała bispecyficzne (*55*) oraz limfocyty CAR-T (*56*, *57*). Oprócz klasycznych leków wykorzystywanych w nowoczesnej terapii MM do dyspozycji lekarzy klinicystów jest szereg związków o działaniu immunomodulującym, w ramach tzw. terapii adjuwantowej. Na szczególną uwagę zasługują tu witaminy 25(OH)D3 (ang. vitamin D, VD) oraz VK2MK7 (ang. vitamin K, VK) ze względu na ich plejotropowe, w tym immunomodulujące działania (*58*) oraz szeroką dostępność.

Cele pracy

Mając na uwadze powyższe zagadnienia celem niniejszej pracy było:

- poszerzenie wiedzy na temat epigenetycznych aspektów mechanizmu działania BTZ na komórki MM,
- zdefiniowanie, zależnych od modyfikacji epigenetycznych, mechanizmów rozwoju lekooporności na BTZ,
- poszukiwanie potencjalnych związków w ramach terapii adjuwantowej, zwiększających skuteczność BTZ w terapii szpiczaka plazmocytowego.

Metodologia

Publikacja 1

Pierwsza praca z cyklu jest artykułem poglądowym. Selekcja artykułów została przeprowadzona poprzez przeszukanie następujących repozytoriów literatury naukowej: PubMed, PubMed Central, Scopus, Web of Science, Embase oraz Google Scholar. Wykorzystano następujące słowa kluczowe: bortezomib epigenetics; bortezomib methylation; bortezomib RNA; bortezomib miRNA; bortezomib non-

coding RNA; bortezomib circular RNA; bortezomib chromatin remodeling; proteasome inhibitor epigenetics; proteasome inhibitor methylation, proteasome inhibitor RNA; proteasome inhibitor miRNA; proteasome inhibitor non-coding RNA; proteasome inhibitor circular RNA; proteasome inhibitor chromatin remodeling. W publikacji zostały uwzględnione wszystkie prace oryginalne traktujące o epigenetycznych aspektach mechanizmu działania BTZ, rozwoju oporności na BTZ oraz przezwyciężaniu oporności na BTZ opublikowanych do 2023 roku.

Publikacja 2

Do badania wykorzystano komórki MM linii U266 (ATCC, Manassas, VA, USA). Do hodowli wykorzystano medium RPMI-1640 (ATCC, Manassas, VA, USA, cat no. 30-2001) z dodatkiem 2 mM L-glutaminy, 10 mM HEPES, 1 mM pirogronianu sodu, 4500 mg/L glukozy, 1500 mg/L wodorowęglanu sodu oraz 15% surowicy wołowej. Medium zmieniano co trzy dni.

Komórki MM linii U266 były inkubowane z BTZ w stężeniu 2,75 nM (Cell Signaling Technology, Danvers, MA, USA) oraz inhibitorem metylacji, 5-Aza-2-deoksycytydyną (AZA), w stężeniu 1μM (Sigma Aldrich, St. Louis, MO, USA) trzykrotnie przez okres dwudziestu czterech godzin w dziesięciodniowych interwałach. Szczegółowy protokół doboru dawki BTZ oraz ustalenie linii MM opornej na lek jest opisany w artykule autorstwa naszego zespołu, niebędącym częścią niniejszego cyklu (*59*).

Szczegółowy protokół ustalenia dawki AZA jest opisany w sekcji Materiały i Metody niniejszej publikacji.

Przeprowadzono izolację DNA oraz analizę proliferacji po każdej inkubacji komórek z BTZ lub AZA. Dodatkowo, aby sprawdzić, czy zmiany epigenetyczne wywołane ekspozycją na leki są przekazywane komórkom potomnym, wyizolowano DNA z komórek pasażowanych do medium pozbawionego BTZ oraz AZA, w którym pozostawały przez 10 dni po trzeciej inkubacji z w/w lekami.

Przeprowadzono izolację DNA z wykorzystaniem zestawu PureLink Genomic DNA Mini Kit (Thermo Fisher, Waltham, MA, USA). Szczegółowy opis procedury znajduje się w sekcji Materiały i Metody niniejszej publikacji.

Analizę mikromacierzy metylacji wykonano przy użyciu zestawu Infinium Methylation EPIC BeadChip kit (Illumina, San Diego, CA, USA) zgodne z instrukcją

producenta. Do skanowania macierzy wykorzystano urządzenie NextSeq550 (Illumina, San Diego, CA, USA).

Wykonano dokładną analizę bioinformatyczną z wykorzystaniem środowiska do obliczeń statystycznych R, implementując odpowiednie pakiety. Szczegółowy opis analizy bioinformatycznej znajduje się w sekcji Materiały i Metody niniejszej publikacji.

Publikacja 3

Do badania wykorzystano komórki MM linii U266 (ATCC, Manassas, VA, USA). Do hodowli wykorzystano medium RPMI-1640 (ATCC, Manassas, VA, USA, cat no. 30-2001) z dodatkiem 2 mM L-glutaminy, 10 mM HEPES, 1 mM pirogronianu sodu, 4500 mg/L glukozy, 1500 mg/L wodorowęglanu sodu oraz 15% surowicy wołowej. Medium zmieniano co trzy dni.

Komórki MM linii U266 były inkubowane z BTZ w stężeniu 2,75 nM (Cell Signaling Technology, Danvers, MA, USA) trzykrotnie przez okres dwudziestu czterech godzin w dziesięciodniowych interwałach.

Szczegółowy protokół doboru dawki BTZ oraz ustalenie linii MM opornej na lek jest opisany w artykule autorstwa naszego zespołu, niebędącym częścią niniejszego cyklu (*59*).

Analizę mikromacierzy metylacji wykonano przy użyciu zestawu Infinium Methylation EPIC BeadChip kit (Illumina, San Diego, CA, USA) zgodne z instrukcją producenta. Do skanowania macierzy wykorzystano urządzenie NextSeq550 (Illumina, San Diego, CA, USA).

Wykonano dokładną analizę bioinformatyczną z wykorzystaniem środowiska do obliczeń statystycznych R, implementując odpowiednie pakiety. Szczegółowy opis analizy bioinformatycznej znajduje się w sekcji Materiały i Metody niniejszej publikacji.

Wyniki zostały zwalidowane przy pomocy qRT-PCR (Thermo Fisher Scientific, Waltham, MA, USA; Bio-Rad, Hercules, California, USA). Szczegółowy opis walidacji jest zawarty w niniejszej publikacji.

Wyniki

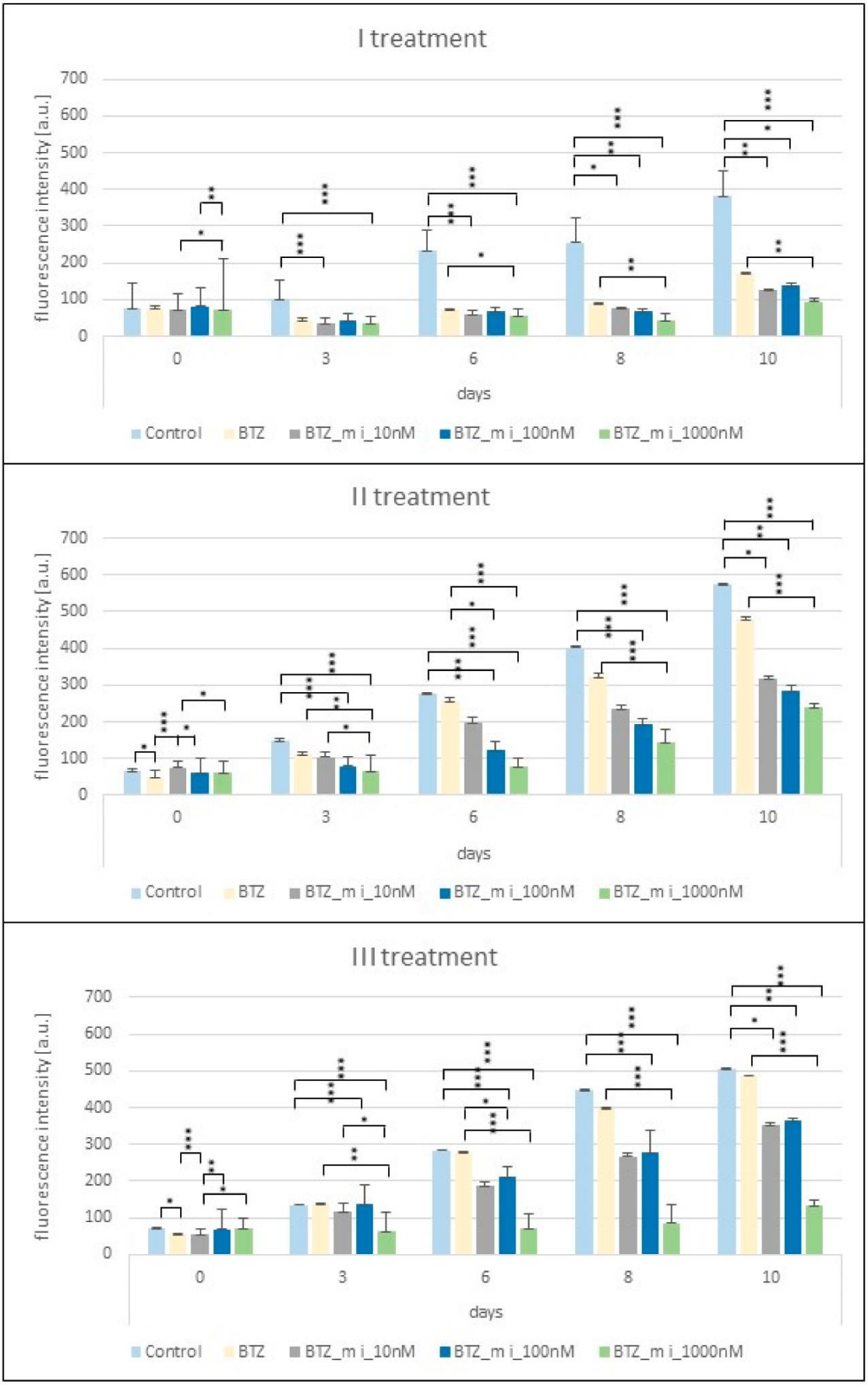
Publikacja 1

Publikacja nr 1 jest artykułem poglądowym. Wnioski na podstawie przeglądu literatury zostały zawarte w sekcji Dyskusja.

Publikacja 2

Analiza potencjału proliferacyjnego

Analiza potencjału proliferacyjnego komórek MM linii U266 wykazała, że po trzecim pasażu, komórki inkubowane z BTZ wykształciły oporność na lek. Ich poziom proliferacji był zbliżony do kontrolnych komórek linii U266 ($p>0,05$). Następnie, oporne na BTZ komórki linii U266 traktowano jednocześnie BTZ i inhibitorem metylacji (AZA), aby sprawdzić jego wpływ rozwój fenotypu opornego. Uzyskane wyniki wyraźnie wskazują na spadek proliferacji wraz ze wzrostem dawki AZA. Po pierwszym pasażu, gdy fenotyp komórek U266 nie był jeszcze oporny na BTZ, różnice w poziomie proliferacji sięgały kilku procent przy porównaniu różnych dawek AZA. Najniższy poziom proliferacji zaobserwowano przy zastosowaniu najwyższej dawki AZA tj. 1000 nM. Po drugim traktowaniu wyraźnie zaobserwowano malejący poziom proliferacji po zastosowaniu AZA, zarówno w stosunku do komórek kontrolnych, jak i komórek inkubowanych z BTZ. Dodatkowo, AZA w dawce 1000 nM najskuteczniej spowalniała proliferację komórek. Trzykrotne inkubowanie komórek szpiczaka AZA w dawce 1000 nM zmniejszyło proliferację komórek opornych na BTZ o około 72% ($p<0,0004$). Na podstawie uzyskanych wyników do dalszych części eksperymentu wybrano dawkę 1000 nM AZA.



Rycina 1. Analiza potencjału proliferacyjnego komórek szpiczaka plazmocytowego linii U266. Wykresy przedstawiają poziom proliferacji komórek U266 po każdym

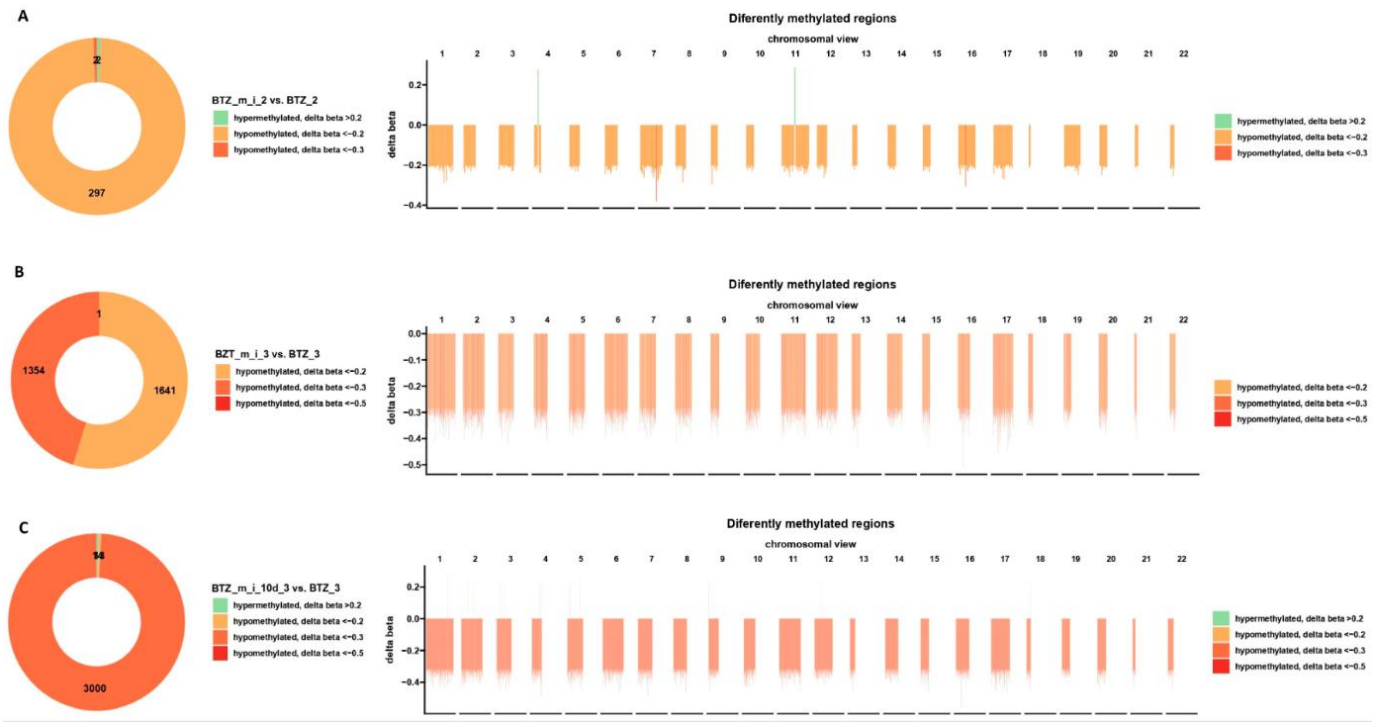
traktowaniu BTZ i/lub inhibitorem metylacji (BTZ_m i). Dane nie miały rozkładu normalnego, w związku z tym różnice między grupami analizowano za pomocą testu Kruskala–Wallisa. Analiza post-hoc została wykonana przy zastosowaniu testu Dunna z poprawką Bonferroniego dla wielokrotnych porównań. $p < 0,05$ uznano za istotne statystycznie; * $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$. Control – komórki kontrolne; BTZ – bortezomib; BTZ_m i_ 10nM – bortezomib oraz 10nM inhibitora metylacji; BTZ_m i_ 100nM – bortezomib oraz 100nM inhibitora metylacji; BTZ_m i_ 1000nM – bortezomib oraz 1000nM inhibitora metylacji (źródło: Publikacja nr 2)

Analiza profilu metylacji

Po analizie potencjału proliferacyjnego wykonano analizę metylomu. Po pierwszym traktowaniu nie zaobserwowano istotnych zmian w profilu metylacji, dlatego przedstawiono wyniki uzyskane po drugim (BTZ_m_i_2 i BTZ_2) i trzecim (BTZ_m_i_3 i BTZ_3) pasażu komórek U266. Ponadto, przeprowadzono analizę 10 dni po trzeciej inkubacji (BTZ_m_i_10d_3). W tym czasie nie dodawano nic do medium hodowlanego, aby sprawdzić, czy powstałe zmiany w profilu metylacji były trwałe i zostały przeniesione na komórki potomne, pomimo braku BTZ oraz AZA w medium hodowlanym.

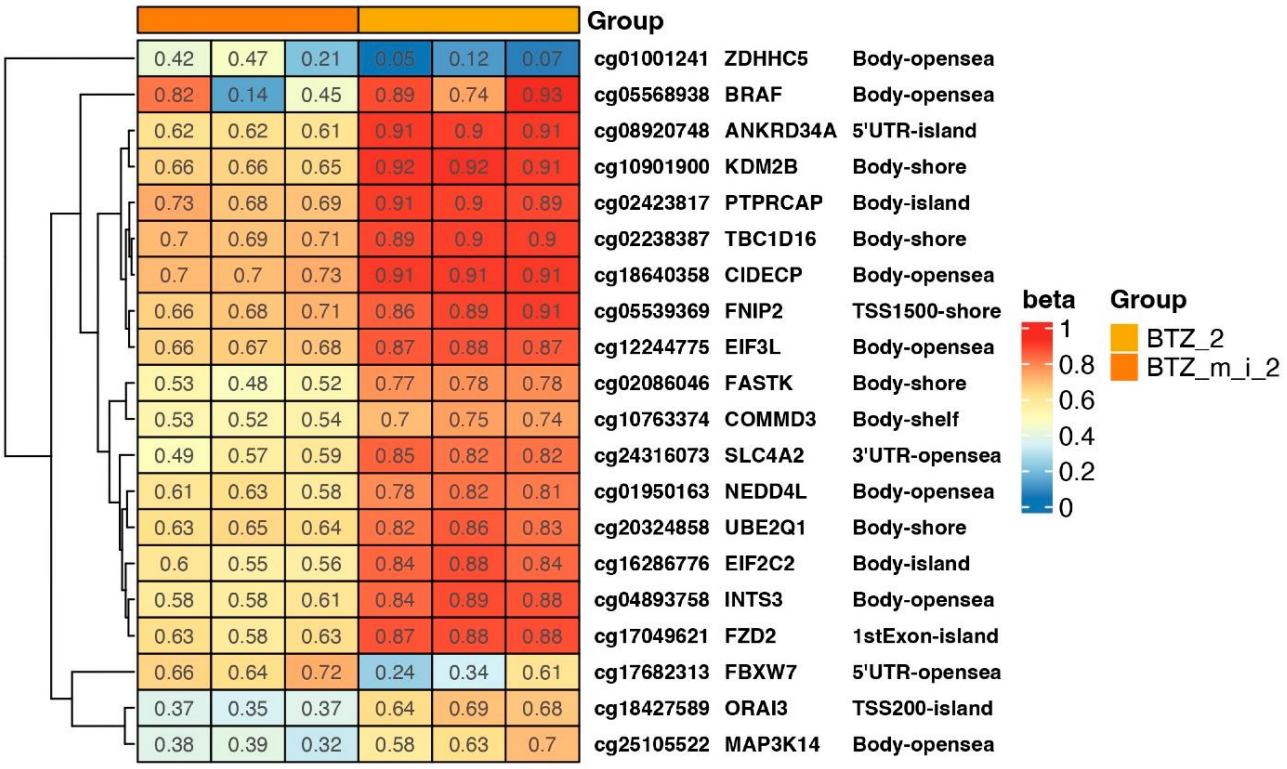
Analiza profilu metylacji po drugim pasażu

Analiza bioinformatyczna wykazała 301 miejsc (299 hipometylowanych i 2 hipermetylowane) ze zmienioną metylacją w komórkach szpiczaka dwukrotnie traktowanych BTZ i inhibitorem metylacji w porównaniu z komórkami traktowanymi dwukrotnie samym BTZ (Ryc. 2A). Wartości beta delta pokazane na wykresach otrzymano poprzez obliczenie stosunku znormalizowanych wartości intensywności fluorescencji sondy pomiędzy sygnałami metylowanymi i niemetylowanymi (0 = całkowicie niemetylowany; 1 = całkowicie metylowany). Rozkład zmian metylacji pokazano osobno na każdym chromosomie na rycinie 2A. Hipermetylację zaobserwowano tylko na chromosomach 4 i 11. Co więcej, analiza bioinformatyczna wykazała 2996 miejsc o zmienionej metylacji (zmiany wyłącznie o charakterze hipometylacji) w komórkach MM trzykrotnie traktowanych BTZ i inhibitorem metylacji w porównaniu z komórkami traktowanymi trzykrotnie samym BTZ (Ryc. 2B). Liczba zmian w poziomie metylacji wzrosła prawie 10-krotnie po trzecim traktowaniu w porównaniu do wyników uzyskanych po drugiej inkubacji (drugie traktowanie: 301 zmienionych miejsc vs trzecie traktowanie: 2996 zmienionych miejsc).



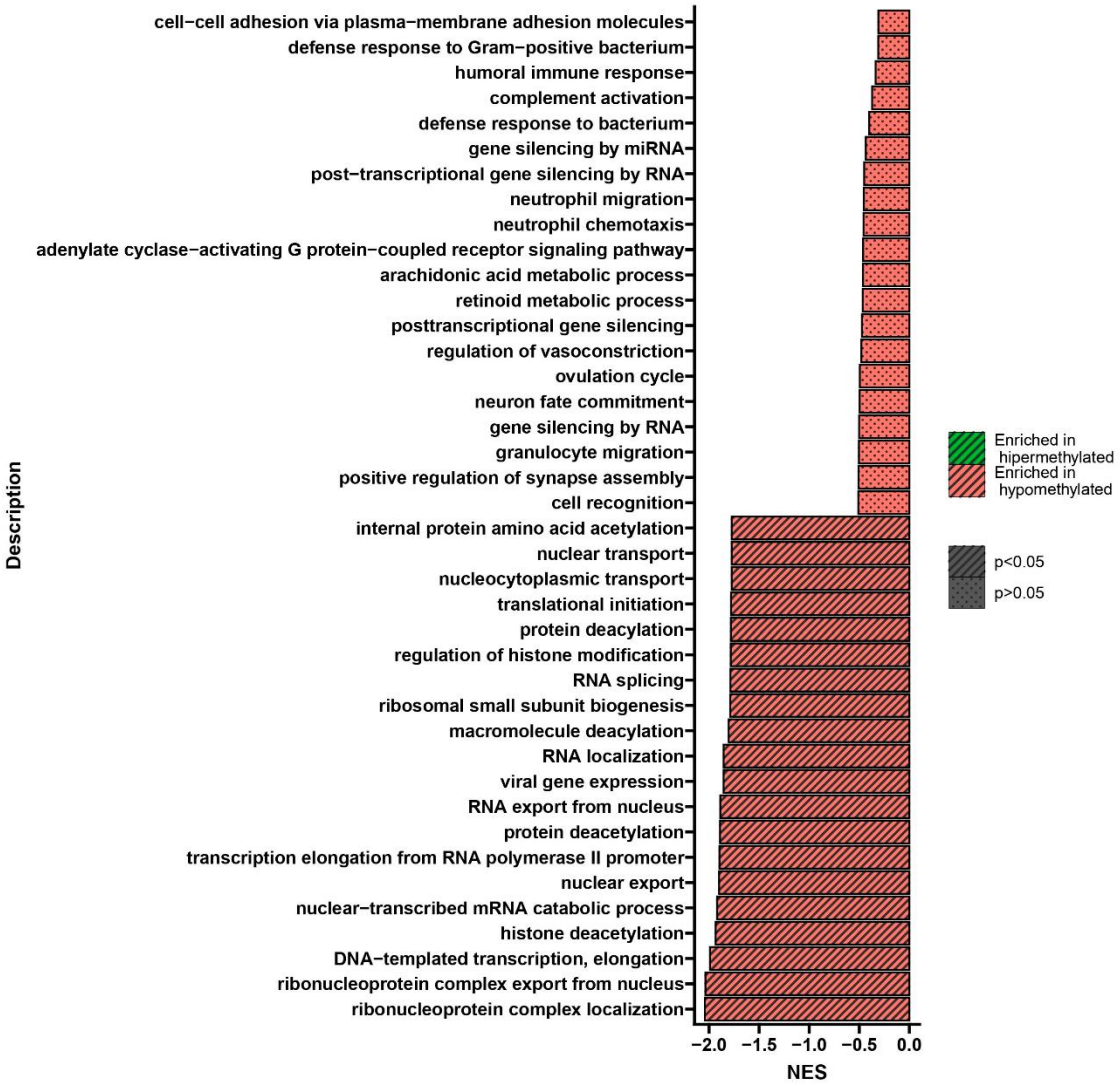
Rycina 2. Analiza profilu metylacji. Wykresy przedstawiają profil metylacji DNA komórek szpiczaka U266 po dwóch (A) i trzech (B) równoczesnych inkubacjach z BTZ i inhibitorem metylacji (BTZ_m_i) w porównaniu z komórkami traktowanymi samym BTZ (BTZ). Dodatkowo wykres (C) przedstawia profil metylacji DNA 10 dni po ostatnim traktowaniu. Wykresy kołowe pokazują różnice w poziomach metylacji w komórkach U266 traktowanych BTZ i inhibitorem metylacji w porównaniu z komórkami traktowanymi BTZ ($p<0,05$). Ponadto, przedstawiono rozkład zmian metylacji DNA na poszczególnych chromosomach (kolor pomarańczowy oznacza hipometylację, kolor zielony hipermetylację; $p < 0,05$). BTZ – bortezomib; BTZ_2 – bortezomib, drugie traktowanie; BTZ_m_i – bortezomib i inhibitor metylacji; BTZ_m_i_2 – bortezomib i inhibitor metylacji, drugie traktowanie; BTZ_3 – bortezomib, trzecie traktowanie; BTZ_m_i_3 – bortezomib i inhibitor metylacji, trzecie traktowanie; BTZ_m_i_10d_3 – bortezomib i inhibitor metylacji 10 dni po trzecim traktowaniu; (źródło: Publikacja nr 2)

Zmiany w metylacji po dwukrotnym traktowaniu komórek U266 BTZ (BTZ_2) lub BTZ i inhibitorem metylacji (BTZ_m_i_2) w wybranych genach przedstawiono na heatmapie (Ryc. 3). Najciekawsze zmiany w metylacji, z punktu widzenia rozwoju oporności, zaobserwowano w genach *FBXW7* oraz *ORAI3*. Zaobserwowane zmiany w w/w genach mogą w istotny sposób wpływać na rozwój oporności na BTZ.



Rycina 3. 20 genów z największymi zmianami w poziomie metylacji. Heatmapa przedstawia wartości beta reprezentujące poziom metylacji wybranych genów w komórkach MM traktowanych dwukrotnie BTZ (BTZ_2) oraz BTZ i inhibitorem metylacji (BTZ_m_i_2). Wartości beta „1" wskazują na pełną metylację (kolor czerwony), a „0" oznacza brak metylacji (kolor niebieski) ($p<0,05$). Symbole genów i miejsca metylacji zaznaczono na heatmapie w następujący sposób: island − wyspa CpG; 5′ UTR − region nieulegający translacji na końcu 5; shore - sekwencje o długości 2 kb bezpośrednio w górę i w dół od wysp CpG; shelf – sekwencje o wielkości 2 kb bezpośrednio sąsiadujące z regionem shore; opensea - poza obszarem shelf; (źródło: Publikacja nr 2)

Analiza GSEA (Gene Set Enrichment Analysis) przeprowadzona po dwukrotnym traktowaniu komórek U266 BTZ i inhibitorem metylacji w porównaniu z komórkami traktowanymi dwukrotnie samym BTZ wykazała zmiany o charakterze hipometylacji (Ryc. 4). GSEA pozwoliła na zidentyfikowanie 20 istotnych statystycznie procesów ($p<0,05$), których geny wykazywały obniżony poziom metylacji w komórkach traktowanych BTZ i inhibitorem metylacji w porównaniu z komórkami traktowanymi samym BTZ. Najciekawszymi, istotnymi statystycznie procesami ($p<0,05$) wydają się być splicing RNA i zmiany epigenetyczne, takie jak deacetylacja białek, regulacja modyfikacji histonów i deacetylacja histonów, które są istotne przy rozważaniu wpływu modyfikacji epigenetycznych na rozwój oporności na BTZ.

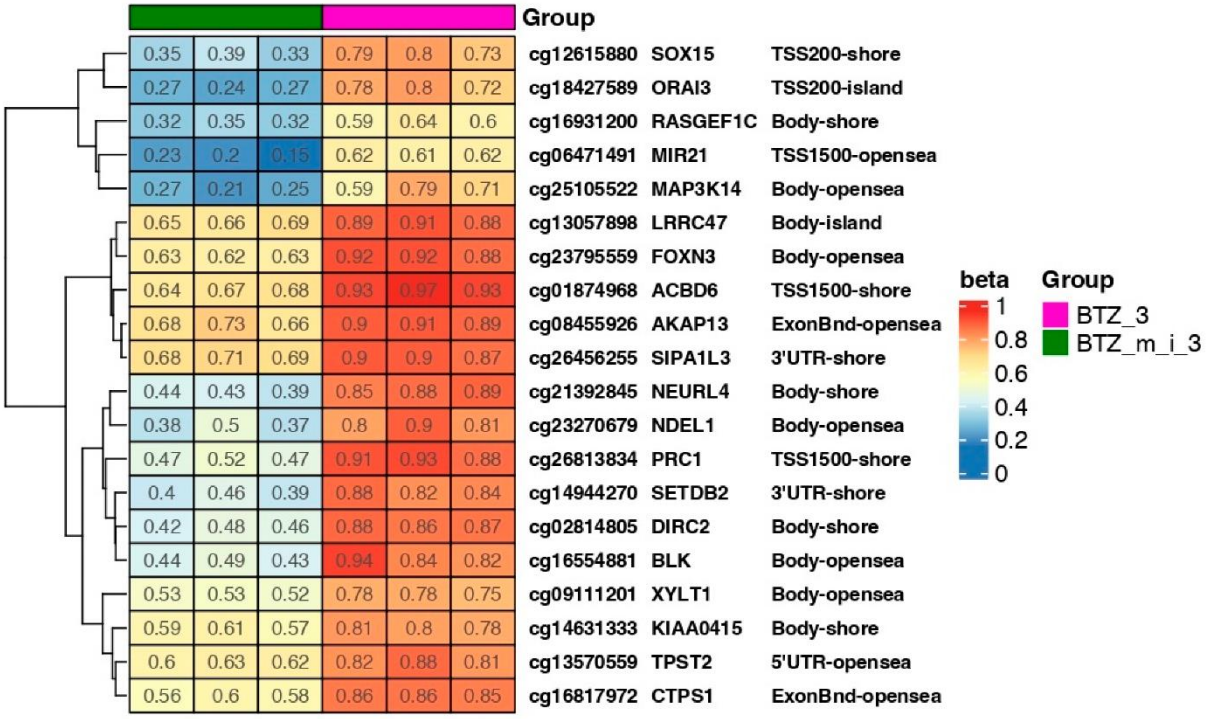


Rycina 4. Gene Set Enrichment Analysis. GSEA przedstawia wartości NES (Normalized Enrichment Score) wskazujące zmiany w metylacji DNA w genach zaangażowanych w regulację wymienionych procesów biologicznych w komórkach U266 traktowanych dwukrotnie BTZ i inhibitorem metylacji w porównaniu z komórkami traktowanymi dwukrotnie samym BTZ. Kolor czerwony oznacza hipometylację, kolor zielony oznacza hipermetylację; (źródło: Publikacja nr 2)

Analiza profilu metylacji po trzecim pasażu

Analiza bioinformatyczna wykazała 2996 miejsc o zmienionej metylacji (wyłącznie o charakterze hipometylacji) w komórkach MM trzykrotnie traktowanych BTZ i inhibitorem metylacji w porównaniu z komórkami traktowanymi trzykrotnie samym BTZ (Ryc. 2B). Liczba zmian w poziomie metylacji wzrosła prawie 10-krotnie po trzecim traktowaniu w porównaniu do wyników uzyskanych po drugiej inkubacji (drugie traktowanie: 301 zmienionych miejsc vs trzecie traktowanie: 2996 zmienionych miejsc).

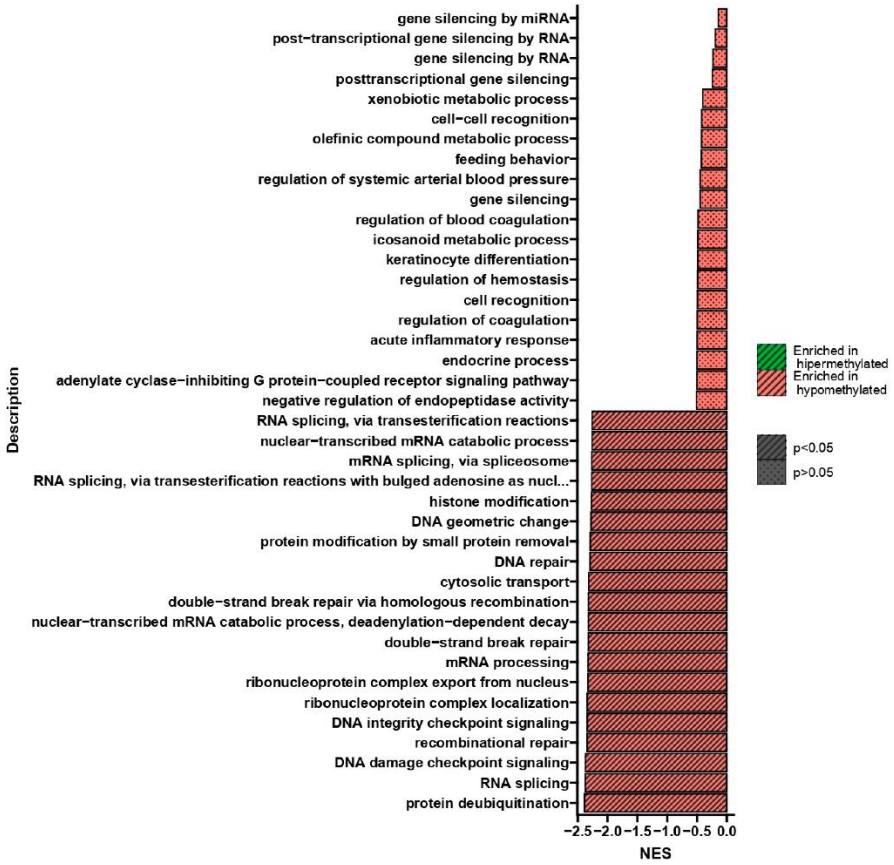
Analiza zmian w poziomach metylacji poszczególnych genów po trzech inkubacjach z BTZ i inhibitorem metylacji w porównaniu do komórek traktowanych samym BTZ wykazała hipometylację następujących genów: *MIR21, PRC1, AKAP13 i ORAI3* (Ryc. 5), które mogą być bezpośrednio powiązane z rozwojem lekooporności.



Rycina 5. 20 genów z największymi zmianami w poziomie metylacji. Heatmapa przedstawia wartości beta reprezentujące poziom metylacji wybranych genów w komórkach traktowanych trzykrotnie BTZ (BTZ_3) oraz BTZ i inhibitorem metylacji (BTZ_m_i_3). Wartości beta „1" wskazują na pełną metylację (kolor czerwony), a „0" oznacza brak metylacji (kolor niebieski) ($p<0,05$). Symbole genów i miejsca metylacji zaznaczono na heatmapie w następujący sposób: island − wyspa CpG; 5′ UTR − region nieulegający translacji na końcu 5; shore - sekwencje o długości 2 kb bezpośrednio w górę i w dół od wysp CpG; shelf – sekwencje o wielkości 2 kb bezpośrednio sąsiadujące z regionem shore; opensea - poza obszarem shelf; (źródło: Publikacja nr 2)

GSEA wykonana po trzecim traktowaniu komórek MM linii U266 BTZ i inhibitorem metylacji w porównaniu z komórkami traktowanymi trzykrotnie samym BTZ wykazała zmiany wyłącznie o charakterze hipometylacji ($p<0,05$) (Ryc. 6). Podobnie jak po drugim traktowaniu, proces splicingu RNA uległ istotnej hipometylacji. Proces ten pogłębiał się wraz z kolejnymi pasażami (drugie traktowanie - NES = −1,78; $p = 3,4\times10^{-15}$; trzecie traktowanie - NES = −2,37; $p = 8,2\times10^{-23}$) (Ryc. 4 i Ryc. 6). Inne procesy, które zwracają uwagę podczas analizy GSEA, dotyczą procesów uszkodzeń i naprawy DNA. Procesy te uczestniczą w rozwoju oporności poprzez modyfikacje DNA, które pozwalają komórkom

nowotworowym przetrwać w środowisku o wysokim poziomie stresu indukowanego przez terapię (*60*). W/w procesy biologiczne zidentyfikowano także 10 dni po ostatnim traktowaniu, potwierdzając zarówno znaczenie tych zmian w procesie rozwoju oporności na BTZ, jak i ich trwałość i transmisję do komórek potomnych.

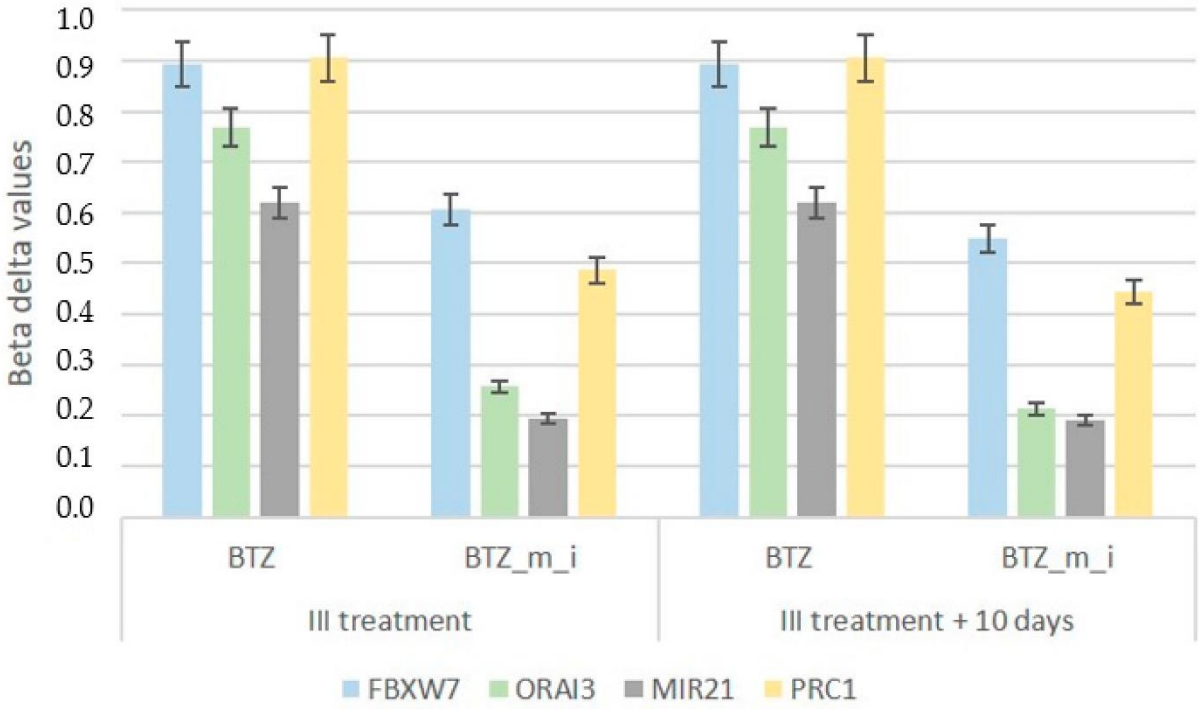


Rycina 6. Gene Set Enrichment Analysis (GSEA). GSEA pokazuje wartości NES (Normalized Enrichment Score) wskazujące zmiany w metylacji DNA w genach zaangażowanych w regulację wymienionych procesów biologicznych w komórkach U266 traktowanych trzykrotnie BTZ i inhibitorem metylacji w porównaniu z komórkami traktowanymi trzykrotnie samym BTZ. Kolor czerwony oznacza hipometylację, kolor zielony oznacza hipermetylację; (źródło: Publikacja nr 2)

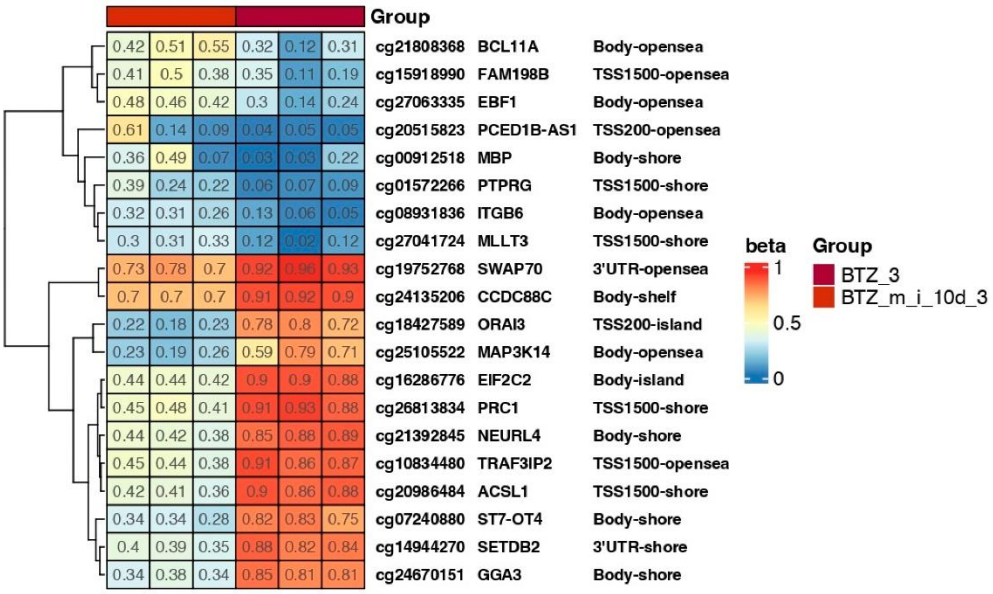
Analiza profilu metylacji 10 dni po trzecim pasażu

Trzykrotna 24-godzinna inkubacja komórek MM z BTZ i inhibitorem metylacji wywołała trwałe zmiany w dużej liczbie genów, szczególnie w tych odpowiedzialnych za rozwój lekooporności, takich jak *FBXW7, ORAI3, MIR21* i PRC1 (Ryc. 7 i Ryc. 8). Analiza danych przedstawionych na rycinie 7, która pokazuje wartości beta wybranych genów bezpośrednio po trzecim pasażu i 10 dni później, wykazała porównywalne poziomy metylacji w obu punktach czasowych. Dodatkowo przeprowadzono analizę bioinformatyczną porównującą wyniki poziomu metylacji DNA bezpośrednio po trzech

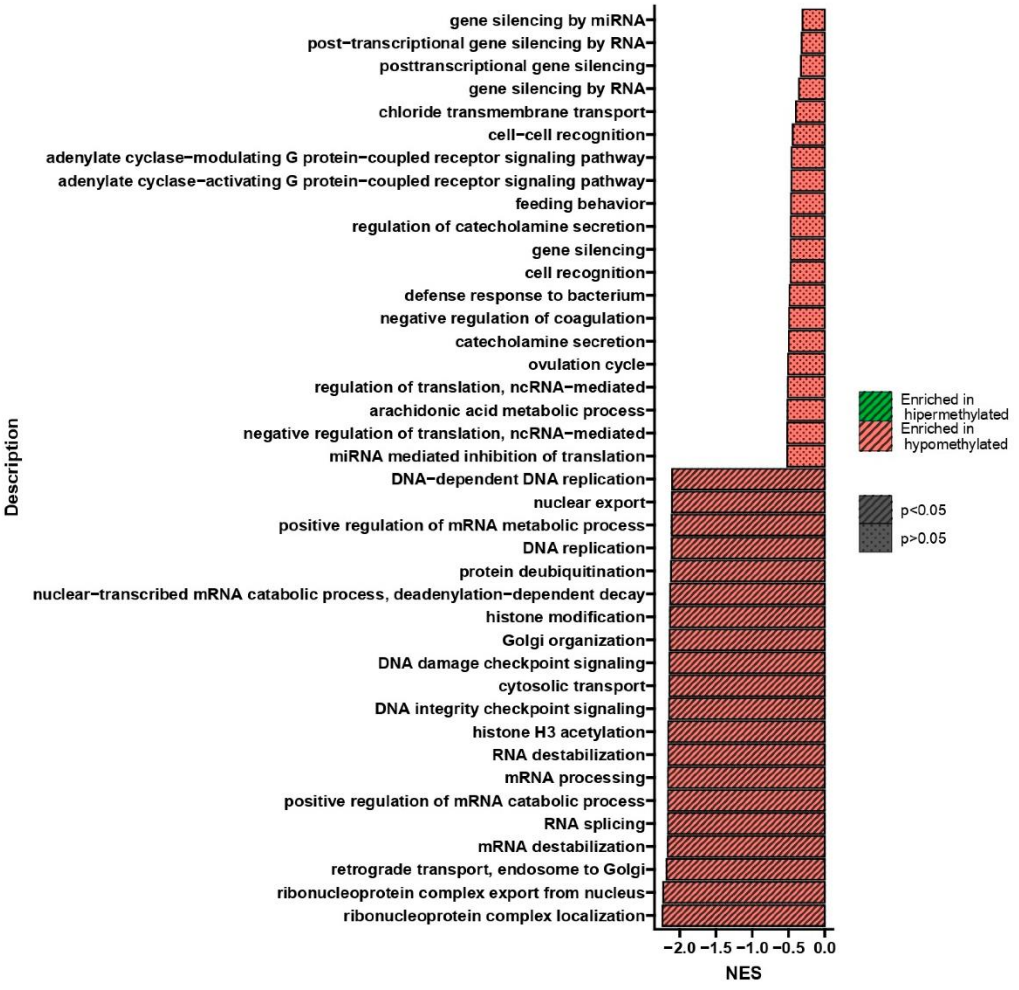
traktowaniach (BTZ_m_i) vs 10 dni później (BTZ_m_i_10days). Analiza nie wykazała znaczących zmian w metylacji DNA. Potwierdza to, że indukowane zmiany w metylacji DNA są trwałe i przekazywane do komórek potomnych, pomimo braku leków w medium hodowlanym. Według analizy GSEA nie stwierdzono wzrostu metylacji (Ryc. 9).



Rycina 7. Poziom metylacji genów związanych z lekoopornością. Wartości beta delta (średnia n = 3; p<0,05 dla wszystkich wyników) dla wybranych genów istotnych dla rozwoju oporności na BTZ mierzone bezpośrednio i 10 dni po trzecim zabiegu. BTZ – bortezomib; BTZ_m_i – bortezomib w połączeniu z inhibitorem metylacji; (źródło: Publikacja nr 2)



Rycina 8. 20 genów z największymi zmianami w poziomie metylacji. Heatmapa przedstawiająca wartości beta reprezentujące poziom metylacji wybranych genów w komórkach traktowanych trzykrotnie BTZ (BTZ_3) oraz BTZ i inhibitorem metylacji 10 dni po trzeciej inkubacji (BTZ_m_i_10d_3). Wartości beta „1" wskazują na pełną metylację (kolor czerwony), a „0" oznacza brak metylacji (kolor niebieski) ($p<0,05$). Symbole genów i miejsca metylacji zaznaczono na heatmapie w następujący sposób: island − wyspa CpG; 5′ UTR − region nieulegający translacji na końcu 5; shore – sekwencje o długości 2 kb bezpośrednio w górę i w dół od wysp CpG; shelf – sekwencje o wielkości 2 kb bezpośrednio sąsiadujące z regionem shore; opensea - poza obszarem shelf; (źródło: Publikacja nr 2)



Rycina 9. Gene Set Enrichment Analysis (GSEA). GSEA pokazuje wartości NES (Normalized Enrichment Score) wskazujące zmiany w metylacji DNA w genach zaangażowanych w regulację wymienionych procesów biologicznych w komórkach U266 10 dni po trzecim traktowaniu BTZ i inhibitorem metylacji w porównaniu z komórkami traktowanymi trzykrotnie samym BTZ. Kolor czerwony oznacza hipometylację, kolor zielony oznacza hipermetylację; (źródło: Publikacja nr 2)

Analiza bioinformatyczna przeprowadzona 10 dni po trzecim leczeniu komórek BTZ i inhibitorem metylacji w porównaniu z komórkami traktowanymi samym BTZ ujawniła 3023 (3009 hipometylowanych i 14 hipermetylowanych) miejsc o zmienionym profilu metylacji (Fig. 2C). Wyniki te nieznacznie różnią się od tych uzyskanych bezpośrednio po trzecim traktowaniu. Obszary hipermetylacji zaobserwowano na chromosomach 1–5, 9, 12 i 18 (Ryc. 2C).

Publikacja 3

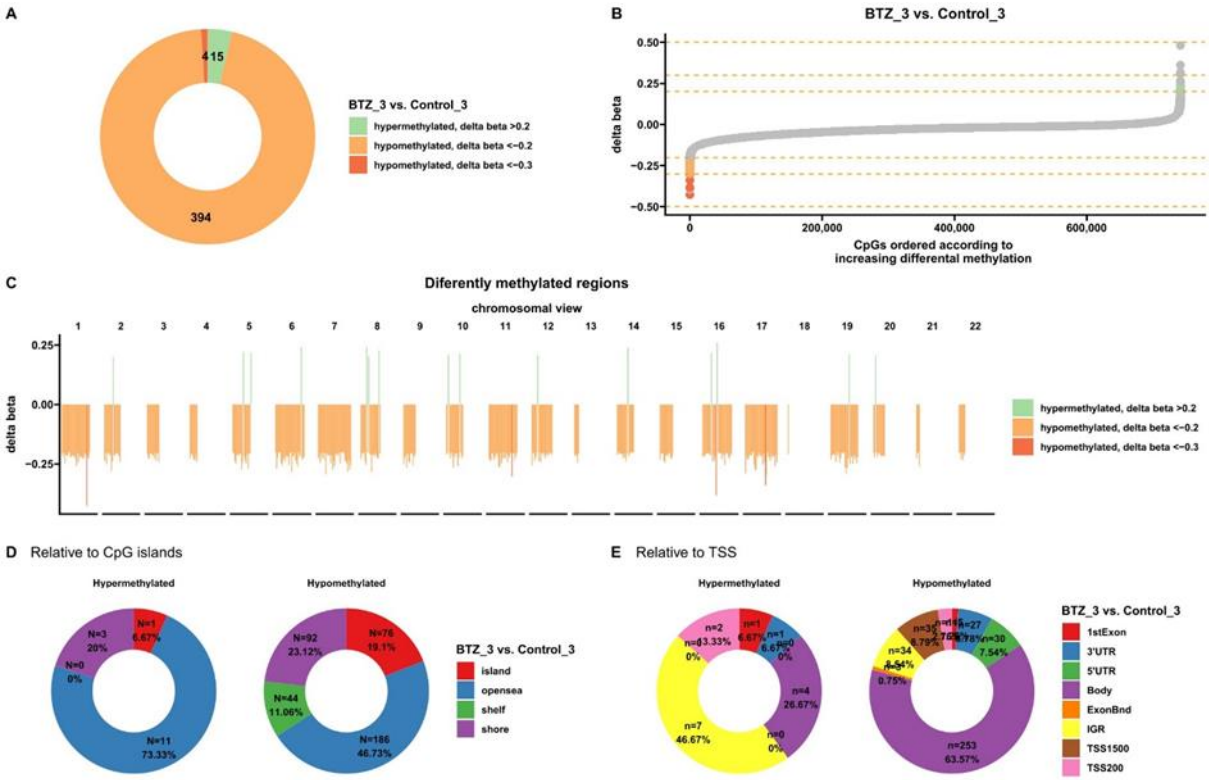
Wpływ witaminy 25(OH)D3 oraz K2MK7 na proliferację komórek szpiczaka plazmocytowego linii U266

Dokładna analiza wpływu witaminy 25(OH)D3 (VD) oraz witaminy K2MK7 (VK) została przedstawiona w publikacji naszego zespołu, niebędącej częścią niniejszej rozprawy doktorskiej (59). W skrócie, po pierwszej inkubacji komórek MM z BTZ zaobserwowano redukcję proliferacji o 70,06% w porównaniu z komórkami kontrolnymi ($p<0,0001$). Po drugim traktowaniu BTZ, zaobserwowano zmniejszenie proliferacji o 24,12% ($p<0,0001$). Trzecia inkubacja spowodowała rozwój fenotypu opornego na BTZ. Dodatek VD do hodowli spowodował zmniejszenie proliferacji komórek MM o ponad 20%. Podobne efekty zaobserwowano po dodaniu VK. W komórkach wrażliwych na BTZ witaminy wykazywały działanie synergistyczne z BTZ. Co szczególnie ważne, dodanie witamin do medium hodowlanego skutkowało zmniejszeniem proliferacji komórek MM U266 opornych na BTZ.

Zmiany w profilu metylacji związane z opornością na BTZ

Wyjściowo zbadano mechanizmy epigenetyczne związane z wykształceniem oporności na BTZ. W tym celu porównano kontrolne komórki MM z opornymi na BTZ. Analiza bioinformatyczna wykazała 413 miejsc ze zmienioną metylacją w komórkach traktowanych trzykrotnie BTZ w porównaniu z komórkami kontrolnymi (Ryc. 10A). Wartości delta beta obliczono zgodnie ze znormalizowanymi stosunkami intensywności fluorescencji sondy pomiędzy sygnałami metylowanymi i niemetylowanymi (wartość 0 = całkowicie niemetylowany, 1 = całkowicie metylowany). Zidentyfikowano 398 miejsc hipometylowanych i 15 hipermetylowanych (Ryc. 10A/B). Różnice dotyczyły wszystkich

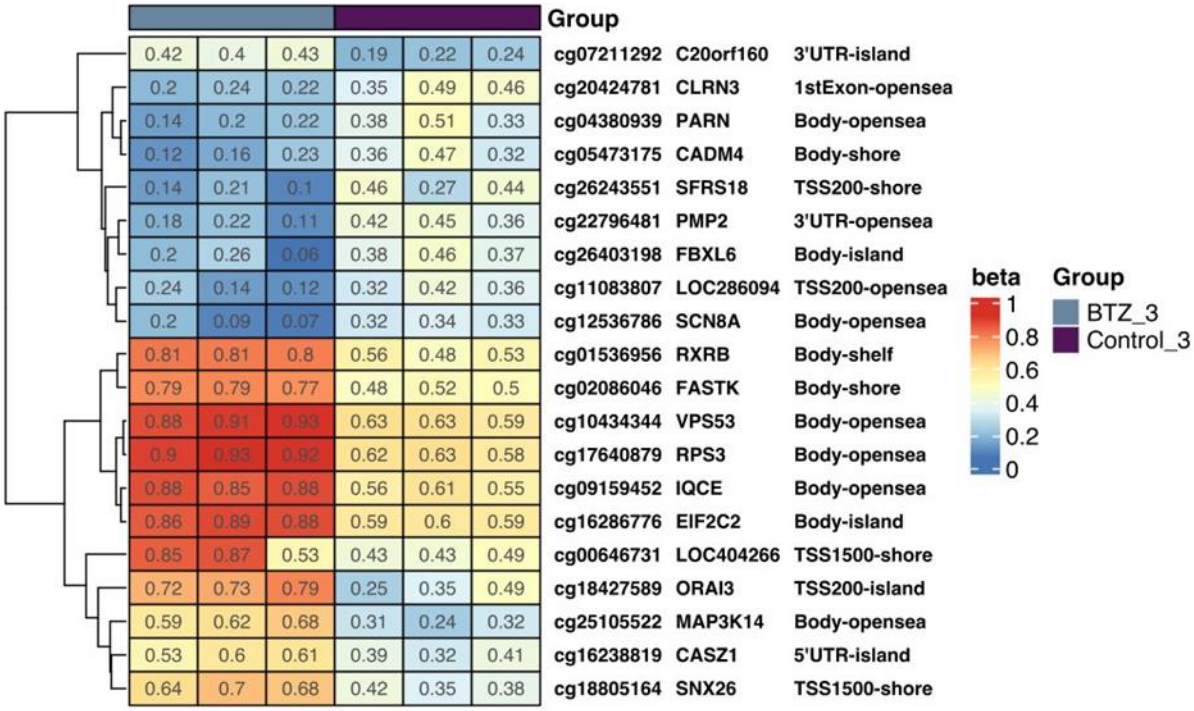
chromosomów (Ryc. 10C). Dalsza analiza umożliwiła klasyfikację zmienionych miejsc według lokalizacji wysp CpG (Ryc. 10D) oraz miejsca inicjacji transkrypcji (TSS) (Ryc. 10E). Zmiany w poziomach metylacji zaobserwowano w następujących rejonach genomu: opensea (izolowane miejsca CpG w genomie, które nie mają konkretnego oznaczenia), shelf (regiony 2–4 kb od wysp CpG), shore (regiony 0–2 kb z wysp CpG). Większość zmienionych miejsc, zarówno hipo-, jak i hipermetylowanych, uwidoczniono w opensea. Na wyspach CpG zaobserwowano 19,1% miejsc hipometylowanych i 6,67% miejsc hipermetylowanych. Podwyższony poziom metylacji w relacji do TSS zaobserwowano głównie w IGR (region międzygenowy), a obniżony w obszarze ulegającym transkrypcji („body") (Ryc. 10E).



Rycina 10. Profil metylacji po trzech kolejnych traktowaniach BTZ komórek szpiczaka linii U266. A, B) Różnice w poziomach metylacji w komórkach U266 traktowanych BTZ w porównaniu z nietraktowanymi komórkami kontrolnymi ($p<0,05$); C) Zmiany poziomu metylacji w poszczególnych chromosomach. Kolor pomarańczowy oznacza hipometylację, a zielony hipermetylację ($p<0,05$); D) Różnice w metylacji miejsc w genomie względem ich położenia w relacji do wysp CpG ($p<0,05$); E) Różnice w metylacji miejsc w genomie według ich położenia względem miejsca startu transkrypcji (TSS) ($p<0,05$). D – traktowanie witaminą 25(OH)D3; BTZ – traktowanie bortezomibem; DK – traktowanie jednocześnie witaminą 25(OH)D3

i K2MK7; BTZ_DK – traktowanie jednocześnie bortezomibem, witaminą 25(OH)D3 i K2MK7; Control - grupa kontrolna; 3 – trzecia inkubacja; (źródło: Publikacja nr 3)

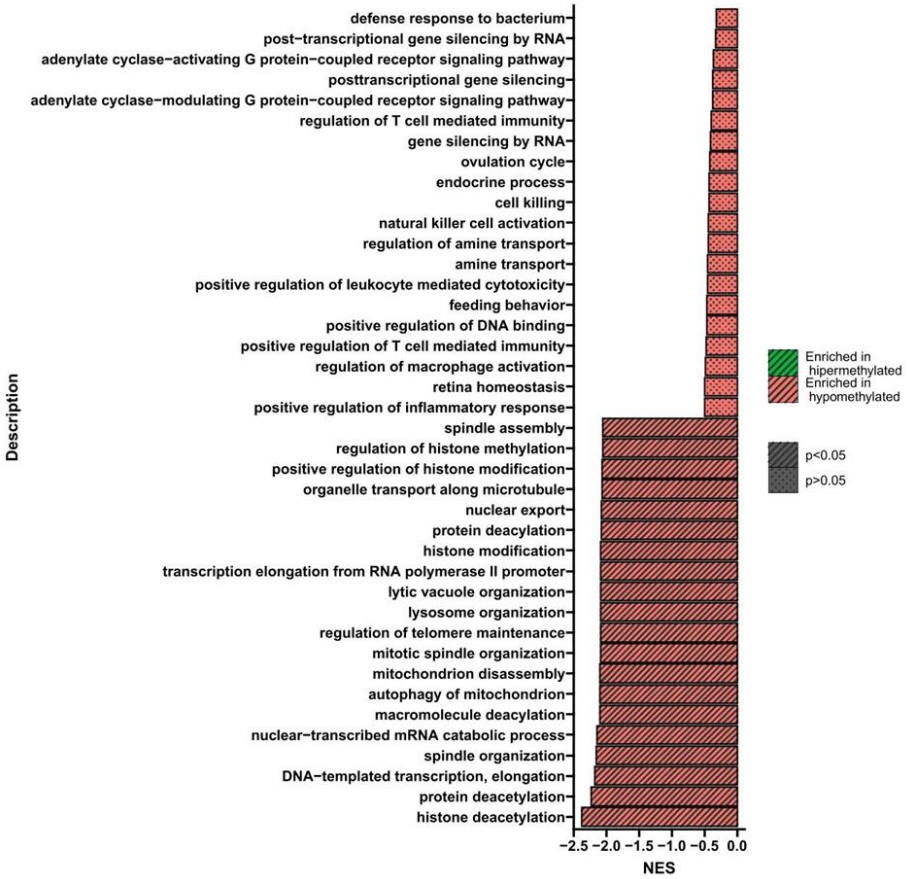
Poziomy metylacji w wybranych genach w komórkach kontrolnych i komórkach trzykrotnie traktowanych BTZ przedstawiono na rycinie 11 w postaci heatmapy. Z istotnych obserwacji wykazano hipometylację genów *FBXL6, CLRN3* i *PMP2* w komórkach opornych na BTZ w porównaniu z komórkami kontrolnymi. Wysoka ekspresja *FBXL6* wiąże się z progresją nowotworu i złym rokowaniem u pacjentów onkologicznych (*61*).



Rycina 11. 20 genów z największymi zmianami w poziomie metylacji. Heatmapa przedstawia wartości beta reprezentujące poziom metylacji wybranych genów w komórkach traktowanych trzykrotnie BTZ (BTZ_3) oraz w komórkach kontrolnych (Control_3). Wartości beta „1" wskazują na pełną metylację (kolor czerwony), a „0" oznacza brak metylacji (kolor niebieski) ($p<0,05$). Symbole genów i miejsca metylacji zaznaczono na heatmapie w następujący sposób: island − wyspa CpG; 5′ UTR − region nieulegający translacji na końcu 5; shore – sekwencje o długości 2 kb bezpośrednio w górę i w dół od wysp CpG; shelf – sekwencje o wielkości 2 kb bezpośrednio sąsiadujące z regionem shore; opensea - poza obszarem shelf; (źródło: Publikacja nr 3)

Rycina 12 przedstawia procesy, których poziomy metylacji uległy zmianie w komórkach MM opornych na BTZ w porównaniu z komórkami nietraktowanymi. Wszystkie zidentyfikowane zmiany miały charakter hipometylacji ($p<0,05$). Do

kluczowych procesów, które uległy hipometylacji zaliczono regulację metylacji histonów, modyfikację histonów, deacylację białek, regulację utrzymania telomerów, transkrypcję na szablonie DNA oraz elongację.

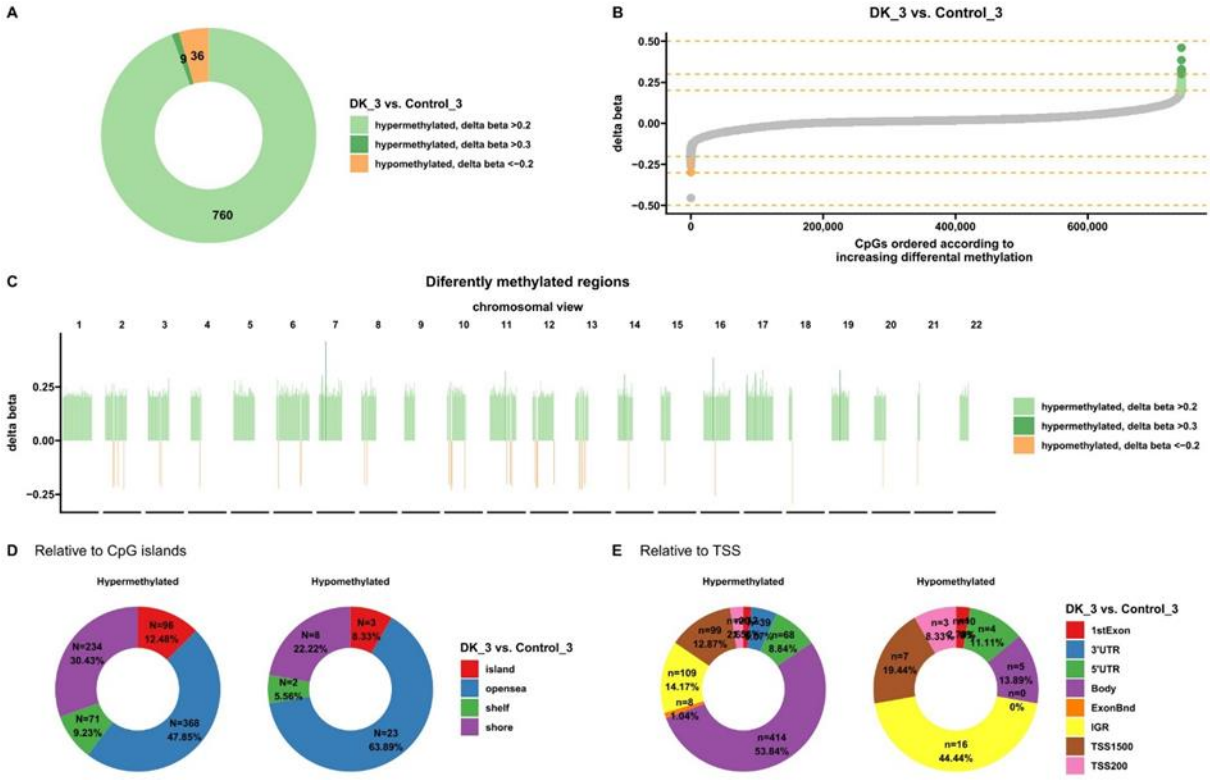


Rycina 12. Gene Set Enrichment Analysis (GSEA). GSEA pokazuje wartości NES (Normalized Enrichment Score) wskazujące zmiany w metylacji DNA w genach zaangażowanych w regulację wymienionych procesów biologicznych w komórkach U266 opornych na BTZ w porównaniu z komórkami kontrolnymi. Kolor czerwony oznacza hipometylację, kolor zielony oznacza hipermetylację; (źródło: Publikacja nr 3)

Wpływ witamin 25(OH)D3 oraz K2MK7 na kontrolne komórki szpiczaka plazmocytowego

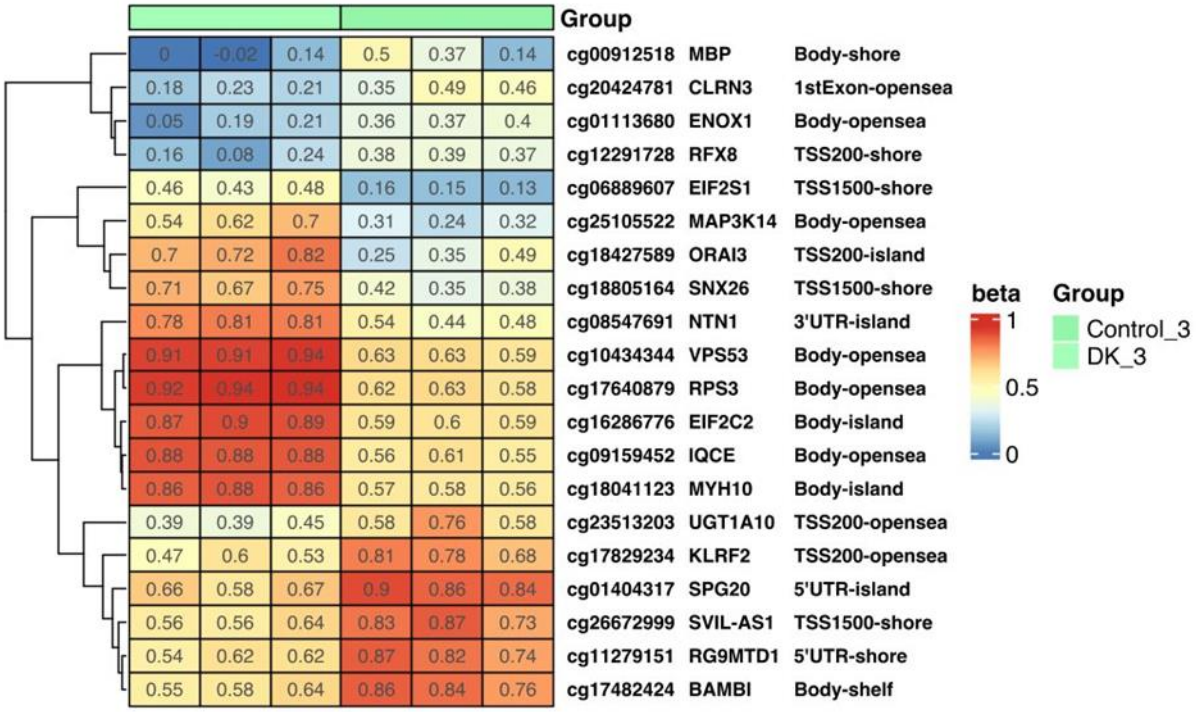
Analiza bioinformatyczna wykazała 805 miejsc ze zmienioną metylacją w komórkach MM inkubowanych trzykrotnie z VD i VK w porównaniu z komórkami kontrolnymi (Ryc. 13A). W analizie zidentyfikowano 36 miejsc hipometylowanych i 769 miejsc hipermetylowanych (Ryc. 13A/B). Różnice dotyczyły wszystkich chromosomów (Ryc. 13C). Miejsca hipermetylowane zaznaczono na zielono, a hipometylowane na pomarańczowo. Dalsza analiza umożliwiła klasyfikację zmienionych miejsc względem lokalizacji wysp CpG (Ryc. 13D) i TSS (Ryc. 13E). Większość zmienionych miejsc, zarówno hipo-, jak i hipermetylowanych, uwidoczniono w obszarze „opensea". Na wyspach

CpG zaobserwowano 8,33% miejsc hipometylowanych i 12,48% miejsc hipermetylowanych. W przypadku TSS zaobserwowano wzrost poziomu metylacji głównie w obszarze ulegającym transkrypcji („body"), a spadek w IGR (Ryc. 13E). W obszarze 3'UTR nie obserwowano hipometylacji.



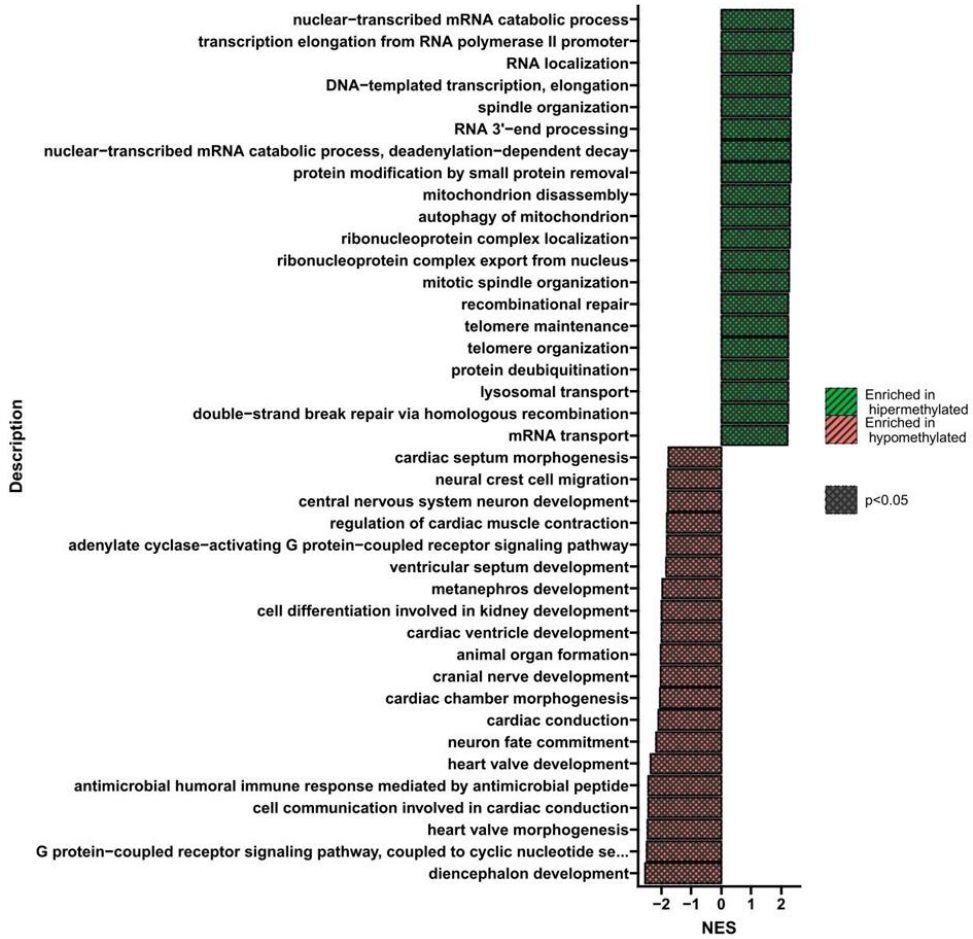
Rycina 13. Profil metylacji po trzech kolejnych traktowaniach VD i VK komórek szpiczaka U266. A, B) Różnice w poziomach metylacji w komórkach U266 inkubowanych z VD i VK w porównaniu z komórkami kontrolnymi ($p<0,05$); C) Zmiany poziomu metylacji w poszczególnych chromosomach. Kolor pomarańczowy oznacza hipometylację, a zielony hipermetylację ($p<0,05$); D) Różnice w metylacji miejsc w genomie względem ich położenia w relacji do wysp CpG ($p<0,05$); E) Różnice w metylacji miejsc w genomie według ich położenia względem miejsca startu transkrypcji (TSS) ($p<0,05$). DK – 25(OH)D3 i K2MK7; Control - komórki kontrolne; 3 – trzecia inkubacja; (źródło: Publikacja nr 3)

Poziomy metylacji w wybranych genach w komórkach kontrolnych i komórkach traktowanych trzykrotnie VD i VK przedstawiono na rycinie 14 jako heatmapę. Zidentyfikowano, m.in. hipometylację genu *RFX8*. Uważa się, że gen ten bierze udział w regulacji transkrypcji przez polimerazę RNA II, a tym samym wpływa na wiele różnych procesów (*62*).



Rycina 14. 20 genów z największymi zmianami w poziomie metylacji. Heatmapa przedstawia wartości beta reprezentujące poziom metylacji wybranych genów w komórkach traktowanych trzykrotnie VD i VK (DK_3) oraz w komórkach kontrolnych (Control_3). Wartości beta „1" wskazują na pełną metylację (kolor czerwony), a „0" oznacza brak metylacji (kolor niebieski) ($p<0,05$). Symbole genów i miejsca metylacji zaznaczono na heatmapie w następujący sposób: island − wyspa CpG; 5′ UTR − region nieulegający translacji na końcu 5; shore – sekwencje o długości 2 kb bezpośrednio w górę i w dół od wysp CpG; shelf – sekwencje o wielkości 2 kb bezpośrednio sąsiadujące z regionem shore; opensea - poza obszarem shelf; (źródło: Publikacja nr 3)

Rycina 15 przedstawia procesy, których geny zmieniły poziomy metylacji w komórkach MM trzykrotnie inkubowanych z VD i VK w porównaniu z komórkami nietraktowanymi. Na podstawie stopnia metylacji genów, zidentyfikowano procesy, które są w ten sposób regulowane ($p<0,05$). Procesy, które uległy hipermetylacji obejmują lokalizację RNA, transkrypcję DNA, elongację DNA oraz organizację telomerów.



Rycina 16. Gene Set Enrichment Analysis (GSEA). GSEA pokazuje wartości NES (Normalized Enrichment Score) wskazujące zmiany w metylacji DNA w genach zaangażowanych w regulację wymienionych procesów biologicznych w komórkach U266 trzykrotnie inkubowanych z VD i VK w porównaniu z komórkami kontrolnymi. Kolor czerwony oznacza hipometylację, kolor zielony oznacza hipermetylację; (źródło: Publikacja nr 3)

Komórki MM inkubowane jednocześnie z VD i VK wykazywały podobny profil metylacji jak komórki kontrolne inkubowane jedynie z VD. Dokładny opis wyników znajduje się w sekcji wyniki Publikacji nr 3. Do najistotniejszych obserwacji poczynionych w trakcie inkubacji komórek MM jedynie z VD zaliczono hipometylację genów *CLEC12B* i *BAMBI*, które odpowiadają za hamowanie proliferacji komórek nowotworowych (*63–65*). Zaobserwowano również wzrost poziomu metylacji genów *NTN1* i *MYH10* związanych z progresją nowotworów (*66–68*).

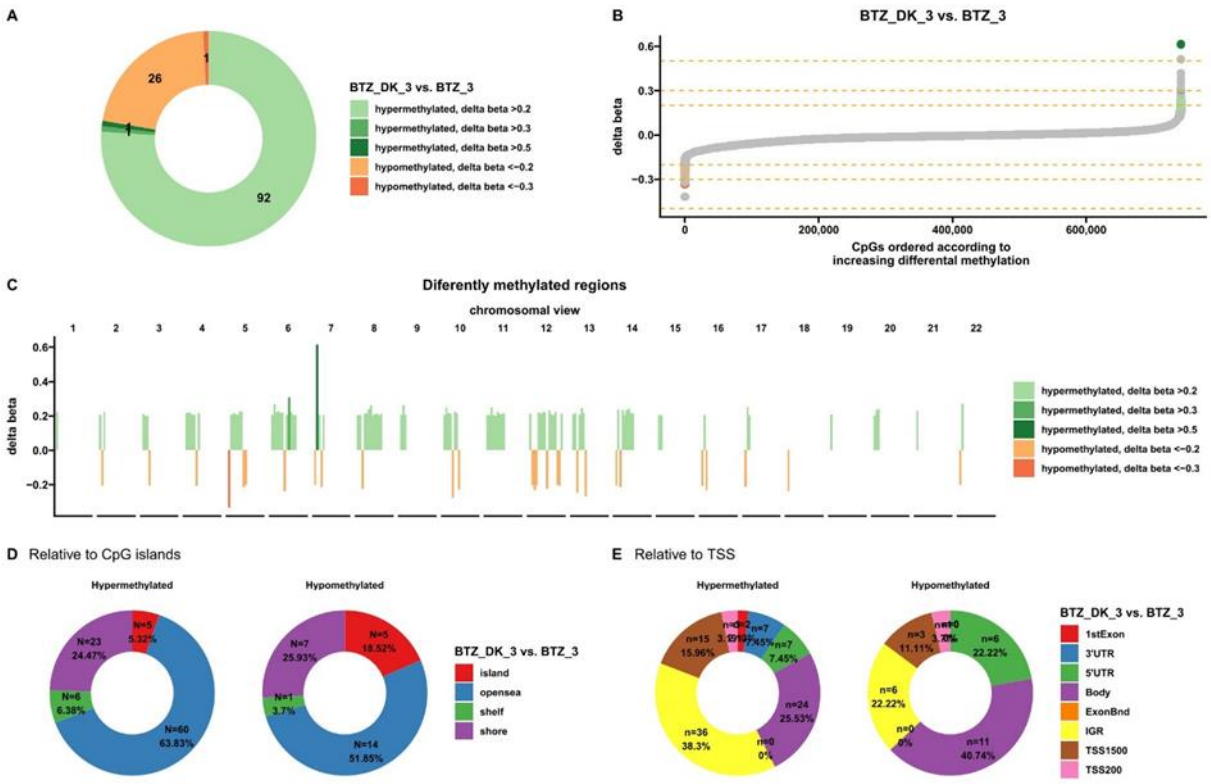
Podsumowując, zidentyfikowano geny odpowiedzialne za różne procesy komórkowe, w których różne regiony były przeważnie hipermetylowane, rzadziej hipometylowane.

Traktowanie komórek MM wyłącznie VD jak i w połączeniu z VK wiązało się z globalnym wzrostem metylacji.

Wpływ witamin 25(OH)D3 oraz K2MK7 na komórki szpiczaka plazmocytowego oporne na BTZ

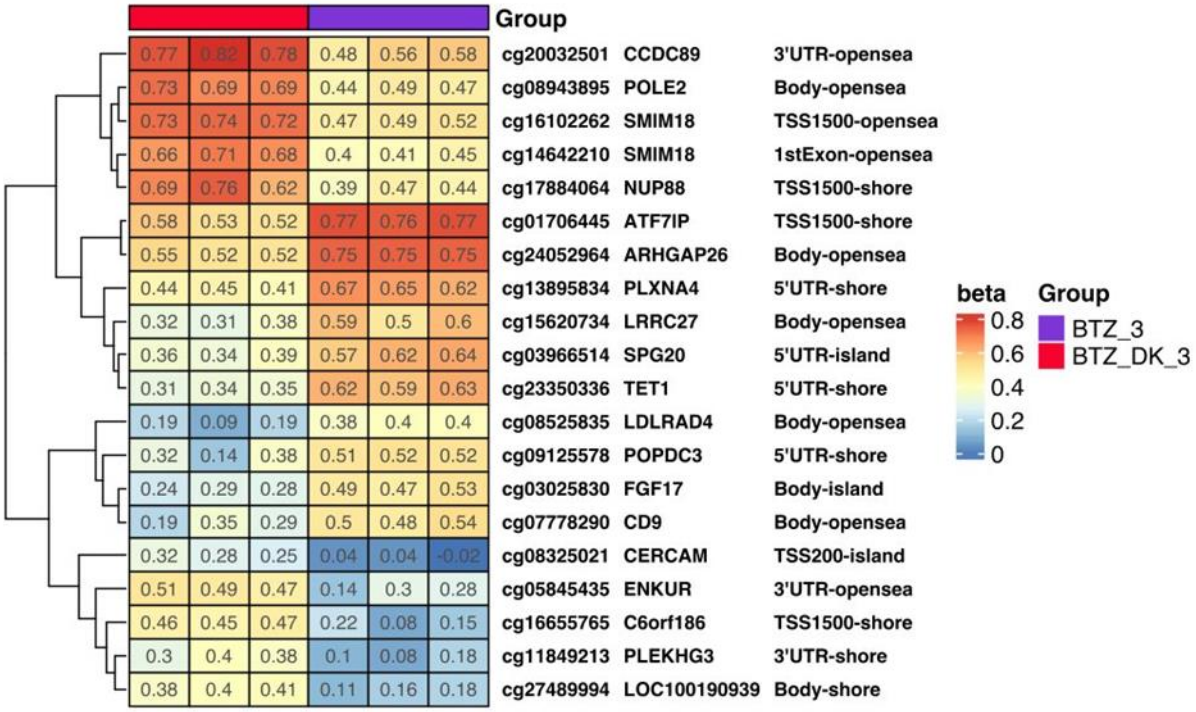
Zarówno sama VD jak i suplementacja w połączeniu z VK może indukować zmiany epigenetyczne w kontrolnych komórkach MM. W związku z tym postawiono hipotezę, że witaminy te mogą wpływać na profil metylacji również w komórkach MM cechujących się fenotypem opornym na BTZ.

Analiza bioinformatyczna wykazała 121 miejsc o zmienionej metylacji w komórkach MM opornych na BTZ traktowanych VD i VK, w porównaniu z komórkami traktowanymi trzykrotnie samym BTZ (Ryc. 17A). W analizie zidentyfikowano 27 miejsc hipometylowanych i 94 miejsca hipermetylowane (Ryc. 17A/B). Różnice dotyczyły wszystkich chromosomów (Ryc. 17C). Miejsca hipermetylowane zaznaczono na zielono, a hipometylowane na pomarańczowo. Dalsza analiza umożliwiła klasyfikację zmienionych miejsc według lokalizacji w odniesieniu do wysp CpG (Ryc. 17D) i TSS (Ryc. 17E). Większość zmienionych miejsc, zarówno hipo-, jak i hipermetylowanych, uwidoczniono na otwartym morzu. Na wyspach CpG zaobserwowano 18,52% miejsc hipometylowanych i 5,32% miejsc hipermetylowanych. Podwyższony poziom metylacji w stosunku do TSS zaobserwowano głównie w IGR i obniżony w obszarze ulegającym transkrypcji („body”). Nie zaobserwowano hipometylacji w 3’UTR (Ryc. 17E).



Rycina 17. Profil metylacji po trzech kolejnych traktowaniach VD i VK komórek szpiczaka U266 opornych na BTZ. A, B) Różnice w poziomach metylacji w komórkach U266 inkubowanych z VD i VK w porównaniu z komórkami opornymi na BTZ ($p<0,05$); C) Zmiany poziomu metylacji w poszczególnych chromosomach. Pomarańczowy oznacza hipometylację, a zielony hipermetylację ($p<0,05$); D) Różnice w metylacji miejsc w genomie względem ich położenia w relacji do wysp CpG ($p<0,05$); E) Różnice w metylacji miejsc w genomie według ich położenia względem miejsca startu transkrypcji (TSS) ($p<0,05$). BTZ – bortezomib; BTZ_DK – jednocześnie bortezomib, 25(OH)D3 i K2MK7; 3 – trzecia inkubacja; (źródło: Publikacja nr 3)

Poziomy metylacji w wybranych genach w komórkach traktowanych trzykrotnie BTZ, VD i VK (BTZ_DK_3) oraz w komórkach traktowanych samym BTZ przedstawiono na Figurze 18 w postaci heatmapy. Z istotnych obserwacji wykazano hipermetylację genu *ARHGAP26* w komórkach opornych na BTZ. Gen ten bierze udział w onkogenezie i progresji nowotworów (69).



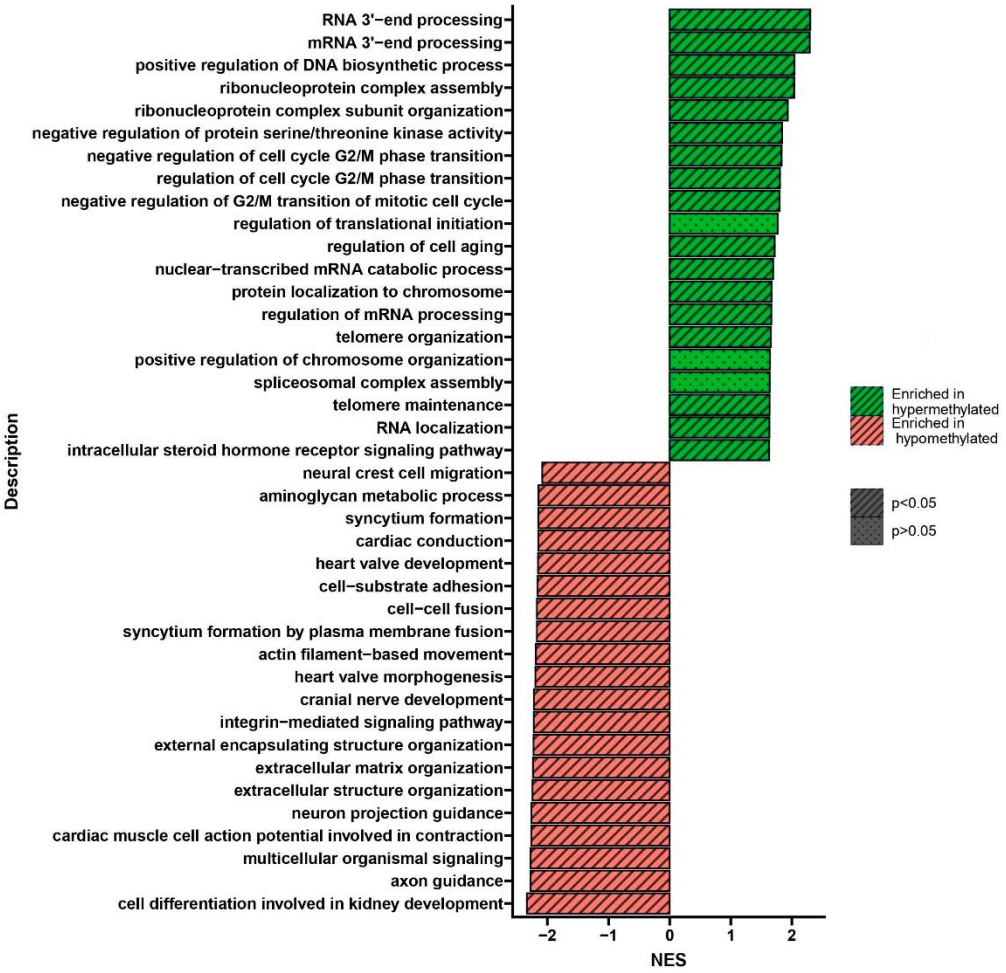
Rycina 18. 20 genów z największymi zmianami w poziomie metylacji. Heatmapa przedstawia wartości beta reprezentujące poziom metylacji wybranych genów w komórkach traktowanych trzykrotnie VD i VK (DK_3) oraz w komórkach opornych na BTZ (BTZ_3). Wartości beta „1" wskazują na pełną metylację (kolor czerwony), a „0" oznacza brak metylacji (kolor niebieski) ($p<0,05$). Symbole genów i miejsca metylacji zaznaczono na heatmapie w następujący sposób: island – wyspa CpG; 5′ UTR – region nieulegający translacji na końcu 5; shore – sekwencje o długości 2 kb bezpośrednio w górę i w dół od wysp CpG; shelf – sekwencje o wielkości 2 kb bezpośrednio sąsiadujące z regionem shore; opensea - poza obszarem shelf; (źródło: Publikacja nr 3)

Rycina 19A przedstawia procesy, których geny zmieniły poziom metylacji w komórkach opornych na BTZ i trzykrotnie traktowanych VD i VK w porównaniu z komórkami opornymi na BTZ. Na podstawie stopnia metylacji genów zidentyfikowano procesy, które są przez nie regulowane ($p<0,05$). Najważniejsze procesy, które uległy hipometylacji po suplementacji witaminami to fuzja komórka-komórka, adhezja komórka-substrat, a hipermetylacji to procesowanie końca 3' RNA, procesowanie końca 3' mRNA, pozytywna regulacja procesu biosyntezy DNA i regulacja starzenia się komórek.

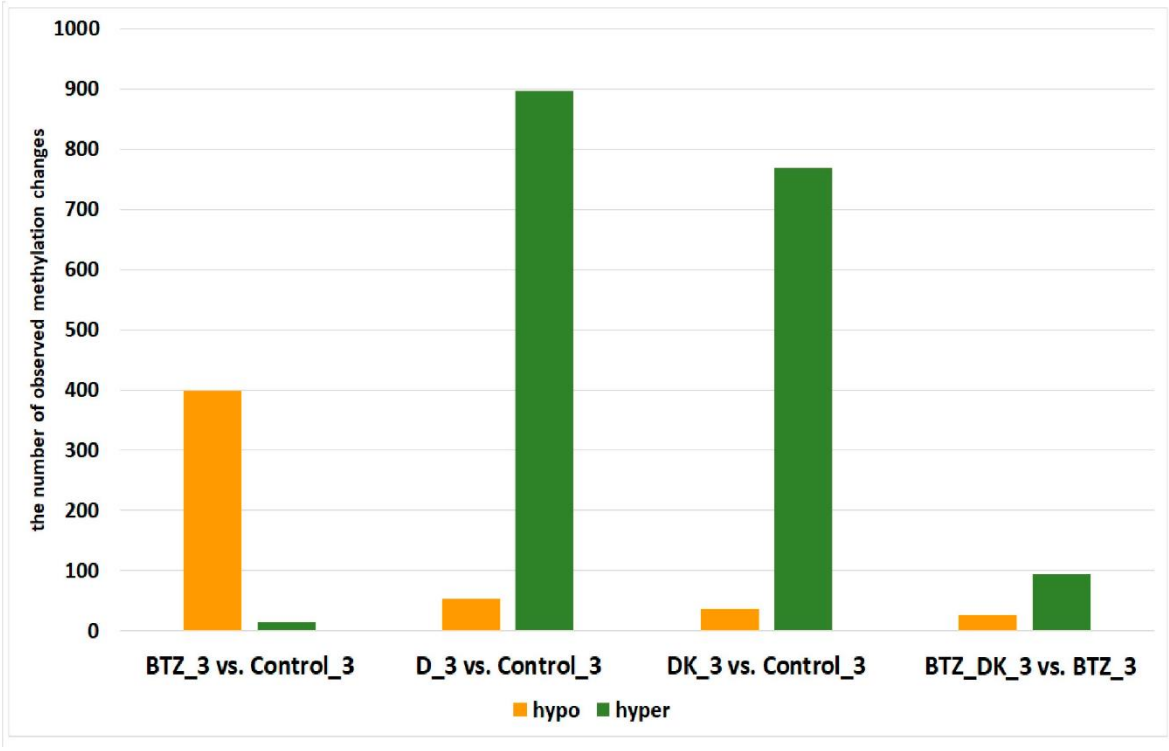
Suplementacja VD i VK wiąże się z globalnym wzrostem metylacji w komórkach MM opornych na BTZ. Należy jednak zauważyć, że efekt ten nie jest aż tak wyraźny, jak w kontrolnych komórkach MM. Niemniej jednak, komórki oporne na BTZ hodowane

wspólnie z samą VD lub jednocześnie z VD i VK, przynajmniej tymczasowo, odzyskiwały wrażliwość na BTZ (Ryc. 19B).

A


RNA 3'−end processing
mRNA 3'−end processing
positive regulation of DNA biosynthetic process
ribonucleoprotein complex assembly
ribonucleoprotein complex subunit organization
negative regulation of protein serine/threonine kinase activity
negative regulation of cell cycle G2/M phase transition
regulation of cell cycle G2/M phase transition
negative regulation of G2/M transition of mitotic cell cycle
regulation of translational initiation
regulation of cell aging
nuclear−transcribed mRNA catabolic process
protein localization to chromosome
regulation of mRNA processing
telomere organization
positive regulation of chromosome organization
spliceosomal complex assembly
telomere maintenance
RNA localization
intracellular steroid hormone receptor signaling pathway
neural crest cell migration
aminoglycan metabolic process
syncytium formation
cardiac conduction
heart valve development
cell−substrate adhesion
cell−cell fusion
syncytium formation by plasma membrane fusion
actin filament−based movement
heart valve morphogenesis
cranial nerve development
integrin−mediated signaling pathway
external encapsulating structure organization
extracellular matrix organization
extracellular structure organization
neuron projection guidance
cardiac muscle cell action potential involved in contraction
multicellular organismal signaling
axon guidance
cell differentiation involved in kidney development
Description
−2
−1
0
1
2
NES
Enriched in hypermethylated
Enriched in hypomethylated
p<0.05
p>0.05

B


1000
900
800
700
600
500
400
300
200
100
0
the number of observed methylation changes
BTZ_3 vs. Control_3
D_3 vs. Control_3
DK_3 vs. Control_3
BTZ_DK_3 vs. BTZ_3
hypo
hyper

Rycina 19. (A) Gene Set Enrichment Analysis (GSEA). GSEA pokazuje wartości NES (Normalized Enrichment Score) wskazujące zmiany w metylacji DNA w genach zaangażowanych w regulację wymienionych procesów biologicznych w komórkach U266 trzykrotnie inkubowanych z VD i VK w porównaniu z komórkami opornymi na BTZ. Kolor czerwony oznacza hipometylację, kolor zielony oznacza hipermetylację. **(B)** Globalne zmiany w metylacji w komórkach kontrolnych MM w porównaniu z komórkami opornymi na BTZ, trzykrotnie traktowanymi VD oraz VD i VK, a także w komórkach opornych na BTZ w porównaniu z fenotypem opornym trzykrotnie traktowanym VD i VK. Kolor pomarańczowy oznacza hipometylację, kolor zielony oznacza hipermetylację. ($p<0,05$). BTZ – inkubowany bortezomibem; D – inkubowany z witaminą 25(OH)D3; DK – inkubowany jednocześnie z witaminą 25(OH)D3 i K2MK7; BTZ_DK – inkubowany jednocześnie z bortezomibem, witaminą 25(OH)D3 i K2MK7; Control – komórki kontrolne; 3 – trzecia inkubacja; (źródło: Publikacja nr 3)

Analiza ekspresji wybranych genów

Na podstawie powyżej przedstawionych wyników zidentyfikowano geny (*ARHGAP26, MYH10, PMP2, RFX8, BAMBI, CLEC12b*) ze znaczącymi zmianami w poziomie metylacji, których ekspresję poddano walidacji za pomocą techniki qRT-PCR (Tab. 1). W stosunku do wyselekcjonowanych genów, potwierdzono rzeczywisty wpływ poziomu metylacji na ekspresję genów. W myśl zasady, im wyższy poziom metylacji, tym niższa ekspresja genu i odwrotnie. Z punktu widzenia wpływu badanych witamin na wrażliwą na BTZ linię szpiczaka U266, istotną obserwacją jest niemal 60% spadek ekspresji genu *MYH10* po trzykrotnej ekspozycji na VD w stosunku do komórek kontrolnych i jednocześnie 73% wzrost ekspresji genu *CLEC12b*. Dodatkowo, po trzykrotnej ekspozycji zarówno na VD i VK, zaobserwowano 70% wzrost ekspresji genu *RFX8* w porównaniu z komórkami kontrolnymi.

Ponadto, zaobserwowano znaczący, 54% wzrost ekspresji genu *ARHGAP26* w komórkach szpiczaka U266 opornych na BTZ inkubowanych trzykrotnie z VD i VK w porównaniu z komórkami opornymi na BTZ bez ekspozycji na witaminy. Wynik walidacji potwierdza, że niższy poziom metylacji tego genu w komórkach BTZ_DK_3 (wartość beta=0,52±0,01) w porównaniu do BTZ_3 (wartość beta=0,75±0,00) koreluje z wyższym poziomem jego ekspresji.

	Control_3 Mean ± SD; 95% CI	**BTZ_3** Mean ± SD; 95% CI	**D_3** Mean ± SD; 95% CI	**DK_3** Mean ± SD; 95% CI	**BTZ_DK_3** Mean ± SD; 95% CI
ARHGAP26	1,00±0.08; 0,75-1,25	0,78±0,09; 0,50-1,06	1,16±0,01; 1,01-1,31	1,34±0,11; 1,00- 1,68	1,69±0,24; 0,94-2,43

MYH10	0,21±0,03; -0,28-0,71	0,06±0,01; 0,01-0,10	0.09±0.02; 0.03-0.16	0,18±0,07; -0,06-0,42	0,19±0,04; 0,06-0,32
PMP2	0,05±0,02; -0,01-0,12	0,11±0,04; -0,02-0,23	0.36±0.24; -0.39-1.11	0,41±0,03; 0,30-0,52	0,44±0,17; -0,09-0,98
RFX8	0,23±0,04; 0,10-0,35	0,13±0,03; 0,02-0,25	0.40±0.12; 0.02-0.78	0,73±0,16; 0,21-1,25	0,33±0,13; -0,09-0,75
BAMBI	11,50±1,94; 5,57-17,42	11,55±1,41; 7,26-15,84	15.46±1.37; 10.98-19.95	29,10±7,02; 7,73-50,46	20,58±4,66; 6,39-34,77
CLEC12b	0,16±0,09; -0,15-0,46	0,10±0,01; 0,04-0,15	0.22±0.07; -0.02-0.45	0,38±0,1; 0,06-0,69	0,35±0,07; 0,12-0,58

Tabela 1. Walidacja ekspresji wybranych genów z zastosowaniem techniki qRT-PCR. Mean – średnia arytmetyczna; SD – odchylenie standardowe; 95% CI – 95% przedział ufności; Control – komórki kontrolne; BTZ – bortezomib; D – witamina 25(OH)D3; DK – witaminy 25(OH)D3 i K2MK7; 3 – trzecia inkubacja; (źródło: Publikacja nr 3)

Dyskusja

Zastosowanie BTZ u chorych ze szpiczakiem plazmocytowym stało się kamieniem milowym w leczeniu tego schorzenia, istotnie przyczyniając się do poprawy rokowania w tej grupie pacjentów. BTZ to pierwszy inhibitor proteasomu wprowadzony na rynek. Lek do dziś jest powszechnie wykorzystywany, będąc często podstawą wielu złożonych schematów terapeutycznych. Dlatego można przypuszczać, iż większość pacjentów chorych na MM była eksponowana na BTZ lub w dalszym ciągu jest nim leczona. Ze względu na dużą populację chorych, którzy mają lub będą mieć styczność z BTZ, niezwykle istotne jest szczegółowe poznanie patomechanizmu działania leku jak i zrozumienie zmian molekularnych przyczyniających się do wystąpienia lekooporności. Taka wiedza może przyczynić się do opracowania nowych schematów lekowych z wykorzystaniem BTZ, które będą cechować się większą skutecznością terapeutyczną oraz przyczynią się do późniejszego wystąpienia oporności na lek.

Terapia BTZ charakteryzuje się wysoką skutecznością względem komórek szpiczaka plazmocytowego. Niemniej jednak, ekspozycja na lek jest istotnym bodźcem wywierającym na komórki nowotworowe silną presję środowiskową, co może skutkować selekcją klonów opornych. W dłuższej perspektywie, nadmierna selekcja oraz następcza proliferacja komórek mniej wrażliwych na BTZ może skutkować nawrotem lub progresją choroby.

Wprowadzenie BTZ do otoczenia komórek MM jest istotną interferencją w środowisko nowotworu, mogącą w nich wywoływać zmiany epigenetyczne. Z jednej strony modyfikacje epigenetyczne mogą być elementem mechanizmu działania cząsteczki, z drugiej strony mogą leżeć u podstaw rozwoju lekooporności. Dowiedziono, że zmiany m.in. w metylomie są istotnym aspektem mechanizmu działania cząsteczki, warunkującym jej cytotoksyczne właściwości. Dostępne badania wskazują, że BTZ indukuje globalny

spadek metylacji w komórkach nowotworowych (*70*). Poza globalną modyfikacją profilu metylacji, ekspozycja na BTZ skutkuje hipometylacją konkretnych genów, m.in. *NOXA,* będącego proapoptytycznym genem z rodziny Bcl-2 (*71*). Co więcej, wykazano, że *NOXA* jest kluczowy dla cytotoksycznej aktywności BTZ, a jego deplecja skutkowała spadkiem skuteczności leku i wystąpieniem oporności na terapię (*71, 72*). Dodatkowo, BTZ wpływa na aktywność deacetylaz histonowych (ang. histone deacetylases, HDACs). Precyzyjniej, BTZ powoduje supresję HDACs oraz hiperacetylację histonów, skutkującą śmiercią złośliwych plazmocytów. Powyższe zjawiska są najbardziej nasilone w stosunku do HDAC1. Co więcej, upregulacja HDAC1 skutkowała wystąpieniem oporności na lek (*73*).

Powyższe przykłady wskazują, że zmiany epigenetyczne indukowane przez BTZ są istotnym aspektem mechanizmu działania cząsteczki oraz w pewnym stopniu warunkują jej cytotoksyczne właściwości. Niemniej jednak, ekspozycja na lek, pomimo początkowej i często długotrwałej skuteczności, przyczynia się do selekcji opornych klonów oraz nawrotu choroby. Wydaje się również słuszną hipoteza, że modyfikacje epigenetyczne, przynajmniej częściowo, przyczyniają się do wystąpienia lekooporności. Przykładowo, spadek ekspresji proteasomu, będącego punktem uchwytu leku, na skutek hipermetylacji promotora genu *PSMD5* kodującego podjednostkę 19S skutkuje opornością na BTZ (*74*). Podobnie zmiany w profilu metylacji odpowiadają za wystąpienie oporności na BTZ również w komórkach neuroblastoma (*75*). Natomiast, hipermetylacja promotora genu kodującego CD9, którego niska ekspresja jest związana z progresją nowotworów, skutkuje wystąpieniem oporności na BTZ w komórkach MM linii NCI-H929 (*76*).

Oprócz zmian w metylomie, inne mechanizmy epigenetyczne, w tym przede wszystkim tzw. niekodujące RNA (ang. non-coding RNA, ncRNA) są zaangażowane zarówno w mediowanie cytotoksycznych właściwości cząsteczki, jak również w wystąpienie oporności na lek. Do ncRNA zaliczamy między innymi mikro RNA (miRNA), małe interferujące RNA (ang. small interfering RNA, siRNA), RNA oddziaływujące na białka piwi (ang. piwi-interacting RNA, piRNA), małe regulatorowe RNA (ang. small regulatory RNA, srRNA) oraz długie niekodujące RNA (ang. long non-coding RNA, lncRNA). ncRNA regulują ekspresję informacji genetycznej na etapie potranskrypcyjnym (*77*). Liczne badania wskazują, że ncRNA, zwłaszcza miRNA oraz lncRNA, są istotnym aspektem mechanizmu działania BTZ, działając z nim synergistycznie lub resensytyzując komórki MM na działanie leku (*78–83*). Z drugiej strony wykazano, że niektóre ncRNA wpływają na oporność na BTZ lub są związane z gorszą odpowiedzią na terapię (*84–86*).

Modyfikacje epigenetyczne są istotnym aspektem mechanizmu działania BTZ, jak również rozwoju jego lekooporności. Dlatego, cząsteczki interferujące w epigenetykę, takie jak inhibitory metylotransferazy DNA (ang. DNA methyltransferase, DNMT), do których należy azacytydyna (AZA) oraz decytabina (DAC), wydają się być obiecującym kierunkiem badań (*51*, *52*, *87*).

W publikacji nr 2 będącej częścią niniejszej rozprawy doktorskiej wykazano, że AZA działa synergistyczne z BTZ. Co istotne, AZA powodowała istotny spadek proliferacji klonów opornych na BTZ. Wyniki analizy profilu metylacji wskazują, że AZA, podobnie jak sam BTZ (*70*), powoduje globalny spadek metylacji w komórkach nowotworowych, stąd zapewne ich wzajemny synergizm. Dodatkowo, poza globalnym spadkiem metylacji, który niejako warunkuje wrażliwość komórek nowotworowych na BTZ, kombinacja AZA z BTZ wywołuje zmiany w statusie metylacji poszczególnych genów w porównaniu do komórek opornych na BTZ. Szczególne znaczenie mają geny związane z onkogenezą, progresją nowotworu oraz lekoopornością. Przykładowo, po drugim pasażu komórek MM z BTZ i AZA wykazano różnice w statusie metylacji regionu „island" genu *ORAI3*, którego nadmierna ekspresja jest związana z opornością na chemioterapię, m.in. w raku piersi (*88*). Po trzecim pasażu ponownie zidentyfikowano hipometylację genu *ORAI3* w komórkach MM inkubowanych z BTZ i AZA oraz hipermetylację w komórkach MM o fenotypie opornym na BTZ. Ponadto, zidentyfikowano różnice w statusie metylacji genu *MIR21*, który jest powiązany z opornością na leczenie w różnych nowotworach, takich jak rak jajnika (*89*) i rak nerki (*90*). Co więcej, metylacja regionu 5′ UTR-opensea genu *FBXW7* istotnie różniła się między komórkami opornymi na BTZ a inkubowanymi jednocześnie z BTZ i AZA. Obecnie wiadomo, że gen *FBXW7* jest związany z opornością na leczenie i odpowiedzią na chemioterapię w różnych nowotworach (*91*–*93*). Analiza GSEA wykazała statystycznie istotne różnice w kilku kluczowych procesach związanych z rozwojem oporności na BTZ. Spośród zidentyfikowanych procesów, najważniejszym wydaje się być splicing RNA. Splicing RNA odgrywa bardzo ważną rolę w rozwoju lekooporności. Tworzenie się nieprawidłowych wariantów lub zaburzenia maszynerii splicingowej mogą powodować rozwój lekooporności i sprzyjać rozwojowi nowotworu (*94*, *95*). Znaczenie splicing RNA w kontekście rozwoju oporności na chemioterapię wykazano dotychczas w wielu chorobach nowotworowych, np. w przewlekłej białaczce szpikowej (*96*) oraz w raku piersi (*97*). Inne zespoły badawcze również wykazały, że inhibitory DNMT, takie jak AZA czy DAC, działają synergistycznie z BTZ oraz posiadają zdolność przywracania wrażliwości na lek w przypadku wystąpienia oporności (*71*, *98*–*102*).

Jak wspomniano we wstępie, immunoterapia znajduje szerokie zastosowanie w terapii MM. W kontekście MM, przeciwciała monoklonalne anty-CD38 (daratumumab, izatuximab) (*54*) oraz IMiDs odgrywają dziś kluczową rolę.

Podobnie VD oraz VK ze względu na swoje plejotropowe, w tym immunomodulujące działania (*58*) pretendują dziś do miana kluczowych cząsteczek w tzw. terapii adjuwantowej szpiczaka plazmocytowego. VD i VK, ze szczególnym uwzględnieniem VD, wykazują wiele tak zwanych „nieklasycznych działań", które są przede wszystkim związane z modulacją układu odpornościowego (*103*). Obecność receptora dla VD (ang. vitamin D receptor, VDR) wykazano na licznych komórkach, zarówno zdrowych jak i nowotworowych (*104*). Dlatego hipoteza, że VD może wpływać na komórki złośliwe, interferując w progresję nowotworu, wydaje się uzasadniona. Rzeczywiście, istnieje wiele dowodów na to, że wzrost nowotworu złośliwego jest podatny na zahamowanie związane z działaniem VD. Przykładowo, Fife i współpracownicy zbadali wpływ VD na linię komórkową ludzkiego raka piersi MDA-MB-435, linię komórkową ludzkiego raka prostaty LNCaP oraz linię komórkową ludzkiego kostniakomięsaka U2OS. W badaniu wykazano, że VD hamuje proliferację i indukuje apoptozę we wszystkich trzech badanych liniach komórkowych (*105*). Działanie przeciwnowotworowe VD nie ogranicza się jedynie do guzów litych. Również wykazano jej korzystny wpływ na nowotwory hematologiczne, w tym MM. Pomimo, że większość dotychczas opublikowanych badań przeprowadzono w warunkach *in vitro* na liniach komórkowych MM, przyniosły one obiecujące i zachęcające wyniki. Według Buscha i współpracowników utrzymanie prawidłowego stężenia VD u pacjentów ze MM wydaje się mieć ogromne znaczenie, zwłaszcza u chorych leczonych IMiDs. Wspomniani badacze wykazali *in vitro*, że VD odgrywa kluczową rolę w przywracaniu i utrzymywaniu funkcji efektorowych makrofagów, a suplementacja VD w połączeniu z IMiDs może zwiększyć skuteczność terapeutyczną przeciwciał anty-CD38 (*106*). Jak każde badanie *in vitro,* doniesienie to wymaga weryfikacji i oceny skuteczności w badaniach klinicznych. VD stosowana samodzielnie oraz w połączeniu z VK ma zdolność do hamowania potencjału proliferacyjnego komórek MM *in vitro* (*59*). Oprócz samej VD również jej analogi, takie jak EB1089, wykazują działanie cytotoksyczne względem komórek MM. Co jest istotne, EB1089 nie wywołuje działań niepożądanych związanych z hiperkalcemią (*107–109*). Stąd, mając na uwadze obiecujące rezultaty *in vitro,* istnieje pilna potrzeba weryfikacji tych rezultatów w warunkach klinicznych.

W publikacji nr 3 poddano ocenie efekt suplementacji VD i VK na zmiany epigenetyczne wpływające na potencjał proliferacyjny komórek MM. W niniejszej publikacji wykazaliśmy globalny wzrost poziomu metylacji w komórkach MM, zarówno kontrolnych jak i opornych na BTZ, inkubowanych z samą VD jak również w połączniu z VK. Poza globalnymi zmianami w metylomie, zidentyfikowaliśmy geny o istotnie zmienionych poziomach metylacji, które są związane z onkogenezą oraz progresją nowotworów. Na przykład, w kontrolnych komórkach MM U266 inkubowanych wyłącznie z VD obserwowano wzrost poziomu metylacji genów *NTN1* i *MYH10*. Geny te biorą udział w rozwoju oraz progresji różnych nowotworów, w tym nowotworów hematologicznych (*66–68*). Podobnie, w komórkach opornych na BTZ zaobserwowaliśmy hipermetylację genu *ARHGAP26*, który bierze udział w kancerogenezie oraz progresji ludzkich nowotworów. Warto jednak nadmienić, że w różnych typach nowotworów związek ekspresji *ARHGAP26* z onkogenezą lub progresją guza jest odmienny. Na przykład Qian i in. wykazali, że hipermetylacja *ARHGAP26* powoduje zmniejszoną ekspresję genu, co może być wczesnym zdarzeniem w patogenezie AML. Podobne zależności zaobserwowali Bojesen i wsp. (*110, 111*). Z drugiej strony, Li i współpracownicy wykazali wzrost ekspresji *ARHGAP26* w raku prostaty (*112*). W przypadku genu *BAMBI*, zarówno jego zmniejszona jak i zwiększona ekspresja, wiążą się z rozwojem i progresją nowotworów (*63, 113*). Natomiast, niska ekspresja genu *CLEC12B* jest związana rozwojem i progresją nowotworów (*64, 65*), co również znalazło odzwierciedlenie w naszych wynikach. W komórkach MM, kontrolnych oraz opornych na BTZ, ekspresja tego genu jest niższa niż w komórkach inkubowanych z VD i VK.

Podsumowując, VD oraz VK zmniejszają potencjał proliferacyjny komórek MM. Powyższy efekt jest przynajmniej częściowo warunkowany zmianami w metylomie indukowanymi przez witaminy. Zmiany w poziomie metylacji są widoczne w skali całego genomu, jak również dotyczą genów, które są związane z onkogenezą i progresją nowotworów.

Wnioski

1. Modyfikacje epigenetyczne, takie jak zmiany w profilu metylacji, ncRNA oraz modyfikacje histonów, są istotnym aspektem mechanizmu działania BTZ.
2. Cytotoksyczne właściwości cząsteczki BTZ są przede wszystkim związane z globalnym spadkiem metylacji w komórkach nowotworowych eksponowanych na lek.

Jednocześnie, rozwój oporność na BTZ jest również związana ze zmianami w metylomie.

3. Zastosowanie inhibitorów metylacji, zwłaszcza AZA, w terapii MM ma podstawy patofizjologiczne. Po pierwsze, ze względu na działanie synergistyczne z BTZ, AZA może stać się składową schematów leczenia opartych o BTZ. Po drugie, AZA ma potencjał do resensytyzacji opornych komórek MM na BTZ oraz wykazuje działanie cytotoksyczne względem opornych klonów, co czyni ją potencjalnym kandydatem do zastosowania w kolejnych liniach leczenia.
4. VD oraz VK ze względu na swoje plejotropowne działania związane z modulacją układu immunologicznego oraz wpływ na epigenetykę, mogą być wkrótce rutynowo wykorzystywane w ramach tzw. terapii adjuwantowej.
5. Powyższe, obiecujące spostrzeżenia wymagają dalszej weryfikacji na alternatywnych liniach komórkowych MM oraz finalnie w modelu badania klinicznego.

Bibliografia

1. Katzmann, J. A., Dispenzieri, A., Kyle, R. A., Snyder, M. R., Plevak, M. F., Larson, D. R., Abraham, R. S., Lust, J. A., Melton, L. J., and Rajkumar, S. V. (2006) Elimination of the Need for Urine Studies in the Screening Algorithm for Monoclonal Gammopathies by Using Serum Immunofixation and Free Light Chain Assays, *Mayo Clinic Proceedings 81*, 1575–1578.
2. Kyle, R. A., Gertz, M. A., Witzig, T. E., Lust, J. A., Lacy, M. Q., Dispenzieri, A., Fonseca, R., Rajkumar, S. V., Offord, J. R., Larson, D. R., Plevak, M. E., Therneau, T. M., and Greipp, P. R. (2003) Review of 1027 Patients With Newly Diagnosed Multiple Myeloma, *Mayo Clinic Proceedings 78*, 21–33.
3. Landgren, O., Kyle, R. A., Pfeiffer, R. M., Katzmann, J. A., Caporaso, N. E., Hayes, R. B., Dispenzieri, A., Kumar, S., Clark, R. J., Baris, D., Hoover, R., and Rajkumar, S. V. (2009) Monoclonal gammopathy of undetermined significance (MGUS) consistently precedes multiple myeloma: a prospective study, *Blood 113*, 5412–5417.
4. Dhodapkar, M. V. (2016) MGUS to myeloma: a mysterious gammopathy of underexplored significance, *Blood 128*, 2599–2606.
5. Siegel, R. L., Miller, K. D., and Jemal, A. (2016) Cancer statistics, 2016, *CA: A Cancer Journal for Clinicians 66*, 7–30.
6. Szpiczak plazmocytowy. Ocena jakości informacyjnej rejestru kontraktowego. https://ezdrowie.gov.pl/portal/home/badania-i-dane/zdrowe-dane/raporty/szpiczak-plazmocytowy.
7. Palumbo, A., Bringhen, S., Ludwig, H., Dimopoulos, M. A., Bladé, J., Mateos, M. V., Rosiñol, L., Boccadoro, M., Cavo, M., Lokhorst, H., Zweegman, S., Terpos, E., Davies, F., Driessen, C., Gimsing, P., Gramatzki, M., Hàjek, R., Johnsen, H. E., Leal Da Costa, F., Sezer, O., Spencer, A., Beksac, M., Morgan, G., Einsele, H., San Miguel, J. F., and Sonneveld, P. (2011) Personalized therapy in multiple myeloma according to patient age and vulnerability: a report of the European Myeloma Network (EMN), *Blood 118*, 4519–4529.

8. Bergsagel, P. L., and Kuehl, W. M. (2003) Critical roles for immunoglobulin translocations and cyclin D dysregulation in multiple myeloma, *Immunological Reviews 194*, 96–104.
9. Bergsagel, P. L., Kuehl, W. M., Zhan, F., Sawyer, J., Barlogie, B., and Shaughnessy, J. (2005) Cyclin D dysregulation: an early and unifying pathogenic event in multiple myeloma, *Blood 106*, 296–303.
10. Lewis, W. D., Lilly, S., and Jones, K. L. (2020) Lymphoma: Diagnosis and Treatment, *American Family Physician 101*, 34–41.
11. Nakaya, A., Fujita, S., Satake, A., Nakanishi, T., Azuma, Y., Tsubokura, Y., Hotta, M., Yoshimura, H., Ishii, K., Ito, T., and Nomura, S. (2017) Impact of CRAB Symptoms in Survival of Patients with Symptomatic Myeloma in Novel Agent Era, *Hematology Reports 9*, 16–18.
12. Terpos, E., Ntanasis-Stathopoulos, I., Gavriatopoulou, M., and Dimopoulos, M. A. (2018) Pathogenesis of bone disease in multiple myeloma: from bench to bedside, *Blood Cancer Journal 8*, 7.
13. Kundu, S., Jha, S. B., Rivera, A. P., Flores Monar, G. V., Islam, H., Puttagunta, S. M., Islam, R., and Sange, I. (2022) Multiple Myeloma and Renal Failure: Mechanisms, Diagnosis, and Management, *Cureus*.
14. Tucci, M., Grinello, D., Cafforio, P., Silvestris, F., and Dammacco, F. (2002) Anemia in Multiple Myeloma: Role of Deregulated Plasma Cell Apoptosis, *Leukemia & Lymphoma 43*, 1527–1533.
15. Thorsteinsdottir, S., Dickman, P. W., Landgren, O., Blimark, C., Hultcrantz, M., Turesson, I., Björkholm, M., and Kristinsson, S. Y. (2018) Dramatically improved survival in multiple myeloma patients in the recent decade: results from a Swedish population-based study, *Haematologica 103*, e412–e415.
16. Speirs, A. . (1962) THALIDOMIDE AND CONGENITAL ABNORMALITIES, *The Lancet 279*, 303–305.
17. Koeppen, S. (2014) Treatment of Multiple Myeloma: Thalidomide-, Bortezomib-, and Lenalidomide-Induced Peripheral Neuropathy, *Oncology Research and Treatment 37*, 506–513.
18. Richardson, P. G., Schlossman, R. L., Weller, E., Hideshima, T., Mitsiades, C., Davies, F., LeBlanc, R., Catley, L. P., Doss, D., Kelly, K., McKenney, M., Mechlowicz, J., Freeman, A., Deocampo, R., Rich, R., Ryoo, J. J., Chauhan, D., Balinski, K., Zeldis, J., and Anderson, K. C. (2002) Immunomodulatory drug CC-5013 overcomes drug resistance and is well tolerated in patients with relapsed multiple myeloma, *Blood 100*, 3063–3067.
19. Lacy, M. Q., and McCurdy, A. R. (2013) Pomalidomide, *Blood 122*, 2305–2309.
20. Richardson, P. G., Mitsiades, C., Hideshima, T., and Anderson, K. C. (2006) Bortezomib: Proteasome Inhibition as an Effective Anticancer Therapy, *Annual Review of Medicine 57*, 33–47.
21. Levêque, D., Carvalho, M. C. M., and Maloisel, F. (2007) Review. Clinical pharmacokinetics of bortezomib, *In Vivo (Athens, Greece) 21*, 273–278.
22. Yee, A. J. (2021) The role of carfilzomib in relapsed/refractory multiple myeloma, *Therapeutic Advances in Hematology 12*, 204062072110196.
23. Bonnet, A., and Moreau, P. (2017) Safety of ixazomib for the treatment of multiple myeloma, *Expert Opinion on Drug Safety 16*, 973–980.
24. Hamilton, A. L., Eder, J. P., Pavlick, A. C., Clark, J. W., Liebes, L., Garcia-Carbonero, R., Chachoua, A., Ryan, D. P., Soma, V., Farrell, K., Kinchla, N., Boyden, J., Yee, H., Zeleniuch-Jacquotte, A., Wright, J., Elliott, P., Adams, J., and Muggia, F. M. (2005) Proteasome Inhibition With Bortezomib (PS-341): A Phase I Study With

Pharmacodynamic End Points Using a Day 1 and Day 4 Schedule in a 14-Day Cycle, *Journal of Clinical Oncology 23*, 6107–6116.

25. Moreau, P., Pylypenko, H., Grosicki, S., Karamanesht, I., Leleu, X., Grishunina, M., Rekhtman, G., Masliak, Z., Robak, T., Shubina, A., Arnulf, B., Kropff, M., Cavet, J., Esseltine, D.-L., Feng, H., Girgis, S., Van De Velde, H., Deraedt, W., and Harousseau, J.-L. (2011) Subcutaneous versus intravenous administration of bortezomib in patients with relapsed multiple myeloma: a randomised, phase 3, non-inferiority study, *The Lancet Oncology 12*, 431–440.
26. Uttamsingh, V., Lu, C., Miwa, G., and Gan, L.-S. (2005) RELATIVE CONTRIBUTIONS OF THE FIVE MAJOR HUMAN CYTOCHROMES P450, 1A2, 2C9, 2C19, 2D6, AND 3A4, TO THE HEPATIC METABOLISM OF THE PROTEASOME INHIBITOR BORTEZOMIB, *Drug Metabolism and Disposition 33*, 1723–1728.
27. Labutti, J., Parsons, I., Huang, R., Miwa, G., Gan, L.-S., and Daniels, J. S. (2006) Oxidative Deboronation of the Peptide Boronic Acid Proteasome Inhibitor Bortezomib: Contributions from Reactive Oxygen Species in This Novel Cytochrome P450 Reaction, *Chemical Research in Toxicology 19*, 539–546.
28. Schwartz, R., and Davidson, T. (2004) Pharmacology, pharmacokinetics, and practical applications of bortezomib, *Oncology (Williston Park, N.Y.) 18*, 14–21.
29. Leal, T. B., Remick, S. C., Takimoto, C. H., Ramanathan, R. K., Davies, A., Egorin, M. J., Hamilton, A., LoRusso, P. A., Shibata, S., Lenz, H.-J., Mier, J., Sarantopoulos, J., Mani, S., Wright, J. J., Ivy, S. P., Neuwirth, R., Von Moltke, L., Venkatakrishnan, K., and Mulkerin, D. (2011) Dose-escalating and pharmacological study of bortezomib in adult cancer patients with impaired renal function: a National Cancer Institute Organ Dysfunction Working Group Study, *Cancer Chemotherapy and Pharmacology 68*, 1439–1447.
30. Richardson, P. G., Barlogie, B., Berenson, J., Singhal, S., Jagannath, S., Irwin, D., Rajkumar, S. V., Srkalovic, G., Alsina, M., Alexanian, R., Siegel, D., Orlowski, R. Z., Kuter, D., Limentani, S. A., Lee, S., Hideshima, T., Esseltine, D.-L., Kauffman, M., Adams, J., Schenkein, D. P., and Anderson, K. C. (2003) A Phase 2 Study of Bortezomib in Relapsed, Refractory Myeloma, *New England Journal of Medicine 348*, 2609–2617.
31. Berenson, J. R., Jagannath, S., Barlogie, B., Siegel, D. T., Alexanian, R., Richardson, P. G., Irwin, D., Alsina, M., Rajkumar, S. V., Srkalovic, G., Singhal, S., Limentani, S., Niesvizky, R., Esseltine, D. L., Trehu, E., Schenkein, D. P., and Anderson, K. (2005) Safety of prolonged therapy with bortezomib in relapsed or refractory multiple myeloma, *Cancer 104*, 2141–2148.
32. Lee, K.-W., Yun, T., Song, E. K., Na, I. I., Shin, H., Bang, S.-M., Lee, J. H., Lee, S. T., Kim, J. H., Yoon, S.-S., Lee, J. S., Park, S., Kim, B. K., and Kim, N. K. (2005) A Pilot Study of Bortezomib in Korean Patients with Relapsed or Refractory Myeloma, *Journal of Korean Medical Science 20*, 598.
33. Reece, D. E., Sanchorawala, V., Hegenbart, U., Merlini, G., Palladini, G., Fermand, J.-P., Vescio, R. A., Liu, X., Elsayed, Y. A., Cakana, A., Comenzo, R. L., and VELCADE CAN2007 Study Group, for the. (2009) Weekly and twice-weekly bortezomib in patients with systemic AL amyloidosis: results of a phase 1 dose-escalation study, *Blood 114*, 1489–1497.
34. Reece, D. E., Hegenbart, U., Sanchorawala, V., Merlini, G., Palladini, G., Bladé, J., Fermand, J.-P., Hassoun, H., Heffner, L., Vescio, R. A., Liu, K., Enny, C., Esseltine, D.-L., Van De Velde, H., Cakana, A., and Comenzo, R. L. (2011) Efficacy and safety of once-weekly and twice-weekly bortezomib in patients with relapsed systemic AL amyloidosis: results of a phase 1/2 study, *Blood 118*, 865–873.

35. Besse, B., Planchard, D., Veillard, A.-S., Taillade, L., Khayat, D., Ducourtieux, M., Pignon, J.-P., Lumbroso, J., Lafontaine, C., Mathiot, C., and Soria, J.-C. (2012) Phase 2 study of frontline bortezomib in patients with advanced non-small cell lung cancer, *Lung Cancer 76*, 78–83.
36. Di Bella, N., Taetle, R., Kolibaba, K., Boyd, T., Raju, R., Barrera, D., Cochran, E. W., Dien, P. Y., Lyons, R., Schlegel, P. J., Vukelja, S. J., Boston, J., Boehm, K. A., Wang, Y., and Asmar, L. (2010) Results of a phase 2 study of bortezomib in patients with relapsed or refractory indolent lymphoma, *Blood 115*, 475–480.
37. Hrusovsky, I., Emmerich, B., Von Rohr, A., Voegeli, J., Taverna, C., Olie, R. A., Pliskat, H., Frohn, C., and Hess, G. (2010) Bortezomib Retreatment in Relapsed Multiple Myeloma – Results from a Retrospective Multicentre Survey in Germany and Switzerland, *Oncology 79*, 247–254.
38. Hulin, C., De La Rubia, J., Dimopoulos, M. A., Terpos, E., Katodritou, E., Hungria, V., De Samblanx, H., Stoppa, A., Aagesen, J., Sargin, D., Sioni, A., Belch, A., Diels, J., Olie, R. A., Robinson, D., Potamianou, A., Van De Velde, H., and Delforge, M. (2019) Bortezomib retreatment for relapsed and refractory multiple myeloma in real‐world clinical practice, *Health Science Reports 2*, e104.
39. Sood, R., Carloss, H., Kerr, R., Lopez, J., Lee, M., Druck, M., Walters, I. B., and Noga, S. J. (2009) Retreatment with bortezomib alone or in combination for patients with multiple myeloma following an initial response to bortezomib, *American Journal of Hematology 84*, 657–660.
40. Palumbo, A., Chanan-Khan, A., Weisel, K., Nooka, A. K., Masszi, T., Beksac, M., Spicka, I., Hungria, V., Munder, M., Mateos, M. V., Mark, T. M., Qi, M., Schecter, J., Amin, H., Qin, X., Deraedt, W., Ahmadi, T., Spencer, A., and Sonneveld, P. (2016) Daratumumab, Bortezomib, and Dexamethasone for Multiple Myeloma, *New England Journal of Medicine 375*, 754–766.
41. Grosicki, S., Simonova, M., Spicka, I., Pour, L., Kriachok, I., Gavriatopoulou, M., Pylypenko, H., Auner, H. W., Leleu, X., Doronin, V., Usenko, G., Bahlis, N. J., Hajek, R., Benjamin, R., Dolai, T. K., Sinha, D. K., Venner, C. P., Garg, M., Gironella, M., Jurczyszyn, A., Robak, P., Galli, M., Wallington-Beddoe, C., Radinoff, A., Salogub, G., Stevens, D. A., Basu, S., Liberati, A. M., Quach, H., Goranova-Marinova, V. S., Bila, J., Katodritou, E., Oliynyk, H., Korenkova, S., Kumar, J., Jagannath, S., Moreau, P., Levy, M., White, D., Gatt, M. E., Facon, T., Mateos, M. V., Cavo, M., Reece, D., Anderson, L. D., Saint-Martin, J.-R., Jeha, J., Joshi, A. A., Chai, Y., Li, L., Peddagali, V., Arazy, M., Shah, J., Shacham, S., Kauffman, M. G., Dimopoulos, M. A., Richardson, P. G., and Delimpasi, S. (2020) Once-per-week selinexor, bortezomib, and dexamethasone versus twice-per-week bortezomib and dexamethasone in patients with multiple myeloma (BOSTON): a randomised, open-label, phase 3 trial, *The Lancet 396*, 1563–1573.
42. Teachey, D. T., Devidas, M., Wood, B. L., Chen, Z., Hayashi, R. J., Hermiston, M. L., Annett, R. D., Archer, J. H., Asselin, B. L., August, K. J., Cho, S. Y., Dunsmore, K. P., Fisher, B. T., Freedman, J. L., Galardy, P. J., Harker-Murray, P., Horton, T. M., Jaju, A. I., Lam, A., Messinger, Y. H., Miles, R. R., Okada, M., Patel, S. I., Schafer, E. S., Schechter, T., Singh, N., Steele, A. C., Sulis, M. L., Vargas, S. L., Winter, S. S., Wood, C., Zweidler-McKay, P., Bollard, C. M., Loh, M. L., Hunger, S. P., and Raetz, E. A. (2022) Children's Oncology Group Trial AALL1231: A Phase III Clinical Trial Testing Bortezomib in Newly Diagnosed T-Cell Acute Lymphoblastic Leukemia and Lymphoma, *Journal of Clinical Oncology 40*, 2106–2118.
43. Irvin, W. J., Orlowski, R. Z., Chiu, W.-K., Carey, L. A., Collichio, F. A., Bernard, P. S., Stijleman, I. J., Perou, C., Ivanova, A., and Dees, E. C. (2010) Phase II Study of

Bortezomib and Pegylated Liposomal Doxorubicin in the Treatment of Metastatic Breast Cancer, *Clinical Breast Cancer 10*, 465–470.
44. Dirix, L. (2011) A phase II study of the combination of endocrine treatment and bortezomib in patients with endocrine-resistant metastatic breast cancerA phase II study of the combination of endocrine treatment and bortezomib in patients with endocrine-resistant metastatic breast cancer, *Oncology Reports*.
45. Lee, Y. J., Seol, A., Lee, M., Kim, J.-W., Kim, H. S., Kim, K., Suh, D. H., Kim, S., Kim, S. W., and Lee, J.-Y. (2022) A Phase II Trial to Evaluate the Efficacy of Bortezomib and Liposomal Doxorubicin in Patients With BRCA Wild-type Platinum-resistant Recurrent Ovarian Cancer (KGOG 3044/EBLIN), *In Vivo 36*, 1949–1958.
46. Lara, P. N., Longmate, J., Reckamp, K., Gitlitz, B., Argiris, A., Ramalingam, S., Belani, C. P., Mack, P. C., Lau, D. H. M., Koczywas, M., Wright, J. J., Shepherd, F. A., Leighl, N., and Gandara, D. R. (2011) Randomized Phase II Trial of Concurrent Versus Sequential Bortezomib Plus Docetaxel in Advanced Non–Small-Cell Lung Cancer: A California Cancer Consortium Trial, *Clinical Lung Cancer 12*, 33–37.
47. Raedler, L. (2015) Velcade (Bortezomib) Receives 2 New FDA Indications: For Retreatment of Patients with Multiple Myeloma and for First-Line Treatment of Patients with Mantle-Cell Lymphoma, *American Health & Drug Benefits 8*, 135–140.
48. Hamilton, J. P. (2011) Epigenetics: Principles and Practice, *Digestive Diseases 29*, 130–135.
49. Metere, A., and Graves, C. E. (2020) Factors Influencing Epigenetic Mechanisms: Is There A Role for Bariatric Surgery?, *High-Throughput 9*, 6.
50. Wu, Y.-L., Lin, Z.-J., Li, C.-C., Lin, X., Shan, S.-K., Guo, B., Zheng, M.-H., Li, F., Yuan, L.-Q., and Li, Z. (2023) Epigenetic regulation in metabolic diseases: mechanisms and advances in clinical study, *Signal Transduction and Targeted Therapy 8*, 98.
51. Moreno Vanegas, Y., and Badar, T. (2022) Clinical Utility of Azacitidine in the Management of Acute Myeloid Leukemia: Update on Patient Selection and Reported Outcomes, *Cancer Management and Research Volume 14*, 3527–3538.
52. Khan, C., Pathe, N., Fazal, S., Lister, J., and Rossetti, J. M. (2012) Azacitidine in the management of patients with myelodysplastic syndromes, *Therapeutic Advances in Hematology 3*, 355–373.
53. Abramson, H. N. (2023) Immunotherapy of Multiple Myeloma: Current Status as Prologue to the Future, *International Journal of Molecular Sciences 24*, 15674.
54. Petrucci, M. T., and Vozella, F. (2019) The Anti-CD38 Antibody Therapy in Multiple Myeloma, *Cells 8*, 1629.
55. Raje, N., Anderson, K., Einsele, H., Efebera, Y., Gay, F., Hammond, S. P., Lesokhin, A. M., Lonial, S., Ludwig, H., Moreau, P., Patel, K., Ramasamy, K., and Mateos, M.-V. (2023) Monitoring, prophylaxis, and treatment of infections in patients with MM receiving bispecific antibody therapy: consensus recommendations from an expert panel, *Blood Cancer Journal 13*, 116.
56. Firestone, R. S., and Mailankody, S. (2023) Current use of CAR T cells to treat multiple myeloma, *Hematology 2023*, 340–347.
57. Skorka, K., Ostapinska, K., Malesa, A., and Giannopoulos, K. (2020) The Application of CAR-T Cells in Haematological Malignancies, *Archivum Immunologiae et Therapiae Experimentalis 68*, 34.
58. Baeke, F., Takiishi, T., Korf, H., Gysemans, C., and Mathieu, C. (2010) Vitamin D: modulator of the immune system, *Current Opinion in Pharmacology 10*, 482–496.
59. Łuczkowska, K., Kulig, P., Baumert, B., and Machaliński, B. (2022) The Evidence That 25(OH)D3 and VK2 MK-7 Vitamins Influence the Proliferative Potential and Gene Expression Profiles of Multiple Myeloma Cells and the Development of Resistance to Bortezomib, *Nutrients 14*, 5190.

60. Salehan, M. R., and Morse, H. R. (2013) DNA damage repair and tolerance: a role in chemotherapeutic drug resistance, *British Journal of Biomedical Science 70*, 31–40.
61. Li, Y., Cui, K., Zhang, Q., Li, X., Lin, X., Tang, Y., Prochownik, E. V., and Li, Y. (2021) FBXL6 degrades phosphorylated p53 to promote tumor growth, *Cell Death & Differentiation 28*, 2112–2125.
62. Sugiaman-Trapman, D., Vitezic, M., Jouhilahti, E.-M., Mathelier, A., Lauter, G., Misra, S., Daub, C. O., Kere, J., and Swoboda, P. (2018) Characterization of the human RFX transcription factor family by regulatory and target gene analysis, *BMC Genomics 19*, 181.
63. Khin, S. S., Kitazawa, R., Win, N., Aye, T. T., Mori, K., Kondo, T., and Kitazawa, S. (2009) BAMBI gene is epigenetically silenced in subset of high‐grade bladder cancer, *International Journal of Cancer 125*, 328‐338.
64. Montaudié, H., Sormani, L., Dadone-Montaudié, B., Heim, M., Cardot-Leccia, N., Tulic, M. K., Beranger, G., Gay, A.-S., Debayle, D., Cheli, Y., Raymond, J. H., Sohier, P., Petit, V., Rocchi, S., Gesbert, F., Larue, L., and Passeron, T. (2022) CLEC12B Decreases Melanoma Proliferation by Repressing Signal Transducer and Activator of Transcription 3, *Journal of Investigative Dermatology 142*, 425–434.
65. Chi, D., Wang, D., Zhang, M., Ma, H., Chen, F., and Sun, Y. (2021) CLEC12B suppresses lung cancer progression by inducing SHP-1 expression and inactivating the PI3K/AKT signaling pathway, *Experimental Cell Research 409*, 112914.
66. Nakayama, H., Ohnuki, H., Nakahara, M., Nishida-Fukuda, H., Sakaue, T., Fukuda, S., Higashiyama, S., Doi, Y., Mitsuyoshi, M., Okimoto, T., Tosato, G., and Kusumoto, C. (2022) Inactivation of axon guidance molecule netrin-1 in human colorectal cancer by an epigenetic mechanism, *Biochemical and Biophysical Research Communications 611*, 146–150.
67. Huang, L., An, X., Zhu, Y., Zhang, K., Xiao, L., Yao, X., Zeng, X., Liang, S., and Yu, J. (2022) Netrin-1 induces the anti-apoptotic and pro-survival effects of B-ALL cells through the Unc5b-MAPK axis, *Cell Communication and Signaling 20*, 122.
68. Jin, Q., Cheng, M., Xia, X., Han, Y., Zhang, J., Cao, P., and Zhou, G. (2021) Down‐regulation of MYH10 driven by chromosome 17p13.1 deletion promotes hepatocellular carcinoma metastasis through activation of the EGFR pathway, *Journal of Cellular and Molecular Medicine 25*, 11142–11156.
69. Zhang, L., Zhou, A., Zhu, S., Min, L., Liu, S., Li, P., and Zhang, S. (2022) The role of GTPase-activating protein ARHGAP26 in human cancers, *Molecular and Cellular Biochemistry 477*, 319–326.
70. Liu, S., Liu, Z., Xie, Z., Pang, J., Yu, J., Lehmann, E., Huynh, L., Vukosavljevic, T., Takeki, M., Klisovic, R. B., Baiocchi, R. A., Blum, W., Porcu, P., Garzon, R., Byrd, J. C., Perrotti, D., Caligiuri, M. A., Chan, K. K., Wu, L.-C., and Marcucci, G. (2008) Bortezomib induces DNA hypomethylation and silenced gene transcription by interfering with Sp1/NF-κB–dependent DNA methyltransferase activity in acute myeloid leukemia, *Blood 111*, 2364–2373.
71. Leshchenko, V. V., Kuo, P.-Y., Jiang, Z., Weniger, M. A., Overbey, J., Dunleavy, K., Wilson, W. H., Wiestner, A., and Parekh, S. (2015) Harnessing Noxa demethylation to overcome Bortezomib resistance in mantle cell lymphoma, *Oncotarget 6*, 27332–27342.
72. Wirth, M., Stojanovic, N., Christian, J., Paul, M. C., Stauber, R. H., Schmid, R. M., Häcker, G., Krämer, O. H., Saur, D., and Schneider, G. (2014) MYC and EGR1 synergize to trigger tumor cell death by controlling NOXA and BIM transcription upon treatment with the proteasome inhibitor bortezomib, *Nucleic Acids Research 42*, 10433–10447.
73. Kikuchi, J., Wada, T., Shimizu, R., Izumi, T., Akutsu, M., Mitsunaga, K., Noborio-Hatano, K., Nobuyoshi, M., Ozawa, K., Kano, Y., and Furukawa, Y. (2010) Histone

deacetylases are critical targets of bortezomib-induced cytotoxicity in multiple myeloma, *Blood 116*, 406–417.
74. Tsvetkov, P., Sokol, E., Jin, D., Brune, Z., Thiru, P., Ghandi, M., Garraway, L. A., Gupta, P. B., Santagata, S., Whitesell, L., and Lindquist, S. (2017) Suppression of 19S proteasome subunits marks emergence of an altered cell state in diverse cancers, *Proceedings of the National Academy of Sciences 114*, 382–387.
75. Łuczkowska, K., Sokolowska, K. E., Taryma-Lesniak, O., Pastuszak, K., Supernat, A., Bybjerg-Grauholm, J., Hansen, L. L., Paczkowska, E., Wojdacz, T. K., and Machaliński, B. (2021) Bortezomib induces methylation changes in neuroblastoma cells that appear to play a significant role in resistance development to this compound, *Scientific Reports 11*, 9846.
76. Hu, X., Xuan, H., Du, H., Jiang, H., and Huang, J. (2014) Down-Regulation of CD9 by Methylation Decreased Bortezomib Sensitivity in Multiple Myeloma, *PLoS ONE* (Agoulnik, I. U., Ed.) *9*, e95765.
77. Wei, J.-W., Huang, K., Yang, C., and Kang, C.-S. (2017) Non-coding RNAs as regulators in epigenetics, *Oncology Reports 37*, 3–9.
78. Chu, Y.-Y., Ko, C.-Y., Wang, S.-M., Lin, P.-I., Wang, H.-Y., Lin, W.-C., Wu, D.-Y., Wang, L.-H., and Wang, J.-M. (2017) Bortezomib-induced miRNAs direct epigenetic silencing of locus genes and trigger apoptosis in leukemia, *Cell Death & Disease 8*, e3167–e3167.
79. Amodio, N., Di Martino, M. T., Foresta, U., Leone, E., Lionetti, M., Leotta, M., Gullà, A. M., Pitari, M. R., Conforti, F., Rossi, M., Agosti, V., Fulciniti, M., Misso, G., Morabito, F., Ferrarini, M., Neri, A., Caraglia, M., Munshi, N. C., Anderson, K. C., Tagliaferri, P., and Tassone, P. (2012) miR-29b sensitizes multiple myeloma cells to bortezomib-induced apoptosis through the activation of a feedback loop with the transcription factor Sp1, *Cell Death & Disease 3*, e436–e436.
80. Liu, Y., Cheng, P., Zhao, W., Zhu, L., Sui, J., Dai, Y., and Lai, Y. (2022) MiR-197-3p reduces bortezomib resistance in multiple myeloma by inhibiting IL-6 expression in a MEAF6-dependent manner, *Leukemia Research 114*, 106785.
81. Xi, H., Li, L., Du, J., An, R., Fan, R., Lu, J., Wu, Y.-X., Wu, S.-X., Hou, J., and Zhao, L.-M. (2017) hsa-miR-631 resensitizes bortezomib-resistant multiple myeloma cell lines by inhibiting UbcH10, *Oncology Reports 37*, 961–968.
82. Tian, F., Zhan, Y., Zhu, W., Li, J., Tang, M., Chen, X., and Jiang, J. (2018) MicroRNA-497 inhibits multiple myeloma growth and increases susceptibility to bortezomib by targeting Bcl-2, *International Journal of Molecular Medicine*.
83. Yuan, X., Ma, R., Yang, S., Jiang, L., Wang, Z., Zhu, Z., and Li, H. (2019) miR-520g and miR-520h overcome bortezomib resistance in multiple myeloma via suppressing APE1, *Cell Cycle 18*, 1660–1669.
84. Nian, F., Zhu, J., and Chang, H. (2019) Long non-coding RNA ANGPTL1-3 promotes multiple myeloma bortezomib resistance by sponging miR-30a-3p to activate c-Maf expression, *Biochemical and Biophysical Research Communications 514*, 1140–1146.
85. Liu, D., Wang, Y., Li, H., Peng, S., Tan, H., and Huang, Z. (2022) Circular RNA circ-CCT3 promotes bortezomib resistance in multiple myeloma via modulating miR-223-3p/BRD4 axis, *Anti-Cancer Drugs 33*, e145–e154.
86. Saltarella, I., Lamanuzzi, A., Desantis, V., Di Marzo, L., Melaccio, A., Curci, P., Annese, T., Nico, B., Solimando, A. G., Bartoli, G., Tolomeo, D., Storlazzi, C. T., Mariggiò, M. A., Ria, R., Musto, P., Vacca, A., and Frassanito, M. A. (2022) Myeloma cells regulate MIRNA transfer from fibroblast‐derived exosomes by expression of lncRNAs, *The Journal of Pathology 256*, 402‐413.
87. Gore, S. D., Jones, C., and Kirkpatrick, P. (2006) Decitabine, *Nature Reviews Drug Discovery 5*, 891–892.

88. Hasna, J., Hague, F., Rodat-Despoix, L., Geerts, D., Leroy, C., Tulasne, D., Ouadid-Ahidouch, H., and Kischel, P. (2018) Orai3 calcium channel and resistance to chemotherapy in breast cancer cells: the p53 connection, *Cell Death & Differentiation 25*, 693–707.
89. Sheng, S., Su, W., Mao, D., Li, C., Hu, X., Deng, W., Yao, Y., and Ji, Y. (2022) MicroRNA-21 induces cisplatin resistance in head and neck squamous cell carcinoma, *PLOS ONE* (El-Ashram, S., Ed.) *17*, e0267017.
90. Gaudelot, K., Gibier, J.-B., Pottier, N., Hémon, B., Van Seuningen, I., Glowacki, F., Leroy, X., Cauffiez, C., Gnemmi, V., Aubert, S., and Perrais, M. (2017) Targeting miR-21 decreases expression of multi-drug resistant genes and promotes chemosensitivity of renal carcinoma, *Tumor Biology 39*, 101042831770737.
91. Fan, J., Bellon, M., Ju, M., Zhao, L., Wei, M., Fu, L., and Nicot, C. (2022) Clinical significance of FBXW7 loss of function in human cancers, *Molecular Cancer 21*, 87.
92. Chen, S., Lin, J., Zhao, J., Lin, Q., Liu, J., Wang, Q., Mui, R., and Ma, L. (2023) FBXW7 attenuates tumor drug resistance and enhances the efficacy of immunotherapy, *Frontiers in Oncology 13*, 1147239.
93. Mun, G.-I., Choi, E., Lee, Y., and Lee, Y.-S. (2020) Decreased expression of FBXW7 by ERK1/2 activation in drug-resistant cancer cells confers transcriptional activation of MDR1 by suppression of ubiquitin degradation of HSF1, *Cell Death & Disease 11*, 395.
94. Wu, T., Hu, E., Xu, S., Chen, M., Guo, P., Dai, Z., Feng, T., Zhou, L., Tang, W., Zhan, L., Fu, X., Liu, S., Bo, X., and Yu, G. (2021) clusterProfiler 4.0: A universal enrichment tool for interpreting omics data, *The Innovation 2*, 100141.
95. Wang, B.-D., and Lee, N. (2018) Aberrant RNA Splicing in Cancer and Drug Resistance, *Cancers 10*, 458.
96. Lee, T.-S., Ma, W., Zhang, X., Giles, F., Cortes, J., Kantarjian, H., and Albitar, M. (2008) BCR-ABL alternative splicing as a common mechanism for imatinib resistance: evidence from molecular dynamics simulations, *Molecular Cancer Therapeutics 7*, 3834–3841.
97. Wang, Y., Bernhardy, A. J., Cruz, C., Krais, J. J., Nacson, J., Nicolas, E., Peri, S., Van Der Gulden, H., Van Der Heijden, I., O'Brien, S. W., Zhang, Y., Harrell, M. I., Johnson, S. F., Candido Dos Reis, F. J., Pharoah, P. D. P., Karlan, B., Gourley, C., Lambrechts, D., Chenevix-Trench, G., Olsson, H., Benitez, J. J., Greene, M. H., Gore, M., Nussbaum, R., Sadetzki, S., Gayther, S. A., Kjaer, S. K., Investigators, kConFab, D'Andrea, A. D., Shapiro, G. I., Wiest, D. L., Connolly, D. C., Daly, M. B., Swisher, E. M., Bouwman, P., Jonkers, J., Balmaña, J., Serra, V., and Johnson, N. (2016) The BRCA1-Δ11q Alternative Splice Isoform Bypasses Germline Mutations and Promotes Therapeutic Resistance to PARP Inhibition and Cisplatin, *Cancer Research 76*, 2778–2790.
98. Łuczkowska, K., Kulig, P., Rusińska, K., Baumert, B., and Machaliński, B. (2023) 5-Aza-2′ -Deoxycytidine Alters the Methylation Profile of Bortezomib-Resistant U266 Multiple Myeloma Cells and Affects Their Proliferative Potential, *International Journal of Molecular Sciences 24*, 16780.
99. Jin, Y., Xu, L., Wu, X., Feng, J., Shu, M., Gu, H., Gao, G., Zhang, J., Dong, B., and Chen, X. (2019) Synergistic Efficacy of the Demethylation Agent Decitabine in Combination With the Protease Inhibitor Bortezomib for Treating Multiple Myeloma Through the Wnt/β-Catenin Pathway, *Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics 27*, 729–737.
100. Li, M., Qi, L., Xu, J.-B., Zhong, L.-Y., Chan, S., Chen, S.-N., Shao, X.-R., Zheng, L.-Y., Dong, Z.-X., Fang, T.-L., Mai, Z.-Y., Li, J., Zheng, Y., and Zhang, X.-D. (2020) Methylation of the Promoter Region of the Tight Junction Protein-1 by DNMT1 Induces EMT-like Features in Multiple Myeloma, *Molecular Therapy - Oncolytics 19*, 197–207.

101. Qi, W., White, M. C., Choi, W., Guo, C., Dinney, C., McConkey, D. J., and Siefker-Radtke, A. (2013) Inhibition of Inducible Heat Shock Protein-70 (Hsp72) Enhances Bortezomib-Induced Cell Death in Human Bladder Cancer Cells, *PLoS ONE* (Kozak, K. R., Ed.) *8*, e69509.
102. Cao, Y., Qiu, G.-Q., Wu, H.-Q., Wang, Z.-L., Lin, Y., Wu, W., Xie, X.-B., and Gu, W.-Y. (2016) Decitabine enhances bortezomib treatment in RPMI 8226 multiple myeloma cells, *Molecular Medicine Reports 14*, 3469–3475.
103. Baeke, F., Takiishi, T., Korf, H., Gysemans, C., and Mathieu, C. (2010) Vitamin D: modulator of the immune system, *Current Opinion in Pharmacology 10*, 482–496.
104. Campbell, M. J., and Trump, D. L. (2017) Vitamin D Receptor Signaling and Cancer, *Endocrinology and Metabolism Clinics of North America 46*, 1009–1038.
105. Fife, R. ., Sledge, G. ., and Proctor, C. (1997) Effects of vitamin D3 on proliferation of cancer cells in vitro, *Cancer Letters 120*, 65–69.
106. Busch, L., Mougiakakos, D., Büttner-Herold, M., Müller, M. J., Volmer, D. A., Bach, C., Fabri, M., Bittenbring, J. T., Neumann, F., Boxhammer, R., Nolting, J., Bisht, S., Böttcher, M., Jitschin, S., Hoffmann, M. H., Balzer, H., Beier, F., Gezer, D., Dudziak, D., Gelse, K., Hennig, F. F., Pallasch, C. P., Spriewald, B., Mackensen, A., and Bruns, H. (2018) Lenalidomide enhances MOR202-dependent macrophage-mediated effector functions via the vitamin D pathway, *Leukemia 32*, 2445–2458.
107. Park, W. H., Seol, J. G., Kim, E. S., Jung, C. W., Lee, C. C., Binderup, L., Koeffler, H. P., Kim, B. K., and Lee, Y. Y. (2000) Cell Cycle Arrest Induced by the Vitamin D3 Analog EB1089 in NCI-H929 Myeloma Cells Is Associated with Induction of the Cyclin-Dependent Kinase Inhibitor p27, *Experimental Cell Research 254*, 279–286.
108. Park, W. H., Seol, J. G., Kim, E. S., Hyun, J. M., Jung, C. W., Lee, C. C., Binderup, L., Koeffler, H. P., Kim, B. K., and Lee, Y. Y. (2000) Induction of apoptosis by vitamin D _3 analogue EB1089 in NCI‐H929 myeloma cells via activation of caspase 3 and p38 MAP kinase, *British Journal of Haematology 109*, 576–583.
109. Park, W. H., Seol, J. G., Kim, E. S., Binderup, L., Koeffler, H. P., Kim, B. K., and Lee, Y. Y. (2002) The induction of apoptosis by a combined 1,25(OH)2D3 analog, EB1089 and TGF-beta1 in NCI-H929 multiple myeloma cells, *International Journal of Oncology 20*, 533–542.
110. Qian, J., Qian, Z., Lin, J., Yao, D., Chen, Q., Li, Y., Ji, R., Yang, J., Xiao, G., and Wang, Y. (2011) Abnormal methylation of GRAF promoter Chinese patients with acute myeloid leukemia, *Leukemia Research 35*, 783–786.
111. Bojesen, S. E., Ammerpohl, O., Weinhäusl, A., Haas, O. A., Mettal, H., Bohle, R. M., Borkhardt, A., and Fuchs, U. (2006) Characterisation of the GRAF gene promoter and its methylation in patients with acute myeloid leukaemia and myelodysplastic syndrome, *British Journal of Cancer 94*, 323–332.
112. Li, J., Xu, Y.-H., Lu, Y., Ma, X.-P., Chen, P., Luo, S.-W., Jia, Z.-G., Liu, Y., and Guo, Y. (2013) Identifying Differentially Expressed Genes and Small Molecule Drugs for Prostate Cancer by a Bioinformatics Strategy, *Asian Pacific Journal of Cancer Prevention 14*, 5281–5286.
113. Wang, H., and Cui, Z. (2015) The Distribution and Expression of BAMBI in Breast Cancer Cell Lines, *OALib 02*, 1–7.

1. Streszczenie w języku polskim

Szpiczak plazmocytowy (MM) jest drugim co do częstości występowania nowotworem hematologicznym i stanowi około 10% wszystkich chorób rozrostowych układu krwiotwórczego. Pomimo istotnych postępów w terapii w ostatnich latach, wyniki leczenia MM

nadal pozostają niezadowalające. Zastosowanie inhibitorów proteasomów było jednym z ważniejszych kamieni milowych w leczeniu choroby. Pierwszą cząsteczką, w tej klasie leków, był bortezomib (BTZ). Cytotoksyczne działanie BTZ jest wynikiem hamowania proteasomów, co skutkuje późniejszą akumulacją nieprawidłowo sfałdowanych lub w inny sposób wadliwych białek. Poza głównymi mechanizmami działania, BTZ indukuje jednocześnie różne zmiany epigenetyczne w komórkach docelowych. Co ważne, zmiany epigenetyczne również pośredniczą w oporności na BTZ. Wystąpienie lekooporności ostatecznie prowadzi do progresji choroby i wiąże się z gorszym rokowaniem.

Dlatego niezwykle istotne jest opracowanie i wdrożenie nowych terapii, w tym także adjuwantowych, które będą w stanie zadziałać prewencyjnie lub przezwyciężyć lekooporność. Niektóre leki wpływające na mechanizmy epigenetyczne, takie jak inhibitor metylacji, 5-Aza-2′-deoksycytydyna (AZA), działają synergicznie z BTZ oraz mają potencjał przywracania wrażliwości na lek w opornych komórkach złośliwych. W pierwszej części niniejszej dysertacji badano wpływ *in vitro* AZA na potencjał proliferacyjny linii komórkowej MM i rozwój oporności na BTZ. Na podstawie analizy mikromacierzy metylacji wykazano, że zmiany w profilu metylacji DNA są powiązane z opornością na BTZ. Ponadto, traktowanie AZA komórek MM opornych na BTZ doprowadziło do uwrażliwienia tego fenotypu na działanie BTZ oraz wpłynęło hamująco na poziom proliferacji komórek.

Aktualnie BTZ praktycznie nie jest stosowany w monoterapii MM. Zwykle podaje się go w skojarzeniu z innymi lekami, takimi jak glikokortykosteroidy, leki immunomodulujące oraz przeciwciała monoklonalne. Cząsteczki te biorą udział w interakcji z układem odpornościowym, przekierowując jego funkcje efektorowe do zwalczania komórek MM. Dlatego terapie adjuwantowe, współdziałające z układem odpornościowym, mogą być potencjalnie korzystne w terapii MM. Witamina 25(OH)D3 (VD) i K2MK7 (VK) wykazują wiele tak zwanych „nieklasycznych" działań, m.in. immunomodulujących oraz przeciwnowotworowych. W drugiej części pracy zbadano wpływ *in vitro* VD i VK na zmiany epigenetyczne związane z potencjałem proliferacyjnym komórek MM i rozwojem oporności na BTZ. Uzyskane wyniki wykazały, że rozwój oporności komórek MM na BTZ jest związany z globalnym spadkiem metylacji DNA w porównaniu z komórkami kontrolnymi. Przeciwnie, zarówno kontrolne komórki MM, jak i komórki MM oporne na BTZ, eksponowane na działanie samej VD oraz kombinacji VD i VK, wykazują globalny wzrost metylacji, co supresyjnie wpływa na potencjał proliferacyjny komórek MM.

Podsumowując, zarówno AZA, jak i VD oraz VK w warunkach *in vitro* indukują występowanie zmian epigenetycznych, które hamują potencjał proliferacyjny złośliwych

komórek plazmatycznych, częściowo zapobiegają rozwojowi oporności na BTZ oraz uwrażliwiają fenotyp oporny komórek MM na działanie BTZ. Konieczne są jednak dalsze badania *ex vivo* oraz *in vivo*, aby uzyskane wyniki mogły stać się podstawą do sformułowania rekomendacji dotyczących rutynowego zastosowania AZA oraz terapii adjuwantowej w MM.

2. Streszczenie w języku angielskim

Multiple myeloma (MM) is the second most common hematological malignancy and accounts for approximately 10% of all cancers within hematopoietic system. Despite significant progress in therapy in recent years, the results of MM treatment remain unsatisfactory. The introduction of proteasome inhibitors was one of the most important milestones in the treatment of MM. The first molecule in this class of drugs was bortezomib (BTZ). The cytotoxic effects of BTZ are the result of proteasome inhibition, which results in the subsequent accumulation of misfolded or otherwise defective proteins. In addition to its main mechanisms of action, BTZ simultaneously induces various epigenetic changes in target cells. Importantly, epigenetic changes also mediate BTZ resistance. Drug resistance eventually leads to disease progression and is associated with worse clinical outcome.

Therefore, it is crucial to develop and implement new therapies, including adjuvant ones that will be able to act preventively or overcome drug resistance. Some drugs that affect epigenetic mechanisms, such as the methylation inhibitor, 5-Aza-2′-deoxycytidine (AZA), act synergistically with BTZ and have the potential to restore drug sensitivity in resistant malignant cells. The first part of this dissertation examined the *in vitro* effect of AZA on the proliferative potential of the MM cell line and the development of resistance to BTZ. Methylation microarray analysis showed that changes in the DNA methylation profile are associated with BTZ resistance. Moreover, AZA treatment of BTZ-resistant MM cells led to sensitization of this phenotype to BTZ and subsequently reduced its proliferative potential.

Currently, BTZ is practically not used in monotherapy of MM. It is usually administered in combination with other drugs such as glucocorticoids, immunomodulatory drugs and monoclonal antibodies. These molecules interact with the immune system, redirecting its effector functions to combat MM cells. Therefore, adjuvant therapies that interact with the immune system may be potentially beneficial in the treatment of MM. Vitamin 25(OH)D3 (VD) and vitamin K2MK7 (VK) have many so-called "non-classical" actions, including immunomodulatory and anticancer. In the second part of the study, the *in vitro* impact of VD and VK on epigenetic changes related to the proliferative potential of MM cells and the

development of resistance to BTZ was examined. The obtained results showed that the development of resistance of MM cells to BTZ is associated with a global decrease in DNA methylation compared to control cells. In contrast, both control MM cells and BTZ-resistant MM cells exposed to VD alone and a combination of VD and VK show a global increase in methylation, which suppresses the proliferative potential of MM cells.

In conclusion, both AZA, VD and VK have the potential to induce *in vitro* epigenetic alterations that inhibit the proliferative potential of malignant plasma cells, partially prevent the development of resistance to BTZ and sensitize the resistant phenotype of MM cells to BTZ. However, further *ex vivo* and *in vivo* studies are necessary so that the obtained results can become the basis for formulating recommendations regarding the routine use of AZA and adjuvant therapy in MM.

cancers

MDPI

Review

Epigenetic Alterations as Vital Aspects of Bortezomib Molecular Action

Piotr Kulig [1], Karolina Łuczkowska [1], Estera Bakinowska [1], Bartłomiej Baumert [2,*] and Bogusław Machaliński [1,2,*]

[1] Department of General Pathology, Pomeranian Medical University, 70-111 Szczecin, Poland; piotrkulig@interia.eu (P.K.); karolina.luczkowska@pum.edu.pl (K.Ł.); esterabakinowska@gmail.com (E.B.)
[2] Department of Hematology and Transplantology, Pomeranian Medical University, 71-252 Szczecin, Poland
* Correspondence: bartlomiej.baumert@pum.edu.pl (B.B.); boguslaw.machalinski@pum.edu.pl (B.M.)

Simple Summary: The implantation of proteasome inhibitors was a milestone in the treatment of multiple myeloma (MM). One such first-in-class molecule was bortezomib (BTZ). Its cytotoxic effects are exerted through proteasome inhibition and the subsequent accumulation of misfolded or otherwise defective proteins. In addition to its main mechanisms of action, BTZ elicits various epigenetic alterations within target cells which are part of its mechanism of action. Importantly, epigenetic changes also participate in mediating resistance to BTZ. Some epigenetic agents such as azacitidine act synergically with BTZ or have the potential to restore sensitivity to the drug in resistant malignant cells. In this paper, we reviewed the epigenetic aspects of BTZ molecular action with a particular emphasis on drug resistance mechanisms and potential clinical implications.

Abstract: Bortezomib (BTZ) is widely implemented in the treatment of multiple myeloma (MM). Its main mechanism of action is very well established. BTZ selectively and reversibly inhibits the 26S proteasome. More precisely, it interacts with the chymotryptic site of the 20S proteasome and therefore inhibits the degradation of proteins. This results in the intracellular accumulation of misfolded or otherwise defective proteins leading to growth inhibition and apoptosis. As well as interfering with the ubiquitin–proteasome complex, BTZ elicits various epigenetic alterations which contribute to its cytotoxic effects as well as to the development of BTZ resistance. In this review, we summarized the epigenetic alterations elicited by BTZ. We focused on modifications contributing to the mechanism of action, those mediating drug-resistance development, and epigenetic changes promoting the occurrence of peripheral neuropathy. In addition, there are therapeutic strategies which are specifically designed to target epigenetic changes. Herein, we also reviewed epigenetic agents which might enhance BTZ-related cytotoxicity or restore the sensitivity to BTZ of resistant clones. Finally, we highlighted putative future perspectives regarding the role of targeting epigenetic changes in patients exposed to BTZ.

Keywords: bortezomib; proteasome inhibitor; drug resistance; epigenetic; methylation; non-coding RNA

Citation: Kulig, P.; Łuczkowska, K.; Bakinowska, E.; Baumert, B.; Machaliński, B. Epigenetic Alterations as Vital Aspects of Bortezomib Molecular Action. *Cancers* **2024**, *16*, 84. https://doi.org/10.3390/cancers16010084

Academic Editor: Shingen Nakamura

Received: 29 November 2023
Revised: 20 December 2023
Accepted: 21 December 2023
Published: 23 December 2023

1. Introduction

Proteins are crucial components for maintaining intracellular homeostasis. Their role is multifaceted, as they participate in numerous, if not all, biological processes in cells. Due to the complexity of the interactions in which proteins are involved, it is extremely important to regulate their metabolism, in particular, their synthesis and turnover. Protein synthesis is closely related to the regulation of translation and the bioavailability of various RNA molecules [1], whereas protein degradation and turnover are controlled through the lysosomes or the ubiquitin–proteosome system (UPS). The lysosome-based degradation pathways involve the degradation of cytoplasmic proteins and damaged organelles. This process occurs through autophagy, known as the autophagy-lysosomal

pathway, and the degradation of extracellular proteins through endocytosis, referred to as the endosome-lysosomal pathway [2]. A particular pathway is the phagocytosis-lysosome pathway, where the phagosome merges with the lysosome, leading to the degradation of its contents [3,4]. Lysosomal pathways play a crucial role in breaking down long-lived proteins, insoluble protein aggregates, entire organelles, macromolecular compounds, and intracellular microbes (e.g., certain bacteria). This degradation can occur through endocytosis, phagocytosis, or autophagy pathways [5,6]. The UPS consists of proteasome that is a protease, ubiquitin ligases, and deubiquitinating enzymes (DUBs) [2]. Proteins condemned to decay are labeled with a covalently attached polyubiquitin chain and delivered to the proteasome for degradation. Ubiquitination is catalyzed by specific enzymes called E1 (activating enzyme), E2 (conjugating enzyme), and E3 (ubiquitin ligase) [3]. Monoubiquitinated proteins are degraded within lysosomes whilst polyubiquitinated ones are degraded in the UPS [4,5]. It was demonstrated that at least four ubiquitin molecules are needed for the proteasome to recognize it as a target protein [3]. Ubiquitylation is reversible. The removal of the polyubiquitin chain is catalyzed by DUBs [6]. Proteins marked for degradation are redundant, misfolded, or otherwise damaged (Figure 1A). The 26S proteasome, in which protein degradation takes place, can be divided into two subcomplexes: the 19S regulatory particle (RP) and the 20S core particle (CP). The function of the RP is to recognize, unfold, deubiquitylate, and translocate substrates to the CP—the site of proteolysis [7]. The 26S proteasome is crucial for maintaining protein and amino acid homeostasis, serving as a proteome modulator by degrading regulatory proteins. It plays a vital role in controlling various cellular processes such as the cell cycle, DNA replication, transcription, signal transduction, and stress responses [8]. Therefore, interference with the UPS may disturb cellular metabolism and even induce cell death.

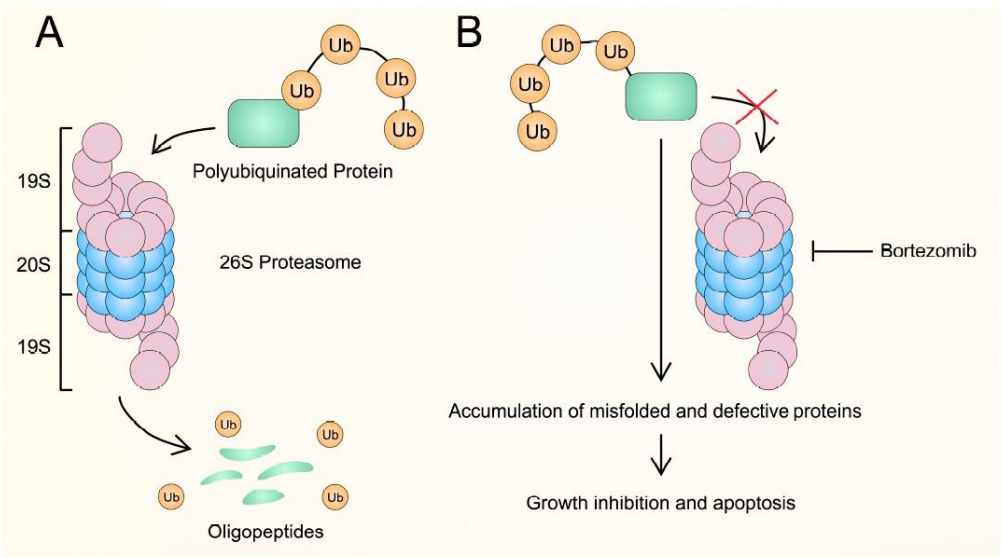


Figure 1. Proteasome activity without and with the presence of BTZ. (**A**) Proteasome degrades polyubiquitinated proteins into oligopeptides. (**B**) BTZ inhibits the 26S proteasome, leading to an intracellular accumulation of misfolded or otherwise defective proteins, and subsequent growth inhibition and apoptosis.

It was hypothesized that proteasome inhibition may be a promising therapeutic approach. The first molecule designed to interfere with UPS was bortezomib (BTZ). Next generation proteasome inhibitors are carfilzomib and ixazomib [9,10]. Although the three molecules have a different chemical structure, BTZ and ixazomib structurally resemble each other as they are modified boronic acid derivatives [11,12]. Carfilzomib is a synthetic tetrapeptide epoxyketone [13]. It should be noted that only ixazomib can be administered orally [14].

BTZ turned out to be a potent chemotherapeutic agent that selectively and reversibly binds to the chymotryptic site located in the 20S proteasome, and therefore inhibits the degradation of ubiquitin-tagged proteins [11]. The blockage of these molecular pathways hampers protein degradation, leading to an accumulation of misfolded or otherwise defective polypeptides. These series of events ultimately lead to growth inhibition and apoptosis [15,16] (Figure 1B). BTZ can be administered intravenously or subcutaneously with the same efficiency. It was demonstrated that subcutaneous administration is as effective as intravenous. However, it is associated with a limited number of complications and adverse effects [17]. Once the drug reaches the circulation, it is rapidly removed from the plasma and distributed to the cellular compartment. The half-life of BTZ elimination is relatively long and is estimated to exceed 10 h [15]. BTZ undergoes oxidative metabolism in the liver via the cytochrome P450 complex to inactive deborated metabolites [18,19] and is excreted both through the kidneys and bile [20]. Despite its potent anticancer activity, patients treated with BTZ are at risk of developing certain adverse reactions. Multiple 1/2 phase clinical trials have been conducted investigating the safety and feasibility of BTZ in various indications, with particular emphasis on multiple myeloma (MM). Among the most frequent adverse reactions were gastrointestinal symptoms, fatigue, thrombocytopenia, neutropenia, and peripheral neuropathy [21–27]. There were no reports of BTZ cumulative toxicity [28–31]. BTZ alone and in combination with other agents was tested in both hematologic malignancies [32–34] and solid tumors such as breast, lung, and ovarian cancer [35–38]. BTZ is currently approved for the treatment of MM and mantle cell lymphoma [39].

The main mechanism of action of BTZ, i.e., proteasome inhibition, is relatively well described. Less is known about the epigenetic alterations that BTZ causes when administered to the organism. Epigenetics involves modifications in gene expression that are self-directed and resist explanation solely through changes in the nucleotide sequence [40]. These modifications may arise from external environmental influences or exposure to various factors, including drugs [41]. There are four main epigenetic mechanisms: DNA methylation, histone modification, chromatin remodeling, and non-coding RNA (ncRNA) [42]. The aim of this review is to provide greater insight into the epigenetic alterations induced by proteasome inhibitors, with particular emphasis on BTZ and its implications in the mechanism of action, the pathophysiology of adverse events, the development of resistance, and clinical implications. We decided to focus on BTZ as we have already investigated BTZ-resistance mechanisms and the role of epigenetic mechanisms in dodging BTZ-induced cytotoxicity [43–46]. Furthermore, it remains the most widely utilized proteasome inhibitor globally. Hence, we hypothesized that the vast majority of MM patients worldwide have been and will continue to be exposed to BTZ in the foreseeable future. Consequently, a more profound comprehension of the mechanisms of action of BTZ and the development of BTZ-resistance is of paramount importance. Such understanding may enhance patients' outcomes, improve prognosis, and contribute to optimizing BTZ-based treatment regimens.

2. Epigenetic Alterations as an Important Part of BTZ's Mechanism of Action

BTZ's major mechanism of action, i.e., the inhibition of the 20S proteasome, is very well established and thoroughly described in the existing literature. However, less is known about its collateral mechanisms that also contribute to BTZ-related cell death. Since BTZ is an external stimulus for cells, it can be hypothesized that its presence, in addition to intracellular protein accumulation, has the proclivity to alter the methylome or

promote other epigenetic alterations. Liu et al. showed that proteasome inhibition leads to protein aggregation, particularly affecting the Sp1 protein. Further analysis revealed that BTZ influences Sp1—the zinc finger protein—that transactivates the *DNMT1* gene (DNA methyltransferase) and is functionally regulated by its abundance. By inducing intracellular protein aggregation, BTZ reduces the levels of Sp1, disrupts its physical interaction with the NF-κB transcription factor, and consequently prevents the binding of the Sp1/NF-κB complex to the *DNMT1* gene promoter. The abrogation of the Sp1/NF-κB complex by BTZ causes the transcriptional repression of the *DNMT1* gene and the downregulation of the DNMT1 protein, leading to global DNA hypomethylation [47]. In a study conducted on mantle cell lymphoma (MCL) cell lines and in a mouse xenograft model, Leshchenko et al. demonstrated in a genome-wide analysis that BTZ administration causes a global loss of methylation, including the *Noxa* gene (a pro-apoptotic member of the Bcl-2 family) [48]. The *Noxa* gene is essential for BTZ cytotoxicity and BTZ-induced apoptosis, as *Noxa* depletion has been shown to abrogate BTZ efficacy [48,49].

Kikuchi et al. conducted an interesting study in which they demonstrated that histone deacetylases (HDACs) are critical targets of BTZ. Their results depicted that BTZ appeared to induce cytotoxicity in MM cells by suppressing HDACs. Furthermore, this phenomenon was accompanied by histone hyperacetylation, both occurring in a dose- and time-dependent manner. The most prominent effect was evident in the suppression of HDAC1. Conversely, the overexpression of HDAC1 rescued MM cells from BTZ-induced apoptosis [50]. Histone ubiquitylation is a potent epigenetic mechanism regulating gene expression and DNA damage repair [51]. BTZ has been shown to deplete histone H2B ubiquitination, triggering various downstream mechanisms that contribute to its cytotoxic activity [52,53].

3. Epigenetic Aspects of the Resistance to BTZ

Exposure to anticancer agents exerts tremendous environmental pressure on malignant cells and, over the course of time, selects for drug-resistant clones. Due to the fact that resistance develops as a consequence of an external trigger, such as anticancer treatment, it can be hypothesized that epigenetic alterations contribute, at least partially, to the development of drug resistance. Recent evidence suggests that this assumption also applies to BTZ. The proteasome serves as the molecular target for BTZ. Consequently, the notion that its downregulation could mitigate BTZ's anti-tumor potential appears reasonable. This hypothesis was adopted by Tsvetkow and colleagues. They investigated the correlation between proteasome expression and sensitivity to BTZ, demonstrating that proteasome suppression, particularly the 19S subunit, was associated with BTZ resistance. Furthermore, the analysis of the underlying mechanisms revealed, among other factors, that the downregulation of *PSMD5* (the gene encoding one of the 19S subunits) due to promoter hypermethylation conferred resistance to BTZ [54]. In a neuroblastoma cell line, we demonstrated that exposure to BTZ elicited alterations in the methylome of cells that survived the treatment, i.e., cells exhibiting a resistant phenotype. The analysis of methylation profiles revealed that BTZ induced genome-wide changes in the methylome of those cells in comparison to both untreated and lenalidomide-treated controls. The alternations were not limited to CpG islands; in fact, the vast majority, approximately 90%, did not involve CpG islands. Most of the observed changes in methylation involved the loss of methylation. It is intriguing, however, that when the analysis is restricted to only significant changes in methylation, most of the observed alternations in the dataset appear to involve a loss or gain of methylation at around 50%. This may suggest that only one allele was affected. Overall, the results indicate that methylation changes may play a major role in the development of BTZ resistance [43].

In another in vitro study, Hu at al. investigated the relationship between the expression of CD9, a membrane molecule whose downregulation plays a role in cancer development and progression, and BTZ sensitivity, with particular emphasis on the epigenetic aspects of the underlying mechanisms. They demonstrated that BTZ-resistant MM cells exhibited

significantly lower CD9 expression compared to cells with a sensitive phenotype. Since the CD9 promoter region includes a CpG island, a further analysis of the methylation profile was performed. The authors demonstrated an increase in the level of methylation in the promoter region of U266 and NCI-H929 MM cells with silenced CD9 expression. Moreover, CD9 expression was significantly induced after treatment with 5-Aza-2-deoxycytidine (AZA), a methylation inhibitor. In the aftermath, MM cells regained sensitivity to BTZ [55]. The deleted in colorectal cancer gene was shown to be involved in the carcinogenesis of various neoplasms. Rodrigues-Junior and colleagues investigated the role of DCC in myelomagenesis. They conducted an in vitro study on three different MM cell lines. The results showed that the hypermethylation of the promoter was associated with a better response to BTZ compared to SKO007 and U266, which were characterized by a low degree of *DCC* methylation and, consequently, its high expression. They not only demonstrated the role of *DCC* in the pathophysiology of MM, but also provided further evidence for the role of epigenetic changes in the sensitivity and resistance to proteasome inhibitors [56].

4. Targeting Epigenetic Mechanisms Restores Sensitivity to BTZ and Represents a Promising Therapeutic Strategy

4.1. Methylation Inhibitors Act Synergically with BTZ and Restore Sensitivity to This Compound

The aforementioned studies have shown that BTZ has the potential to induce epigenetic changes. Alterations in the methylome contribute to BTZ-related cytotoxicity on one hand and are an important aspect of BTZ's mechanism of action. On the other hand, they have been demonstrated to confer BTZ resistance. Therefore, exploring the combination of BTZ with agents that modulate epigenetic changes may be an intriguing research area with potential clinical implications. Indeed, such combinations have been studied in vitro and in vivo. It may be challenging to compose an optimal treatment regimen due to the complexity of interactions and various treatment escape mechanisms. In order to facilitate the design of an effective chemotherapy regimen, Rashid et al. developed the quadratic phenotypic optimization platform (QPOP), a tool to aid in the design of optimal drug combinations in MM. The application of QPOP to BTZ-resistant MM cell lines identified drug combinations that collectively optimized treatment efficacy. The QPOP project determined a drug combination countering DNA methylation, with decitabine (DAC) being one of the selected agents. The results were validated in vivo using a mouse model. DAC and mitomycin C were demonstrated as a potent drug combination in the treatment of BTZ-resistant MM [57].

We demonstrated that AZA acts synergically with BTZ, and a combination of AZA and BTZ exhibited cytotoxic effects against BTZ-resistant U266 MM cells [45]. A similar effect regarding AZA and BTZ was demonstrated by Li and colleagues [58]. Qi et al. provided further evidence supporting the effectiveness of the combination of BTZ and a methylation inhibitor. They conducted an in vitro experiment on bladder cancer cell lines, demonstrating that BTZ-resistant cells had a low expression of *HSPA1A* which was associated with the revealed hypermethylation of the *HSPA1A* promoter. This gene, next to *HSPA1B*, encodes heat shock protein 72 (HSP72), or more precisely, various isoforms of HSP72. The combination of AZA and BTZ restored BTZ sensitivity in previously resistant bladder cancer cells [59]. Similarly, BTZ and another DNMT inhibitor, decitabine (DAC), have been shown to act synergically against BTZ-resistant clones both in vitro and in a xenograft animal model [48]. Another study investigated the effects of DAC alone, BTZ alone, and DAC combined with BTZ on MM cell viability. The results showed that DAC alone inhibited the growth of MM cells, while the combination of BTZ and DAC showed a synergistic effect. The primary molecular anti-MM effects of DAC and BTZ were shown to be induced by the modulation of the Wnt/β-catenin pathway. These observations were first established in MM cell lines and subsequently confirmed in a mouse xenograft model [60]. RRx-001 (1-bromoacetyl-3,3-dinitroazetidine) is an innovative epigenetic modulator that operates differently from classic epigenetic drugs, such as AZA or DAC. It allosterically modifies hemoglobin and, under hypoxic conditions, catalyzes the conversion of nitrite

to bioavailable nitric oxide (NO), which accumulates in poorly oxygenated tumors. NO further generates free radicals and causes oxidative stress. Thus, RRx-001 exerts stress on malignant cells, leading to the inhibition of DNMT and global hypermethylation, along with the restoration of tumor suppressor gene function. RRx-001 has been shown to inhibit growth, induce apoptosis, and overcome BTZ resistance in MM cells [61]. The effectiveness of methylation inhibition was also confirmed in other studies [62].

Although methylation is typically associated with well-known regulatory regions of a gene, such as the promoter, it also applies to other areas of the nucleotide sequence. For instance, Xu and colleagues investigated the role of a relatively novel epigenetic regulatory mechanism, N^6-methyladenosine (m6A), which involves methylation at the N6 position of adenosine. They showed that fat mass and the obesity-associated protein (FTO), m6A demethylator, are upregulated in MM, particularly with extramedullary location, and FTO inhibition was toxic towards MM cells. Moreover, cytotoxicity was significantly enhanced when the FTO inhibitor was administered together with BTZ, so the combination of a proteasome inhibitor and drug interfering with the epigenetic mechanism exerted synergistic anti-MM effects [63]. Similarly, Jia et al. showed that WTAP, a key component of the m6A methyltransferase complex, was methylated by Protein Arginine Methyltransferase 1 (PRMT1), and the combination of a PRMT1 inhibitor and BTZ synergistically inhibited MM progression [64].

4.2. Therapeutic Interference with Histone Modifications

Histone modifications are another epigenetic mechanism that has been studied in BTZ-treated MM. Sun and colleagues investigated Nexturastat A (NexA), a selective histone deacetylase 6 (HDAC6) inhibitor. In vitro NexA inhibited the growth and induced the apoptosis of RPMI-8226 and U266 MM cells, including cells that were resistant to BTZ. Those results were further confirmed in a mouse xenograft model [65]. Cytogenetic abnormalities are a hallmark of MM and serve to stratify patient risk and thus predict prognosis and clinical outcomes to some extent [66,67]. Translocation t(4;14) is relatively common and is associated with a poor prognosis even in the era of novel anti-MM agents, including monoclonal antibodies [68]. Jiang and colleagues demonstrated that Aurora kinase A phosphorylates NSD2 at the S56 residue to enhance NSD2 methyltransferase activity, conferring resistance to BTZ. A selective Aurora kinase A inhibitor (MLN8237) presented a prominent synergistic effect with BTZ on MM cells with t(4;14). Interestingly, such observations were limited to MM cells with t(4;14) translocation. The methylation of Aurora A and the phosphorylation of histone methyltransferase NSD2 bilaterally form a positive regulatory loop that promotes BTZ resistance in MM cells. It should be emphasized that these observations were further confirmed in an in vivo model [69]. Similarly, Liu and co-workers demonstrated the overexpression of NSD2 in BTZ-resistant MM cells and in cells obtained from patients with the t(4;14) translocation. It was found that there was a significant upregulation of NSD2 resulting in an increase in steroid receptor coactivator-3 (SRC-3). Elevated levels of both SRC-3 and NSD2 were confirmed in BTZ-resistant MM cells, irrespective of cytogenetic background. Importantly, the SRC-3 inhibitor, SI-2, restored BTZ sensitivity in vitro and in a xenograft model. Notably, SI-2 promoted bone-lesion recovery in mice. The study concluded that the histone methyltransferase NSD2 stabilized SRC-3 protein levels, and pharmacological interference with SRC-3 abrogated this interaction, resynthesizing MM cells to BTZ in both in vitro and in vivo models [70].

The synergic effect of histone deacetylase inhibitors and BTZ is not limited to MM. For instance, Bollmann and colleagues conducted an important and interesting study in which they demonstrated that a novel selective histone deacetylase inhibitor, named YAK540, and BTZ enhance each other's cytotoxic effects on leukemia cell lines. These effects were exerted through the increased expression of pro-apoptotic genes, increased p21 expression, and caspase 3/7-mediated apoptosis [71]. Chidamide is a novel benzamide inhibitor of HDAC. Xu et al. demonstrated that chidamide repressed autophagy, which, similarly to UPS, participates in intracellular protein degradation, and synergically with BTZ inhibits

MM cell growth. They provided compelling evidence that excessive proteotoxic stress could, at least in part, explain the underlying molecular effects of chidamide in combination with BTZ against MM [72]. The bone marrow microenvironment and its interaction with MM cells plays a vital role in mediating acquired resistance to BTZ, for instance, through Jagged1-induced Notch activation in myeloma cells (Jagged1 is widely expressed in various types of cells within the bone marrow MM niche) [73]. In the context of overcoming bone marrow microenvironment-dependent BTZ resistance through epigenetic mechanisms, Sripayap et al. showed that the HDAC inhibitor Romidepsin can counteract cell adhesion-mediated drug resistance [74].

5. The Role of Non-Coding RNAs

In addition to DNA methylation, non-coding RNAs such as mi-RNA or long non-coding RNA (lncRNA) serve as potent epigenetic regulators of gene expression and protein synthesis. Related processes were also investigated regarding the BTZ's mechanism of action and the development of resistance to this compound. Non-coding RNAs mediate BTZ-induced cytotoxicity and while this area is not entirely elucidated, several underlying mechanisms have been identified. For example, following BTZ exposure, the transcription factor CEBPD is activated, which triggers the transcriptional activation of miR-744, miR-3154, and miR-3162. These miRNAs form a complex with Ago2 and move into the nucleus to target their complementary DNA sequence-binding sites on the promoter regions of four important genes—*CEBPD*, *PRKDC*, *MCM4*, and *UBE2V2*. The initiator miRNAs/Ago2 complex interacts with YY1 and recruits the epigenetic regulators, the PcG complex/DNMTs, to silence the four gene loci, including CEBPD itself. The inactivation of these potent oncogenes, *PRKDC*, *MCM4*, and *UBE2V2*, causes leukemic cell death through epigenetic silencing mediated by CEBPD-responsive miRNA [75]. Another non-coding RNA being investigated is circ_0007841. First, its overexpression was established in MM compared to healthy controls. In addition, patients with a low expression of circ_0007841 had a higher survival rate compared to those with high circ_0007841 levels. Subsequently, circ_0007841 depletion was shown to impede MM cell proliferation and promote apoptosis. The knock out of circ_0007841 reduced the BTZ resistance of MM cells in vitro and MM growth in a mouse xenograft model. Hence, it can be hypothesized that the overexpression of circ_0007841 confers, at least to some extent, resistance to BTZ [76].

Another non-coding RNA being investigated in MM is miR-29b. It has been shown to inhibit DNMT and thus reduce global DNA methylation in MM cells [77]. Moreover, miR-29b was demonstrated to impede MM cell migration [78]. Of particular interest regarding MM treatment, miR-29b was upregulated by BTZ and was involved in BTZ-related cytotoxicity [79]. Therefore, molecules mimicking miR-29b or its analogues may represent a potential novel therapeutic approach in the treatment of MM. Pan et al. showed that lncRNA H19 mediates BTZ resistance in MM cell lines and promotes tumor growth in vivo. First, they showed that lncRNA is highly expressed in the serum of BTZ-resistant patients [80]. Subsequently, they conducted another study to elucidate the underlying mechanisms. They found that BTZ resistance is mediated by lncRNA H19 through the overexpression of MLC-1, an anti-apoptotic protein belonging to the Bcl-2 family. To be more specific, lncRNA H19 interacts with miR-29b-3p, a physiological regulator of MLC-1 expression. The interaction between H19 and miR-29b-3p upregulates MLC-1, enhancing its anti-apoptotic properties and thus promoting BTZ resistance [81]. The role of miR-29b in pathogenesis was also highlighted by Fu et al. The authors demonstrated that lncRNA myocardial infarction-associated transcripts (MIATs) were highly expressed in patients with MM and interacted with miR-29b to negatively regulate its expression. Moreover, experimental evidence demonstrated that MIATs increased BTZ resistance in MM cells by targeting miR-29b [82].

Qin et al. showed that miR-137 is epigenetically silenced by promoter methylation in MM, and the entire process is reversible after using AZA. What is particularly interesting is that the overexpression of miR-137 sensitized cells to BTZ (in vitro and in a murine

xenograft model) and overcame chromosomal instability [83]. Wu et al. demonstrated miR145-3p to be downregulated in MM patients compared to healthy donors. Moreover, its expression was correlated with markers of disease progression. The researchers further demonstrated that induced miR145-3p expression inhibited cell proliferation and promoted apoptosis in MM cells by inducing autophagy. The underlying mechanism was associated with HDAC4 inhibition. Importantly, the upregulation of miR-145-3p enhanced the anti-MM activity of BTZ. The latter has also been demonstrated in a mouse xenograft model [84]. Consistent with the results obtained by Nian and colleagues, lncRNA ANGPTL1-3 expression was correlated with MM International Staging System (ISS) and OS. Furthermore, they demonstrated that this molecule mediates resistance to BTZ via interaction with miR-30a-3p and the transcription factor c-Maf [85]. Other non-coding RNAs that have been proven to mediate BTZ resistance are circ-CCT3 by modulating the miR-223-3p/BRD4 axis [86], miR-214-3p, miR-5100 [87], and several others [88–93].

Moreover, Malek et al. identified an entire panel of deregulated lncRNAs mediating acquired resistance to three different clinically relevant proteasome inhibitors, i.e., BTZ, carfilzomib, and ixazomib in MM [94]. Additionally, the knockdown of lncRNA PCAT-1 inhibits myeloma cell growth and enhances sensitivity to BTZ [95]. Conversely, some non-coding RNAs such as miR-197-3p [96], miR-631 [97], miR-497 [98], miR-155 [99], and miR-200c [100] have been shown to reduce BTZ resistance. Another particular aspect is the synergistic interference with proteasome function and autophagy, enhancing BTZ's anti-MM properties. For example, non-coding RNAs, including lncRNA MEG3, have demonstrated the ability to influence autophagy, thus acting synergistically with BTZ to promote sensitivity in MM [101]. A similar observation regarding the inhibition of autophagy by chidamide was mentioned above, further suggesting the importance of this finding [72]. In addition, Jagannathan et al. showed that concomitant interference with proteasome and autophagosome through miR-29b replacement enhances the anti-MM effect of BTZ [102].

All of the above-mentioned molecules and mechanisms involved in mediating BTZ resistance or enhancing its cytotoxic effects against MM (Figure 2) are highly significant, given their possible clinical implications and targetability. This could potentially translate into therapeutic strategies in the future.

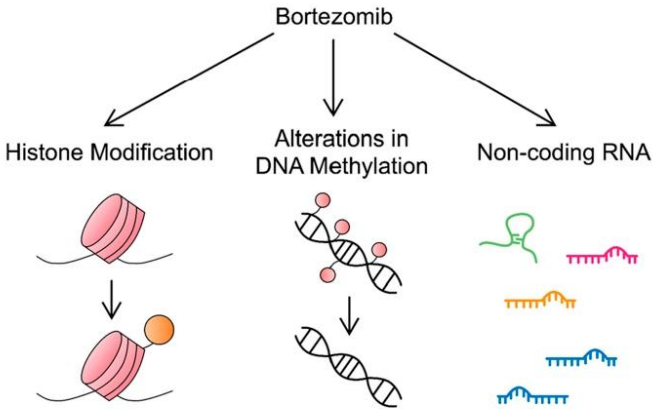


Figure 2. Epigenetic alterations elicited by BTZ. In addition to proteasome inhibition, BTZ exerts its cytotoxic effects through histone modifications, alterations in DNA methylation (mainly, loss of methylation), and non-coding RNs. Similar epigenetic alterations contribute to the development of resistance to this compound.

6. Peripheral Neuropathy

In addition to contributing to the development of BTZ resistance, epigenetic alterations may play a role in the pathogenesis of its adverse reactions. Łuczkowska et al. investigated the pathophysiology of BTZ-induced peripheral neuropathy. As neuropathic symptoms may partially resolve upon discontinuation of BTZ, the researchers hypothesized that epigenetic changes may, at least in part, mediate the development of peripheral neuropathy. First, they demonstrated that BTZ induces global hypomethylation in neuronal cells. Interestingly, their results revealed an increase in methylation at a particular subset of CpG sites. Nevertheless, they were present outside the CGI and gene regulatory regions. Further GSEA analysis revealed that these changes appeared to affect genes involved in morphogenesis, neurogenesis, and neurotransmission. Moreover, the identified methylation changes are significantly enriched within the binding sites of transcription factors, including EBF, PAX, DLX, LHX, and HNF family members. The study concluded that alterations in the methylome are likely to be involved in BTZ-mediated neurotoxicity [44].

In addition to alterations in the methylome, the researchers investigated other epigenetic alterations putatively being involved in BTZ-induced peripheral neuropathy. The obtained results showed a decrease in global histone H3 acetylation. Furthermore, miR-6810-5p has been shown to interfere with the *MSN*, *FOXM1*, *TSPAN9*, and *SLC1A5* genes, which are involved in neuroprotective processes, neuronal differentiation, and signal transduction [103]. Zheng and colleagues demonstrated that the activation of GATA-binding protein 3 (GATA3) mediated the epigenetic upregulation of CCL21 in dorsal horn neurons, which contributed to BTZ-induced neuropathic pain. More precisely, BTZ induced histone hyperacetylation in the *CCL21* gene promoter via GATA3 signaling [104]. The role of histone hyperacetylation in BTZ-induced allodynia was also observed by Chen and colleagues [105] and Liu and co-workers [106]. Similarly, the overexpression of the histone demethylase KDM6A has been shown to participate in BTZ-induced neuropathic pain [107]. Parallel observations regarding the contribution of epigenetic alterations in the pathophysiology of peripheral neuropathy development were made by Liu and colleagues [108]. Furthermore, the analysis of patients with BTZ-induced peripheral neuropathy revealed increased plasma levels of various miRNAs. miR-22-3p, miR-23a-3p, and miR-24-3p have been identified as potential biomarkers of peripheral neuropathy [109].

BTZ-induced peripheral neuropathy has a multifactorial pathogenesis. Several mechanisms were postulated, including inflammatory background [110]. Nonetheless, the above studies depicted a complex interplay between various epigenetic and genetic mechanisms. The hypothesis of the involvement of epigenetic changes in the development of BTZ-induced peripheral neuropathy seems to be convincingly confirmed, yet it needs to be further investigated. It should be emphasized, however, that the development of this complication is also, most likely to a large extent, influenced by other factors.

7. Clinical Implications

In addition to in vitro studies, the significance of epigenetic changes in clinical settings has been investigated. For instance, De Larrea et al. researched the clinical implications of alterations in the methylome in MM. They analyzed the methylation profile of seventy-five MM patients treated with BTZ-based regimens. Bone marrow samples were obtained at the time of relapse. Global methylation was determined using ELISA and the CpG island DNA methylation profile of 30 genes using a PCR system. The results showed that MM patients with more than 3.95% of total DNA methylated achieved better overall survival (OS) than patients with more unmethylated DNA (median 30 versus 15 months, $p = 0.004$). Then, the methylation level of individual genes was analyzed. The results showed that a methylation status lower than 3.97% in CXCR4 was correlated with longer progression-free survival (PFS) after BTZ treatment. Subsequently, cluster analysis of all thirty genes was conducted. It was demonstrated that *NFKB1* was the only gene associated with a differential profile to BTZ, showing that responders to the treatment exhibited a lower methylation status ($p = 0.029$). A low percentage of methylation (less than 1.07%)

in this gene was also associated with longer overall survival (OS) after BTZ exposure. The study concluded that the combination of relatively low levels of global genome methylation (<3.95%) and higher levels of *NFKB1* methylation (≥1.07%) identified a specific subset of patients with extremely short OS [111].

Szudy-Szczyrek et al. investigated the predictive and prognostic value of miR-8074 expression in MM patients. They analyzed 105 patients with newly diagnosed MM treated with thalidomide (THD) (n = 27), BTZ (n = 41), and both BTZ and THD (n = 37). The obtained results showed that a high expression of miR-8074 was associated with a worse clinical outcome, more precisely with a higher risk of death (HR = 4.12, 95% CI: 2.20–7.70; p = 0.0009) and with a significant reduction of PFS. This renders miR-8074 a useful tool for predicting the prognosis for MM patients [112]. Another non-coding RNA associated with clinical outcomes is miR-137. It was demonstrated that the expression of this molecule is negatively correlated with PFS and OS [83].

As mentioned in the sections above, there is ample evidence that epigenetic alterations contribute to the development of BTZ resistance. Furthermore, targeting epigenetic mechanisms either resynthesized cells to BTZ or exhibited a synergistic effect with the drug. In addition, changes in methylome influenced prognosis.

Therefore, it seems reasonable to conduct a clinical trial examining the combination of BTZ and a molecule influencing epigenetic mechanisms. Panobinostat, a first-in-class pandeacetylase inhibitor (DACi), is a molecule interfering with epigenetic mechanisms. More specifically, it prevents deacetylation, a process involved in epigenetic regulation [113]. It was demonstrated that panobinostat is a viable therapeutic option for MM patients. Results from PANORAMA1, a multicenter, randomized, double-blind, placebo-controlled, phase 3 trial, demonstrated that patients treated with the combination of panobinostat, BTZ, and dexamethasone (PAN-BTZ-Dex) benefited in terms of OS compared to placebo (BTZ and dexamethasone alone) [114]. Therefore, the inhibition of a proteasome and interference with epigenetic alterations exert synergistic anti-MM effects. A subgroup analysis included patients who had received a prior immunomodulatory drug (IMiD) or BTZ plus IMiD or ≥2 prior regimens including BTZ and IMiD. This analysis demonstrated a clear benefit in terms of PFS with PAN-BTZ-Dex among patients who had received ≥2 prior regimens containing BTZ and IMiD, a subgroup of patients with limited therapeutic options and a worse prognosis [115]. Studies conducted in a clinical setting are summarized in Table 1.

Table 1. Clinical implications of epigenetic alterations in MM treated with BTZ-based regimens.

Qin et al. [83]	miR-137 increases sensitivity to BTZ whilst the low expression of miR-137 is associated with shorter OS and PFS.
De Larrea et al. [111]	The hypomethylation of *NFKB1* is associated with good response to BTZ and better OS. More than 3.95% of total methylated DNA correlates with better OS.
Szudy-Szczyrek et al. [112]	The high expression of miR-8074 is associated with a higher risk of death and shorter PFS in MM exposed to BTZ and THD.
San-Miguel et al. [113,114]; Richardson et al. [115]	The combination of Panobinostat (epigenetic drug) with BTZ and dexamethasone is an effective treatment regimen.

8. Conclusions

The implementation of BTZ, a first-in-class proteasome inhibitor, was a gamechanger in the treatment of MM. It not only improved the clinical outcomes of MM patients, but also laid the foundation for the further development of next-generation molecules, i.e., carfilzomib and ixazomib. In addition to proteasome inhibition, the effects of BTZ are closely related to epigenetic changes. First, BTZ cytotoxic effects are mediated, among others, through a global decrease in methylation in target malignant cells. Therefore, hypomethy-

lation is a vital aspect of BTZ's mechanism of action. Furthermore, the development of BTZ resistance is also associated with changes in the methylome. In addition, drugs interfering with epigenetic mechanisms, such as AZA or DAC, have been shown to be effective in two different ways. In the first place, it should be mentioned that they have been demonstrated to restore the BTZ sensitivity of BTZ-resistant malignant clones. Moreover, in combination with BTZ, they have a synergistic effect on cells previously not exposed to the proteasome inhibitor. Finally, epigenetic alterations contribute to the development of BTZ adverse effects, such as drug-induced peripheral neuropathy. In addition to various changes in global methylation status, the role of non-coding RNA and histone modifications, particularly in patients with certain cytogenetic abnormalities, is still a subject of ongoing research.

It should be noted that majority of studies were conducted in vitro. Therefore, it is of a paramount importance to further explore this area in a clinical setting. This approach would lead to clinically relevant results that could improve patient outcomes.

9. Future Perspectives

BTZ induces genome-wide methylation changes, which, on one hand, are part of its mechanism of action. On the other hand, they mediate the development of BTZ resistance. The reversibility of methylation changes renders them an interesting research area and a potential therapeutic strategy. Methylation inhibitors have already been shown to act synergistically with BTZ and counteract BTZ resistance when administered to resistant clones. However, there are aspects of epigenetic alterations that need to be further explored. There are novel molecules with a dual mechanism of action, targeting two different epigenetic modifications. They inhibit G9a and DNMT simultaneously. Their efficacy has been confirmed in the treatment of solid tumors such as cholangiocarcinoma and hepatocellular carcinoma [113,114] and several hematologic malignancies, be it acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), or diffuse large B-cell lymphoma (DLBCL), both in cell lines and in mouse xenograft models [115]. A similar molecule that, in addition to inhibiting G9a and DNMT, also interfered with histone deacetylases was tested in MM. The results revealed that the anti-MM effects were achieved through histone-3 acetylation, DNA hypomethylation, and decreased histone-3 methylation at lysine-9. Efficacy was confirmed first in MM cell lines and subsequently in a mouse xenograft model [116]. Despite promising results, these molecules have not been tested in combination with other drugs. The combination of epigenetic agents with BTZ may be investigated due to the fact that both BTZ cytotoxicity and the development of resistance are largely dependent on epigenetic changes, which renders this an interesting research direction.

Author Contributions: Conceptualization, P.K.; methodology, P.K.; investigation, P.K., K.Ł., B.B. and E.B.; figure preparation, E.B.; writing—original draft, P.K.; writing—review and editing, K.Ł., B.B. and B.M.; supervision, B.M.; funding acquisition, B.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was founded by the Minister of Science and Higher Education under the name "Regional Initiative of Excellence" in 2019–2022 (project number 002/RID/2018/19).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in this article.

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

References

1. Merrick, W.C. Mechanism and regulation of eukaryotic protein synthesis. *Microbiol. Rev.* **1992**, *56*, 291–315. [CrossRef] [PubMed]
2. Zhao, L.; Zhao, J.; Zhong, K.; Tong, A.; Jia, D. Targeted protein degradation: Mechanisms, strategies and application. *Signal Transduc. Target Ther.* **2022**, *7*, 113. [CrossRef] [PubMed]
3. Thrower, J.S. Recognition of the polyubiquitin proteolytic signal. *EMBO J.* **2000**, *19*, 94–102. [CrossRef] [PubMed]

4. Glickman, M.H.; Ciechanover, A. The Ubiquitin-Proteasome Proteolytic Pathway: Destruction for the Sake of Construction. *Physiol. Rev.* **2002**, *82*, 373–428. [CrossRef] [PubMed]
5. Hicke, L. Gettin' down with ubiquitin: Turning off cell-surface receptors, transporters and channels. *Trends Cell Biol.* **1999**, *9*, 107–112. [CrossRef] [PubMed]
6. Suresh, B.; Lee, J.; Kim, K.-S.; Ramakrishna, S. The Importance of Ubiquitination and Deubiquitination in Cellular Reprogramming. *Stem Cells Int.* **2016**, *2016*, 6705927. [CrossRef] [PubMed]
7. Budenholzer, L.; Cheng, C.L.; Li, Y.; Hochstrasser, M. Proteasome Structure and Assembly. *J. Mol. Biol.* **2017**, *429*, 3500–3524. [CrossRef]
8. Bard, J.A.M.; Goodall, E.A.; Greene, E.R.; Jonsson, E.; Dong, K.C.; Martin, A. Structure and Function of the 26S Proteasom. *Annu. Rev. Biochem.* **2018**, *87*, 697–724. [CrossRef]
9. Yee, A.J. The role of carfilzomib in relapsed/refractory multiple myeloma. *Ther. Adv. Hematol.* **2021**, *12*, 204062072110196. [CrossRef]
10. Bonnet, A.; Moreau, P. Safety of ixazomib for the treatment of multiple myeloma. *Expert Opin. Drug Saf.* **2017**, *16*, 973–980. [CrossRef]
11. Levêque, D.; Carvalho, M.C.M.; Maloisel, F. Clinical pharmacokinetics of bortezomib. *In Vivo* **2007**, *21*, 273–278. [PubMed]
12. Azab, A.K.; Muz, B.; Ghazarian, R.; Ou, M.; Luderer, M.; Kusdono, H. Spotlight on ixazomib: Potential in the treatment of multiple myeloma. *Drug Des. Dev. Ther.* **2016**, *2016*, 217–226. [CrossRef] [PubMed]
13. Perel, G.; Bliss, J.; Thomas, C.M. Carfilzomib (Kyprolis): A Novel Proteasome Inhibitor for Relapsed and/or Refractory Multiple Myeloma. *P T Peer-Rev. J. Formul. Manag.* **2016**, *41*, 303–307.
14. Offidani, M.; Corvatta, L.; Gentili, S.; Maracci, L.; Leoni, P. Oral ixazomib maintenance therapy in multiple myeloma. *Expert Rev. Anticancer. Ther.* **2016**, *16*, 21–32. [CrossRef] [PubMed]
15. Richardson, P.G.; Mitsiades, C.; Hideshima, T.; Anderson, K.C. Bortezomib: Proteasome Inhibition as an Effective Anticancer Therapy. *Annu. Rev. Med.* **2006**, *57*, 33–47. [CrossRef] [PubMed]
16. Hamilton, A.L.; Eder, J.P.; Pavlick, A.C.; Clark, J.W.; Liebes, L.; Garcia-Carbonero, R.; Chachoua, A.; Ryan, D.; Soma, V.; Farrell, K.; et al. Proteasome Inhibition with Bortezomib (PS-341): A Phase I Study with Pharmacodynamic End Points Using a Day 1 and Day 4 Schedule in a 14-Day Cycl. *J. Clin. Oncol.* **2005**, *23*, 6107–6116. [CrossRef] [PubMed]
17. Moreau, P.; Pylypenko, H.; Grosicki, S.; Karamanesht, I.; Leleu, X.; Grishunina, M.; Rekhtman, G.; Masliak, Z.; Robak, T.; Shubina, A.; et al. Subcutaneous versus intravenous administration of bortezomib in patients with relapsed multiple myeloma: A randomised, phase 3, non-inferiority study. *Lancet Oncol.* **2011**, *12*, 431–440. [CrossRef] [PubMed]
18. Uttamsingh, V.; Lu, C.; Miwa, G.; Gan, L.-S. Relative contributions of the five major human cytochromes P450, 1A2, 2C9, 2C19, 2D6, and 3A4, to the hepatic metabolism of the proteasome inhibitor bortezomib. *Drug Metab. Dispos.* **2005**, *33*, 1723–1728. [CrossRef]
19. Labutti, J.; Parsons, I.; Huang, R.; Miwa, G.; Gan, L.-S.; Daniels, J.S. Oxidative Deboronation of the Peptide Boronic Acid Proteasome Inhibitor Bortezomib: Contributions from Reactive Oxygen Species in This Novel Cytochrome P450 Reactio. *Chem. Res. Toxicol.* **2006**, *19*, 539–546. [CrossRef]
20. Schwartz, R.; Davidson, T. Pharmacology, pharmacokinetics, and practical applications of bortezomib. *Oncology* **2004**, *18* (Suppl. S11), 14–21.
21. Richardson, P.G.; Barlogie, B.; Berenson, J.; Singhal, S.; Jagannath, S.; Irwin, D.; Rajkumar, S.V.; Srkalovic, G.; Alsina, M.; Alexanian, R.; et al. A Phase 2 Study of Bortezomib in Relapsed, Refractory Myeloma. *N. Engl. J. Med.* **2003**, *348*, 2609–2617. [CrossRef] [PubMed]
22. Berenson, J.R.; Jagannath, S.; Barlogie, B.; Siegel, D.T.; Alexanian, R.; Richardson, P.G.; Irwin, D.; Alsina, M.; Rajkumar, S.V.; Srkalovic, G.; et al. Safety of prolonged therapy with bortezomib in relapsed or refractory multiple myeloma. *Cancer* **2005**, *104*, 2141–2148. [CrossRef] [PubMed]
23. Lee, K.-W.; Yun, T.; Song, E.K.; Na, I.I.; Shin, H.; Bang, S.-M.; Lee, J.H.; Lee, S.T.; Kim, J.H.; Yoon, S.-S.; et al. A Pilot Study of Bortezomib in Korean Patients with Relapsed or Refractory Myeloma. *J. Korean Med. Sci.* **2005**, *20*, 598–602. [CrossRef] [PubMed]
24. Reece, D.E.; Sanchorawala, V.; Hegenbart, U.; Merlini, G.; Palladini, G.; Fermand, J.-P.; Vescio, R.A.; Liu, X.; Elsayed, Y.A.; Cakana, A.; et al. Weekly and twice-weekly bortezomib in patients with systemic AL amyloidosis: Results of a phase 1 dose-escalation study. *Blood* **2009**, *114*, 1489–1497. [CrossRef] [PubMed]
25. Reece, D.E.; Hegenbart, U.; Sanchorawala, V.; Merlini, G.; Palladini, G.; Bladé, J.; Fermand, J.P.; Hassoun, H.; Heffner, L.; Vescio, R.A.; et al. Efficacy and safety of once-weekly and twice-weekly bortezomib in patients with relapsed systemic AL amyloidosis: Results of a phase 1/2 study. *Blood* **2011**, *118*, 865–873. [CrossRef] [PubMed]
26. Besse, B.; Planchard, D.; Veillard, A.-S.; Taillade, L.; Khayat, D.; Ducourtieux, M.; Pignon, J.-P.; Lumbroso, J.; Lafontaine, C.; Mathiot, C.; et al. Phase 2 study of frontline bortezomib in patients with advanced non-small cell lung cancer. *Lung Cancer* **2012**, *76*, 78–83. [CrossRef] [PubMed]
27. Di Bella, N.; Taetle, R.; Kolibaba, K.; Boyd, T.; Raju, R.; Barrera, D.; Cochran, E.W., Jr.; Dien, P.Y.; Lyons, R.; Schlegel, P.J.; et al. Results of a phase 2 study of bortezomib in patients with relapsed or refractory indolent lymphoma. *Blood* **2010**, *115*, 475–480. [CrossRef]
28. Oriol, A.; Giraldo, P.; Kotsianidis, I.; Couturier, C.; Olie, R.; Angermund, R.; Corso, A. Efficacy and safety of bortezomib-based retreatment at the first relapse in multiple myeloma patients: A retrospective study. *Hematology* **2015**, *20*, 405–409. [CrossRef]

29. Hrusovsky, I.; Emmerich, B.; Von Rohr, A.; Voegeli, J.; Taverna, C.; Olie, R.A.; Pliskat, H.; Frohn, C.; Hess, G. Bortezomib Retreatment in Relapsed Multiple Myeloma—Results from a Retrospective Multicentre Survey in Germany and Switzerland. *Oncology* **2010**, *79*, 247–254. [CrossRef]
30. Hulin, C.; De La Rubia, J.; Dimopoulos, M.A.; Terpos, E.; Katodritou, E.; Hungria, V.; De Samblanx, H.; Stoppa, A.; Aagesen, J.; Sargin, D.; et al. Bortezomib retreatment for relapsed and refractory multiple myeloma in real-world clinical practice. *Health Sci. Rep.* **2019**, *2*, e104. [CrossRef]
31. Sood, R.; Carloss, H.; Kerr, R.; Lopez, J.; Lee, M.; Druck, M.; Walters, I.B.; Noga, S.J. Retreatment with bortezomib alone or in combination for patients with multiple myeloma following an initial response to bortezomib. *Am. J. Hematol.* **2009**, *84*, 657–660. [CrossRef] [PubMed]
32. Palumbo, A.; Chanan-Khan, A.; Weisel, K.; Nooka, A.K.; Masszi, T.; Beksac, M.; Spicka, I.; Hungria, V.; Munder, M.; Mateos, M.V.; et al. Daratumumab, Bortezomib, and Dexamethasone for Multiple Myeloma. *N. Engl. J. Med.* **2016**, *375*, 754–766. [CrossRef] [PubMed]
33. Grosicki, S.; Simonova, M.; Spicka, I.; Pour, L.; Kriachok, I.; Gavriatopoulou, M.; Pylypenko, H.; Auner, H.W.; Leleu, X.; Doronin, V.; et al. Once-per-week selinexor, bortezomib, and dexamethasone versus twice-per-week bortezomib and dexamethasone in patients with multiple myeloma (BOSTON): A randomised, open-label, phase 3 trial. *Lancet* **2020**, *396*, 1563–1573. [CrossRef] [PubMed]
34. Teachey, D.T.; Devidas, M.; Wood, B.L.; Chen, Z.; Hayashi, R.J.; Hermiston, M.L.; Annett, R.D.; Archer, J.H.; Asselin, B.L.; August, K.J.; et al. Children's Oncology Group Trial AALL1231: A Phase III Clinical Trial Testing Bortezomib in Newly Diagnosed T-Cell Acute Lymphoblastic Leukemia and Lymphoma. *J. Clin. Oncol.* **2022**, *40*, 2106–2118. [CrossRef] [PubMed]
35. Irvin, W.J.; Orlowski, R.Z.; Chiu, W.-K.; Carey, L.A.; Collichio, F.A.; Bernard, P.S.; Stijleman, I.J.; Perou, C.; Ivanova, A.; Dees, E.C. Phase II study of bortezomib and pegylated liposomal doxorubicin in the treatment of metastatic breast cancer. *Clin. Breast Cancer* **2010**, *10*, 465–470. [CrossRef]
36. Trinh, X.B.; Sas, L.; Van Laere, S.J.; Prové, A.; Deleu, I.; Rasschaert, M.; Van de Velde, H.; Vinken, P.; Vermeulen, P.B.; Van Dam, P.A.; et al. A phase II study of the combination of endocrine treatment and bortezomib in patients with endocrine-resistant metastatic breast cancer. *Oncol. Rep.* **2012**, *27*, 657–663.
37. Lee, Y.J.; Seol, A.; Lee, M.; Kim, J.-W.; Kim, H.S.; Kim, K.; Suh, D.H.; Kim, S.; Kim, S.W.; Lee, J.Y. A Phase II Trial to Evaluate the Efficacy of Bortezomib and Liposomal Doxorubicin in Patients with BRCA Wild-type Platinum-resistant Recurrent Ovarian Cancer (KGOG 3044/EBLIN). *Vivo* **2022**, *36*, 1949–1958. [CrossRef]
38. Lara, P.N.; Longmate, J.; Reckamp, K.; Gitlitz, B.; Argiris, A.; Ramalingam, S.; Belani, C.P.; Mack, P.C.; Lau, D.H.; Koczywas, M.; et al. Randomized phase II trial of concurrent versus sequential bortezomib plus docetaxel in advanced non-small-cell lung cancer: A California cancer consortium trial. *Clin. Lung Cancer* **2011**, *12*, 33–37. [CrossRef]
39. Raedler, L. Velcade (Bortezomib) Receives 2 New FDA Indications: For Retreatment of Patients with Multiple Myeloma and for First-Line Treatment of Patients with Mantle-Cell Lymphoma. *Am. Health Drug Benefits* **2015**, *8*, 135–140.
40. Hamilton, J.P. Epigenetics: Principles and Practice. *Dig. Dis.* **2011**, *29*, 130–135. [CrossRef]
41. Metere, A.; Graves, C.E. Factors Influencing Epigenetic Mechanisms: Is There A Role for Bariatric Surgery? *High-Throughput* **2020**, *9*, 6. [CrossRef] [PubMed]
42. Wu, Y.-L.; Lin, Z.-J.; Li, C.-C.; Lin, X.; Shan, S.-K.; Guo, B.; Zheng, M.-H.; Li, F.; Yuan, L.-Q.; Li, Z.-H. Epigenetic regulation in metabolic diseases: Mechanisms and advances in clinical study. *Signal Transduct. Target. Ther.* **2023**, *8*, 98. [CrossRef] [PubMed]
43. Łuczkowska, K.; Sokolowska, K.E.; Taryma-Lesniak, O.; Pastuszak, K.; Supernat, A.; Bybjerg-Grauholm, J.; Hansen, L.L.; Paczkowska, E.; Wojdacz, T.K.; Machaliński, B. Bortezomib induces methylation changes in neuroblastoma cells that appear to play a significant role in resistance development to this compound. *Sci. Rep.* **2021**, *11*, 9846. [CrossRef] [PubMed]
44. Łuczkowska, K.; Taryma-Leśniak, O.; Bińkowski, J.; Sokołowska, K.E.; Strapagiel, D.; Jarczak, J.; Paczkowska, E.; Machaliński, B.; Wojdacz, T.K. Long-Term Treatment with Bortezomib Induces Specific Methylation Changes in Differentiated Neuronal Cells. *Cancers* **2022**, *14*, 3402. [CrossRef] [PubMed]
45. Łuczkowska, K.; Kulig, P.; Rusińska, K.; Baumert, B.; Machaliński, B. 5-Aza-2′-Deoxycytidine Alters the Methylation Profile of Bortezomib-Resistant U266 Multiple Myeloma Cells and Affects Their Proliferative Potential. *Int. J. Mol. Sci.* **2023**, *24*, 16780. [CrossRef] [PubMed]
46. Łuczkowska, K.; Kulig, P.; Baumert, B.; Machaliński, B. The Evidence That 25(OH)D3 and VK2 MK-7 Vitamins Influence the Proliferative Potential and Gene Expression Profiles of Multiple Myeloma Cells and the Development of Resistance to Bortezomib. *Nutrients* **2022**, *14*, 5190. [CrossRef] [PubMed]
47. Liu, S.; Liu, Z.; Xie, Z.; Pang, J.; Yu, J.; Lehmann, E.; Huynh, L.; Vukosavljevic, T.; Takeki, M.; Klisovic, R.B.; et al. Bortezomib induces DNA hypomethylation and silenced gene transcription by interfering with Sp1/NF-κB–dependent DNA methyltransferase activity in acute myeloid leukemia. *Blood* **2008**, *111*, 2364–2373. [CrossRef]
48. Leshchenko, V.V.; Kuo, P.-Y.; Jiang, Z.; Weniger, M.A.; Overbey, J.; Dunleavy, K.; Wilson, W.H.; Wiestner, A.; Parekh, S. Harnessing Noxa demethylation to overcome Bortezomib resistance in mantle cell lymphoma. *Oncotarget* **2015**, *6*, 27332–27342. [CrossRef]
49. Wirth, M.; Stojanovic, N.; Christian, J.; Paul, M.C.; Stauber, R.H.; Schmid, R.M.; Häcker, G.; Krämer, O.H.; Saur, D.; Schneider, G. MYC and EGR1 synergize to trigger tumor cell death by controlling NOXA and BIM transcription upon treatment with the proteasome inhibitor bortezomib. *Nucleic Acids Res.* **2014**, *42*, 10433–10447. [CrossRef]

50. Kikuchi, J.; Wada, T.; Shimizu, R.; Izumi, T.; Akutsu, M.; Mitsunaga, K.; Noborio-Hatano, K.; Nobuyoshi, M.; Ozawa, K.; Kano, Y.; et al. Histone deacetylases are critical targets of bortezomib-induced cytotoxicity in multiple myeloma. *Blood* **2010**, *116*, 406–417. [CrossRef]
51. Jeusset, L.; McManus, K. Developing Targeted Therapies That Exploit Aberrant Histone Ubiquitination in Cancer. *Cells* **2019**, *8*, 165. [CrossRef] [PubMed]
52. Kamens, J.L.; Nance, S.; Koss, C.; Xu, B.; Cotton, A.; Lam, J.W.; Garfinkle, E.A.R.; Nallagatla, P.; Smith, A.M.R.; Mitchell, S.; et al. Proteasome inhibition targets the KMT2A transcriptional complex in acute lymphoblastic leukemia. *Nat. Commun.* **2023**, *14*, 809. [CrossRef] [PubMed]
53. Xu, Q.; Farah, M.; Webster, J.M.; Wojcikiewicz, R.J.H. Bortezomib rapidly suppresses ubiquitin thiolesterification to ubiquitin-conjugating enzymes and inhibits ubiquitination of histones and type I inositol 1,4,5-trisphosphate receptor. *Mol. Cancer Ther.* **2004**, *3*, 1263–1269. [CrossRef] [PubMed]
54. Tsvetkov, P.; Sokol, E.; Jin, D.; Brune, Z.; Thiru, P.; Ghandi, M.; Garraway, L.A.; Gupta, P.B.; Santagata, S.; Whitesell, L.; et al. Suppression of 19S proteasome subunits marks emergence of an altered cell state in diverse cancers. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 382–387. [CrossRef] [PubMed]
55. Hu, X.; Xuan, H.; Du, H.; Jiang, H.; Huang, J. Down-Regulation of CD9 by Methylation Decreased Bortezomib Sensitivity in Multiple Myeloma. *Agoulnik IU, editoPLoS ONE* **2014**, *9*, e95765. [CrossRef] [PubMed]
56. Rodrigues-Junior, D.; Biassi, T.; De Albuquerque, G.; Carlin, V.; Buri, M.; Machado-Junior, J.; Vettore, A.L.; Biassi, T.P. Down-regulation of DCC sensitizes multiple myeloma cells to bortezomib treatment. *Mol. Med. Rep.* **2019**, *19*, 5023–5029. [CrossRef] [PubMed]
57. Rashid, M.B.M.A.; Toh, T.B.; Hooi, L.; Silva, A.; Zhang, Y.; Tan, P.F.; Teh, A.L.; Karnani, N.; Jha, S.; Ho, C.-M.; et al. Optimizing drug combinations against multiple myeloma using a quadratic phenotypic optimization platform (QPOP). *Sci. Transl. Med.* **2018**, *10*, eaan0941. [CrossRef]
58. Li, M.; Qi, L.; Xu, J.-B.; Zhong, L.-Y.; Chan, S.; Chen, S.-N.; Shao, X.-R.; Zheng, L.-Y.; Dong, Z.-X.; Fang, T.-L.; et al. Methylation of the Promoter Region of the Tight Junction Protein-1 by DNMT1 Induces EMT-like Features in Multiple Myeloma. *Mol. Ther.-Oncolytics* **2020**, *19*, 197–207. [CrossRef]
59. Qi, W.; White, M.C.; Choi, W.; Guo, C.; Dinney, C.; McConkey, D.J.; Siefker-Radtke, A. Inhibition of Inducible Heat Shock Protein-70 (Hsp72) Enhances Bortezomib-Induced Cell Death in Human Bladder Cancer Cells. *PLoS ONE* **2013**, *8*, e69509. [CrossRef]
60. Jin, Y.; Xu, L.; Wu, X.; Feng, J.; Shu, M.; Gu, H.; Gao, G.; Zhang, J.; Dong, B.; Chen, X. Synergistic Efficacy of the Demethylation Agent Decitabine in Combination with the Protease Inhibitor Bortezomib for Treating Multiple Myeloma through the Wnt/β-Catenin Pathway. *Oncol. Res. Featur. Preclin. Clin. Cancer Ther.* **2019**, *27*, 729–737. [CrossRef]
61. Das, D.S.; Ray, A.; Das, A.; Song, Y.; Tian, Z.; Oronsky, B.; Richardson, P.; Scicinski, J.; Chauhan, D.; Anderson, K.C. A novel hypoxia-selective epigenetic agent RRx-001 triggers apoptosis and overcomes drug resistance in multiple myeloma cells. *Leukemia* **2016**, *30*, 2187–2197. [CrossRef] [PubMed]
62. Cao, Y.; Qiu, G.-Q.; Wu, H.-Q.; Wang, Z.-L.; Lin, Y.; Wu, W.; Xie, X.-B.; Gu, W.-Y. Decitabine enhances bortezomib treatment in RPMI 8226 multiple myeloma cells. *Mol. Med. Rep.* **2016**, *14*, 3469–3475. [CrossRef] [PubMed]
63. Xu, A.; Zhang, J.; Zuo, L.; Yan, H.; Chen, L.; Zhao, F.; Fan, F.; Xu, J.; Zhang, B.; Zhang, Y.; et al. FTO promotes multiple myeloma progression by posttranscriptional activation of HSF1 in an m6A-YTHDF2-dependent manner. *Mol. Ther.* **2022**, *30*, 1104–1118. [CrossRef] [PubMed]
64. Jia, Y.; Yu, X.; Liu, R.; Shi, L.; Jin, H.; Yang, D.; Zhang, X.; Shen, Y.; Feng, Y.; Zhang, P.; et al. PRMT1 methylation of WTAP promotes multiple myeloma tumorigenesis by activating oxidative phosphorylation via m6A modification of NDUFS6. *Cell Death Dis.* **2023**, *14*, 512. [CrossRef] [PubMed]
65. Sun, X.; Xie, Y.; Sun, X.; Yao, Y.; Li, H.; Li, Z.; Yao, Y.; Xu, K. The selective HDAC6 inhibitor Nexturastat A induces apoptosis, overcomes drug resistance and inhibits tumor growth in multiple myeloma. *Biosci. Rep.* **2019**, *39*, BSR20181916. [CrossRef] [PubMed]
66. Bergsagel, P.L.; Kuehl, W.M. Chromosome translocations in multiple myeloma. *Oncogene* **2001**, *20*, 5611–5622. [CrossRef]
67. Abdallah, N.; Rajkumar, S.V.; Greipp, P.; Kapoor, P.; Gertz, M.A.; Dispenzieri, A.; Baughn, L.B.; Lacy, M.Q.; Hayman, S.R.; Buadi, F.K.; et al. Cytogenetic abnormalities in multiple myeloma: Association with disease characteristics and treatment responsE. *Blood Cancer J.* **2020**, *10*, 82. [CrossRef]
68. Sato, S.; Kamata, W.; Okada, S.; Tamai, Y. Clinical and prognostic significance of t(4;14) translocation in multiple myeloma in the era of novel agents. *Int. J. Hematol.* **2021**, *113*, 207–213. [CrossRef]
69. Jiang, H.; Wang, Y.; Wang, J.; Wang, Y.; Wang, S.; He, E.; Guo, J.; Xie, Y.; Wang, J.; Li, X.; et al. Posttranslational modification of Aurora A-NSD2 loop contributes to drug resistance in t(4;14) multiple myeloma. *Clin. Transl. Med.* **2022**, *12*, e744. [CrossRef]
70. Liu, J.; Xie, Y.; Guo, J.; Li, X.; Wang, J.; Jiang, H.; Peng, Z.; Wang, J.; Wang, S.; Li, Q.; et al. Targeting NSD2-mediated SRC-3 liquid–liquid phase separation sensitizes bortezomib treatment in multiple myeloma. *Nat. Commun.* **2021**, *12*, 1022. [CrossRef]
71. Bollmann, L.M.; Skerhut, A.J.; Asfaha, Y.; Horstick, N.; Hanenberg, H.; Hamacher, A.; Kurz, T.; Kassack, M.U. The Novel Class IIa Selective Histone Deacetylase Inhibitor YAK540 Is Synergistic with Bortezomib in Leukemia Cell Lines. *Int. J. Mol. Sci.* **2022**, *23*, 13398. [CrossRef] [PubMed]

72. Xu, L.; Feng, J.; Tang, H.; Dong, Y.; Shu, M.; Chen, X. Chidamide epigenetically represses autophagy and exerts cooperative antimyeloma activity with bortezomib. *Cell Death Dis.* **2020**, *11*, 297. [CrossRef] [PubMed]
73. Muguruma, Y.; Yahata, T.; Warita, T.; Hozumi, K.; Nakamura, Y.; Suzuki, R.; Ito, M.; Ando, K. Jagged1-induced Notch activation contributes to the acquisition of bortezomib resistance in myeloma cells. *Blood Cancer J.* **2017**, *7*, 650. [CrossRef] [PubMed]
74. Sripayap, P.; Nagai, T.; Hatano, K.; Kikuchi, J.; Furukawa, Y.; Ozawa, K. Romidepsin Overcomes Cell Adhesion-Mediated Drug Resistance in Multiple Myeloma Cells. *Acta Haematol.* **2014**, *132*, 1–4. [CrossRef] [PubMed]
75. Chu, Y.-Y.; Ko, C.-Y.; Wang, S.-M.; Lin, P.-I.; Wang, H.-Y.; Lin, W.-C.; Wu, D.-Y.; Wang, L.-H.; Wang, J.-M. Bortezomib-induced miRNAs direct epigenetic silencing of locus genes and trigger apoptosis in leukemia. *Cell Death Dis.* **2017**, *8*, e3167. [CrossRef] [PubMed]
76. Wang, Y.; Lin, Q.; Song, C.; Ma, R.; Li, X. Depletion of circ_0007841 inhibits multiple myeloma development and BTZ resistance via miR-129-5p/JAG1 axis. *Cell Cycle* **2020**, *19*, 3289–3302. [CrossRef] [PubMed]
77. Amodio, N.; Leotta, M.; Bellizzi, D.; Di Martino, M.T.; D'Aquila, P.; Lionetti, M.; Fabiani, F.; Leone, E.; Gullà, A.M.; Passarino, G.; et al. DNA-demethylating and anti-tumor activity of synthetic miR-29b mimics in multiple myeloma. *Oncotarget* **2012**, *3*, 1246–1258. [CrossRef]
78. Amodio, N.; Bellizzi, D.; Leotta, M.; Raimondi, L.; Biamonte, L.; D'Aquila, P.; Di Martino, M.T.; Calimeri, T.; Rossi, M.; Lionetti, M.; et al. miR-29b induces SOCS-1 expression by promoter demethylation and negatively regulates migration of multiple myeloma and endothelial cells. *Cell Cycle* **2013**, *12*, 3650–3662. [CrossRef]
79. Amodio, N.; Di Martino, M.T.; Foresta, U.; Leone, E.; Lionetti, M.; Leotta, M.; Gullà, A.M.; Pitari, M.R.; Conforti, F.; Rossi, M.; et al. miR-29b sensitizes multiple myeloma cells to bortezomib-induced apoptosis through the activation of a feedback loop with the transcription factor Sp1. *Cell Death Dis.* **2012**, *3*, e436. [CrossRef]
80. Pan, Y.; Chen, H.; Shen, X.; Wang, X.; Ju, S.; Lu, M.; Cong, H. Serum level of long noncoding RNA H19 as a diagnostic biomarker of multiple myeloma. *Clin. Chim. Acta* **2018**, *480*, 199–205. [CrossRef]
81. Pan, Y.; Zhang, Y.; Liu, W.; Huang, Y.; Shen, X.; Jing, R.; Pu, J.; Wang, X.; Ju, S.; Cong, H.; et al. LncRNA H19 overexpression induces bortezomib resistance in multiple myeloma by targeting MCL-1 via miR-29b-3p. *Cell Death Dis.* **2019**, *10*, 106. [CrossRef] [PubMed]
82. Fu, Y.; Liu, X.; Zhang, F.; Jiang, S.; Liu, J.; Luo, Y. Bortezomib-inducible long non-coding RNA myocardial infarction associated transcript is an oncogene in multiple myeloma that suppresses miR-29b. *Cell Death Dis.* **2019**, *10*, 319. [CrossRef] [PubMed]
83. Qin, Y.; Zhang, S.; Deng, S.; An, G.; Qin, X.; Li, F.; Xu, Y.; Hao, M.; Yang, Y.; Zhou, W.; et al. Epigenetic silencing of miR-137 induces drug resistance and chromosomal instability by targeting AURKA in multiple myeloma. *Leukemia* **2017**, *31*, 1123–1135. [CrossRef] [PubMed]
84. Wu, H.; Liu, C.; Yang, Q.; Xin, C.; Du, J.; Sun, F.; Zhou, L. MIR145-3p promotes autophagy and enhances bortezomib sensitivity in multiple myeloma by targeting HDAC4. *Autophagy* **2020**, *16*, 683–697. [CrossRef] [PubMed]
85. Nian, F.; Zhu, J.; Chang, H. Long non-coding RNA ANGPTL1-3 promotes multiple myeloma bortezomib resistance by sponging miR-30a-3p to activate c-Maf expression. *Biochem. Biophys. Res. Commun.* **2019**, *514*, 1140–1146. [CrossRef] [PubMed]
86. Liu, D.; Wang, Y.; Li, H.; Peng, S.; Tan, H.; Huang, Z. Circular RNA circ-CCT3 promotes bortezomib resistance in multiple myeloma via modulating miR-223-3p/BRD4 axis. *Anti-Cancer Drugs* **2022**, *33*, e145–e154. [CrossRef]
87. Saltarella, I.; Lamanuzzi, A.; Desantis, V.; Di Marzo, L.; Melaccio, A.; Curci, P.; Annese, T.; Nico, B.; Solimando, A.G.; Bartoli, G.; et al. Myeloma cells regulate MIRNA transfer from fibroblast-derived exosomes by expression of lncRNAs. *J. Pathol.* **2022**, *256*, 402–413. [CrossRef]
88. Yuan, X.; Ma, R.; Yang, S.; Jiang, L.; Wang, Z.; Zhu, Z.; Li, H. miR-520g and miR-520h overcome bortezomib resistance in multiple myeloma via suppressing APE1. *Cell Cycle* **2019**, *18*, 1660–1669. [CrossRef]
89. Yang, L.-H.; Du, P.; Liu, W.; An, L.-K.; Li, J.; Zhu, W.-Y.; Yuan, S.; Wang, L.; Zang, L. LncRNA ANRIL promotes multiple myeloma progression and bortezomib resistance by EZH2-mediated epigenetically silencing of PTEN. *Neoplasma* **2021**, *68*, 788–797. [CrossRef]
90. Che, F.; Ye, X.; Wang, Y.; Ma, S.; Wang, X. Lnc NEAT1/miR-29b-3p/Sp1 form a positive feedback loop and modulate bortezomib resistance in human multiple myeloma cells. *Eur. J. Pharmacol.* **2021**, *891*, 173752. [CrossRef]
91. Fu, C.; Wang, J.; Hu, M.; Zhou, W. Circ_0005615 contributes to the progression and Bortezomib resistance of multiple myeloma by sponging miR-185-5p and upregulating IRF4. *Anti-Cancer Drugs* **2022**, *33*, 893–902. [CrossRef] [PubMed]
92. Chen, H.; Cao, W.; Chen, J.; Liu, D.; Zhou, L.; Du, F.; Zhu, F. miR-218 contributes to drug resistance in multiple myeloma via targeting LRRC28. *J. Cell. Biochem.* **2021**, *122*, 305–314. [CrossRef] [PubMed]
93. Tang, J.; Chen, Q.; Li, Q.; He, Y.; Xiao, D. Exosomal mRNAs and lncRNAs involved in multiple myeloma resistance to bortezomib. *Cell Biol. Int.* **2021**, *45*, 965–975. [CrossRef] [PubMed]
94. Malek, E.; Kim, B.; Driscoll, J. Identification of Long Non-Coding RNAs Deregulated in Multiple Myeloma Cells Resistant to Proteasome Inhibitors. *Genes* **2016**, *7*, 84. [CrossRef] [PubMed]
95. Shen, X.; Shen, P.; Yang, Q.; Yin, Q.; Wang, F.; Cong, H.; Wang, X.; Ju, S. Knockdown of long non-coding RNA PCAT-1 inhibits myeloma cell growth and drug resistance via p38 and JNK MAPK pathways. *J. Cancer* **2019**, *10*, 6502–6510. [CrossRef] [PubMed]
96. Liu, Y.; Cheng, P.; Zhao, W.; Zhu, L.; Sui, J.; Dai, Y.; Lai, Y. MiR-197-3p reduces bortezomib resistance in multiple myeloma by inhibiting IL-6 expression in a MEAF6-dependent manner. *Leuk. Res.* **2022**, *114*, 106785. [CrossRef] [PubMed]

97. Xi, H.; Li, L.; Du, J.; An, R.; Fan, R.; Lu, J.; Wu, Y.-X.; Wu, S.-X.; Hou, J.; Zhao, L.-M. hsa-miR-631 resensitizes bortezomib-resistant multiple myeloma cell lines by inhibiting UbcH10. *Oncol. Rep.* **2017**, *37*, 961–968. [CrossRef]
98. Tian, F.; Zhan, Y.; Zhu, W.; Li, J.; Tang, M.; Chen, X.; Jiang, J. MicroRNA-497 inhibits multiple myeloma growth and increases susceptibility to bortezomib by targeting Bcl-2. *Int. J. Mol. Med.* **2018**, *43*, 1058–1066. [CrossRef]
99. Rastgoo, N.; Wu, J.; Liu, M.; Pourabdollah, M.; Atenafu, E.G.; Reece, D.; Chen, W.; Chang, H. Targeting CD47/TNFAIP8 by miR-155 overcomes drug resistance and inhibits tumor growth through induction of phagocytosis and apoptosis in multiple myeloma. *Haematologica* **2019**, *105*, 2813–2823. [CrossRef]
100. Lerner, M.; Haneklaus, M.; Harada, M.; Grandér, D. MiR-200c Regulates Noxa Expression and Sensitivity to Proteasomal Inhibitors. *PLoS ONE* **2012**, *7*, e36490. [CrossRef]
101. Zang, X.; Wang, J.; Xia, Y.; Li, J.; Chen, L.; Gu, Y.; Shen, X. LncRNA MEG3 promotes the sensitivity of bortezomib by inhibiting autophagy in multiple myeloma. *Leuk. Res.* **2022**, *123*, 106967. [CrossRef] [PubMed]
102. Jagannathan, S.; Vad, N.; Vallabhapurapu, S.; Vallabhapurapu, S.; Anderson, K.C.; Driscoll, J.J. MiR-29b replacement inhibits proteasomes and disrupts aggresome+autophagosome formation to enhance the antimyeloma benefit of bortezomib. *Leukemia* **2015**, *29*, 727–738. [CrossRef] [PubMed]
103. Łuczkowska, K.; Rogińska, D.; Kulig, P.; Bielikowicz, A.; Baumert, B.; Machaliński, B. Bortezomib-Induced Epigenetic Alterations in Nerve Cells: Focus on the Mechanisms Contributing to the Peripheral Neuropathy Development. *Int. J. Mol. Sci.* **2022**, *23*, 2431. [CrossRef] [PubMed]
104. Zheng, Y.; Sun, Y.; Yang, Y.; Zhang, S.; Xu, T.; Xin, W.; Wu, S.; Zhang, X. GATA3-dependent epigenetic upregulation of CCL21 is involved in the development of neuropathic pain induced by bortezomib. *Mol. Pain* **2019**, *15*, 174480691986329. [CrossRef] [PubMed]
105. Chen, K.; Fan, J.; Luo, Z.-F.; Yang, Y.; Xin, W.-J.; Liu, C.-C. Reduction of SIRT1 epigenetically upregulates NALP1 expression and contributes to neuropathic pain induced by chemotherapeutic drug bortezomib. *J. Neuroinflammation* **2018**, *15*, 292. [CrossRef] [PubMed]
106. Liu, C.-C.; Huang, Z.-X.; Li, X.; Shen, K.-F.; Liu, M.; Ouyang, H.-D.; Zhang, S.-B.; Ruan, Y.-T.; Zhang, X.-L.; Wu, S.-L.; et al. Upregulation of NLRP3 via STAT3-dependent histone acetylation contributes to painful neuropathy induced by bortezomib. *Exp. Neurol.* **2018**, *302*, 104–111. [CrossRef] [PubMed]
107. Rullo, L.; Franchi, S.; Amodeo, G.; Caputi, F.F.; Verduci, B.; Losapio, L.M.; Sacerdote, P.; Romualdi, P.; Candeletti, S. Interplay between Prokineticins and Histone Demethylase KDM6A in a Murine Model of Bortezomib-Induced Neuropathy. *Int. J. Mol. Sci.* **2021**, *22*, 11913. [CrossRef] [PubMed]
108. Liu, M.; Zhao, Y.-T.; Lv, Y.-Y.; Xu, T.; Li, D.; Xiong, Y.-C.; Xin, W.-J.; Lin, S.-Y. Metformin Relieves Bortezomib-Induced Neuropathic Pain by Regulating AMPKa2-Mediated Autophagy in the Spinal Dorsal Horn. *Neurochem. Res.* **2022**, *47*, 1878–1887. [CrossRef]
109. Łuczkowska, K.; Rogińska, D.; Ulańczyk, Z.; Safranow, K.; Paczkowska, E.; Baumert, B.; Milczarek, S.; Osękowska, B.; Górska, M.; Borowiecka, E.; et al. microRNAs as the biomarkers of chemotherapy-induced peripheral neuropathy in patients with multiple myeloma. *Leuk. Lymphoma* **2021**, *62*, 2768–2776. [CrossRef]
110. Łuczkowska, K.; Rutka, M.; Rogińska, D.; Paczkowska, E.; Baumert, B.; Milczarek, S.; Górska, M.; Kulig, P.; Osękowska, B.; Janowski, M.; et al. The Potential Role of Proinflammatory Cytokines and Complement Components in the Development of Drug-Induced Neuropathy in Patients with Multiple Myeloma. *J. Clin. Med.* **2021**, *10*, 4584. [CrossRef]
111. Fernández De Larrea, C.; Martín-Antonio, B.; Cibeira, M.T.; Navarro, A.; Tovar, N.; Díaz, T.; Rosiñol, L.; Monzó, M.; Urbano-Ispizua, A.; Bladé, J. Impact of global and gene-specific DNA methylation pattern in relapsed multiple myeloma patients treated with bortezomib. *Leuk. Res.* **2013**, *37*, 641–646. [CrossRef] [PubMed]
112. Szudy-Szczyrek, A.; Mlak, R.; Mielnik, M.; Mazurek, M.; Chocholska, S.; Podgajna, M.; Szczyrek, M.; Homa-Mlak, I.; Małecka-Massalska, T.; Hus, M. Circulating Serum MiRNA-8074 as a Novel Prognostic Biomarker for Multiple Myeloma. *Cells* **2022**, *11*, 752. [CrossRef] [PubMed]
113. San-Miguel, J.F.; Einsele, H.; Moreau, P. The Role of Panobinostat Plus Bortezomib and Dexamethasone in Treating Relapsed or Relapsed and Refractory Multiple Myeloma: A European Perspective. *Adv. Ther.* **2016**, *33*, 1896–1920. [CrossRef] [PubMed]
114. San-Miguel, J.F.; Hungria, V.T.M.; Yoon, S.-S.; Beksac, M.; Dimopoulos, M.A.; Elghandour, A.; Jedrzejczak, W.W.; Günther, A.; Na Nakorn, T.; Siritanaratkul, N.; et al. Panobinostat plus bortezomib and dexamethasone versus placebo plus bortezomib and dexamethasone in patients with relapsed or relapsed and refractory multiple myeloma: A multicentre, randomised, double-blind phase 3 trial. *Lancet Oncol.* **2014**, *15*, 1195–1206. [CrossRef]
115. Richardson, P.G.; Hungria, V.T.M.; Yoon, S.-S.; Beksac, M.; Dimopoulos, M.A.; Elghandour, A.; Jedrzejczak, W.W.; Guenther, A.; Nakorn, T.N.; Siritanaratkul, N.; et al. Panobinostat plus bortezomib and dexamethasone in previously treated multiple myeloma: Outcomes by prior treatment. *Blood* **2016**, *127*, 713–721. [CrossRef]
116. Rabal, O.; San José-Enériz, E.; Agirre, X.; Sánchez-Arias, J.A.; De Miguel, I.; Ordoñez, R.; Garate, L.; Miranda, E.; Sáez, E.; Vilas-Zornoza, A.; et al. Design and Synthesis of Novel Epigenetic Inhibitors Targeting Histone Deacetylases, DNA Methyltransferase 1, and Lysine Methyltransferase G9a with In Vivo Efficacy in Multiple Myeloma. *J. Med. Chem.* **2021**, *64*, 3392–3426. [CrossRef]

International Journal of
Molecular Sciences

MDPI

Article

5-Aza-2′-Deoxycytidine Alters the Methylation Profile of Bortezomib-Resistant U266 Multiple Myeloma Cells and Affects Their Proliferative Potential

Karolina Łuczkowska [1,†], Piotr Kulig [1,†], Klaudia Rusińska [1], Bartłomiej Baumert [2,*] and Bogusław Machaliński [1,2,*]

[1] Department of General Pathology, Pomeranian Medical University, 70-111 Szczecin, Poland; karolina.luczkowska@pum.edu.pl (K.Ł.); piotrkulig@interia.eu (P.K.); rusinskaklaudia@gmail.com (K.R.)
[2] Department of Hematology and Transplantology, Pomeranian Medical University, 71-252 Szczecin, Poland
* Correspondence: bartlomiej.baumert@pum.edu.pl (B.B.); boguslaw.machalinski@pum.edu.pl (B.M.)
† These authors contributed equally to this work.

Abstract: Multiple myeloma (MM) is a plasma cell malignancy that accounts for 1% of all cancers and is the second-most-common hematological neoplasm. Bortezomib (BTZ) is a proteasome inhibitor widely implemented in the treatment of MM alone or in combination with other agents. The development of resistance to chemotherapy is one of the greatest challenges of modern oncology. Therefore, it is crucial to discover and implement new adjuvant therapies that can bypass therapeutic resistance. In this paper, we investigated the in vitro effect of methylation inhibitor 5-Aza-2′-deoxycytidine on the proliferative potential of MM cells and the development of resistance to BTZ. We demonstrate that alterations in the DNA methylation profile are associated with BTZ resistance. Moreover, the addition of methylation inhibitor 5-Aza-2′-deoxycytidine to BTZ-resistant MM cells led to a reduction in the proliferation of the BTZ-resistant phenotype, resulting in the restoration of sensitivity to BTZ. However, further in vitro and ex vivo studies are required before adjuvant therapy can be incorporated into existing treatment regimens.

Keywords: multiple myeloma; bortezomib; resistance; methylation inhibitor; 5-Aza-2′-deoxycytidine

Citation: Łuczkowska, K.; Kulig, P.; Rusińska, K.; Baumert, B.; Machaliński, B. 5-Aza-2′-Deoxycytidine Alters the Methylation Profile of Bortezomib-Resistant U266 Multiple Myeloma Cells and Affects Their Proliferative Potential. *Int. J. Mol. Sci.* **2023**, *24*, 16780. https://doi.org/10.3390/ijms242316780

Academic Editor: Bernhard Biersack

Received: 18 October 2023
Revised: 15 November 2023
Accepted: 24 November 2023
Published: 26 November 2023

1. Introduction

Multiple myeloma (MM) is the second-most-common hematological malignancy, with a complex and multifaceted pathogenesis. MM affects a significant number of people worldwide, with an age-standardized rate of 1.78 per 100,000 individuals in 2020 [1]. It particularly affects patients over 65 years of age but may also occur in younger individuals [2]. The severity of this medical condition is significant, but progress in pharmacotherapy and a profound comprehension of molecular mechanisms have improved the overall prognosis and survival. B lymphocytes transformed into antibody-producing plasma cells are involved in the pathogenesis of MM. Pathologically altered plasma cells have a proclivity for monoclonal, irreversible, and uncontrolled proliferation. This leads to the destruction and impaired functioning of bone marrow and even end-stage organ damage [3]. A complex array of genetic and epigenetic changes, chromosomal aberrations, and angiogenesis disorders plays a crucial role in the pathogenesis of MM. Cytogenetic abnormalities such as del(17p), t(4;14), t(14;16), t(14;20), 1q gain, and p53 are associated with poor prognosis and high-risk MM [4]. Radiation and exposure to certain chemical substances, i.e., asbestos or benzene, are considered risk factors for MM [5]. MM develops from a premalignant condition known as monoclonal gammopathy of undetermined significance (MGUS) or smoldering myeloma (SMM). MGUS is mostly asymptomatic, discovered accidentally, and manifests itself especially in laboratory tests. MGUS progresses to MM with a risk of 1% per year. SMM is a transitional stage between MGUS and MM, with a 10% risk of

progression per year [6–9]. MM patients mainly present with fatigue and bone pain. About 75% of patients suffer from anemia, which contributes to fatigue, and about 80% exhibit osteolytic changes in the skeleton. Although pathognomonic, hypercalcemia (15%) and renal failure (20%) are slightly less common [10]. The treatment of MM continues to be a challenge for hematologists. Despite significant progress in the pharmacotherapy of MM, which has resulted in prolonged survival, the vast majority of patients experience a relapse of the disease and cannot be cured. The most acclaimed drugs used in the treatment of MM patients are alkylating agents (melphalan and cyclophosphamide), corticosteroids, immunomodulatory drugs (IMiDs (thalidomide, lenalidomide, pomalidomide)), and proteasome inhibitors (bortezomib, carfilzomib, and ixazomib) [11]. Bortezomib (BTZ) is a first-generation proteasome inhibitor and a highly effective agent against MM [12]. BTZ inhibits the ubiquitin–proteasome catalytic pathway in cells by directly binding to the 20S proteasome complex [13]. Such interference leads to the accumulation of misfolded and otherwise defective proteins and induces MM cell death [14,15]. Despite the satisfactory effects of BTZ therapy, many patients acquire resistance. The development of resistance is often associated with disease relapse. Several mechanisms contribute to therapy failure, such as abnormal drug transport, activation of detoxification systems, changes in drug targets, cell cycle or apoptosis dominance, and the distortion of signaling pathways [16]. The abovementioned factors lead to increased BTZ excretion, interfere with the formation of the BTZ–proteasome complex and impair the therapeutic response. The development of resistance to BTZ is the basis for the search for new therapeutic targets or mechanisms to suppress resistance.

Epigenetics deals with mechanisms regulating gene expression that do not result from changes in the DNA sequence. It includes DNA methylation, histone modifications, chromatin-regulating proteins in cells, and non-coding RNA [16–18]. There are several studies reporting methylation changes associated with chemotherapy resistance in solid tumors. For example, the hypermethylated TGBI promoter in breast cancer is associated with trastuzumab resistance in HER2+ patients [19]. Alterations in methylome were shown to mediate resistance to IMiDs in MM. Cereblon (CRBN) enhancer methylation inhibits CRBN expression, which confers resistance to IMiDs. Moreover, in in vitro studies investigating DNA methyltransferase inhibitors (DNTMis), sensitization to lenalidomide treatment was demonstrated in two MM cell lines [20]. DNMT inhibitors are becoming increasingly attractive therapeutic agents. Azacytidine (AZA) and decitabine are the most successful epigenetic drugs used in in vitro studies of MM therapy [21].

We also investigated the role of epigenetic alterations in the development of BTZ resistance. In one of our previous studies, we established a BTZ-resistant neuroblastoma cell line. Subsequently, we analyzed the methylome of both resistant and sensitive cells and demonstrated that changes in the methylation profile contribute to the development of resistance to this compound [22]. Similarly, in another study, we focused on MM cell lines. First, we established the BTZ-resistant U266 MM cell line (cells were cocultured with BTZ; three repeated treatments were required to obtain the BTZ-resistant phenotype); then, implementing next-generation sequencing, we investigated the mechanisms underlying the development of BTZ resistance. In parallel, we demonstrated the contribution of oxidative phosphorylation and the role of SNORD-family genes that mediate epigenetic changes. Moreover, we showed that vitamins D and K act synergically with BTZ [23]. Therefore, we hypothesized that, first, changes in methylome mediate resistance to BTZ and, second, epigenetic agents such as AZA may act synergistically with BTZ and even restore sensitivity to this compound.

The aim of this study was to investigate alterations in methylome affecting the proliferative potential of MM cells associated with the development of resistance to BTZ. In our research, we focused on methylation, which is crucial for the gene regulation process, including both activation and suppression [24]. It is worth emphasizing that methylation is a reversible process and serves as a starting point in therapy. The hypermethylation of

gene promoters may result in the suppression of individual genes, potentially causing the development of multidrug resistance (MDR) [25].

It has been shown several times that resistance to BTZ results from epigenetic alterations, among other causes [23,26]. Therefore, we investigated the impact of changes in the DNA methylation profile on the development of this phenotype. Furthermore, we examined the effect of a methylation inhibitor on the proliferative potential of BTZ-resistant MM cells.

2. Results

2.1. Proliferation Assay

Analysis of the proliferation results showed a fully BTZ-resistant phenotype after the third treatment of U266 cells. BTZ-resistant cells showed a similar proliferation rate to that of control cells; therefore, we can consider them resistant to BTZ (control = 506.3, SD ± 28.4; BTZ = 486.03, SD ± 52.8) (Figure 1). U266 cells were treated simultaneously with BTZ and the methylation inhibitor to examine whether its use would inhibit the development of a resistant phenotype. The obtained results clearly show a decrease in proliferation with increasing dose of the methylation inhibitor. After the first treatment, when the phenotype of U266 cells was not yet resistant to BTZ, the differences in the degree of proliferation reached several percent when comparing different doses of 5-Aza-2′-deoxycytidine to each other. However, the lowest level of proliferation was already observed at the highest dose of 5-Aza-2′-deoxycytidine. After the second treatment, a decreasing level of proliferation was clearly observed after the use of 5-Aza-2′-deoxycytidine in relation to both control cells and cells treated with BTZ alone. Additionally, a dose of 5-Aza-2′-deoxycytidine 1000 nM most effectively slowed down cell proliferation. Proliferation on day 10 was 62.2% lower compared to proliferation at the remaining doses.

Three treatments of myeloma cells with 5-Aza-2′-deoxycytidine at a dose of 1000 nM reduced the proliferation of BTZ-resistant cells by approximately 72% ($p < 0.0004$) (Figure 1). Based on the obtained results, a dose of 1000 nM 5-Aza-2′-deoxycytidine was selected for further procedures.

2.2. Effect of a Methylation Inhibitor on the Development of BTZ Resistance in U266 Myeloma Cells

Methylation analysis provided data immediately after the second (BTZ_m_i_2 and BTZ_2) and third (BTZ_m_i_3 and BTZ_3) treatments of U266 cells. In addition, we performed an analysis 10 days after the third treatment (BTZ_m_i_10d_3) (nothing was added to the medium at that time) to demonstrate whether the resulting changes in methylation levels were permanent and transmitted to daughter cells despite treatment discontinuation. No significant changes in the methylation profile were observed after the first treatment. A detailed analysis of the methylation profile is presented below.

2.3. Results of DNA Methylation Profile Analysis Obtained after Two Treatments

Bioinformatics analysis showed 301 sites (299 hypomethylated and 2 hypermethylated) with altered methylation in myeloma cells treated twice with BTZ and a methylation inhibitor compared to cells treated twice with BTZ alone (Figure 2A). The beta delta values shown in the graphs were obtained by calculating the ratio of the normalized fluorescence intensity values of the probe between the methylated and unmethylated signals (0 = fully unmethylated; 1 = fully methylated). The distribution of methylation changes is shown on each chromosome separately in Figure 2A. Hypermethylation was only observed on chromosomes 4 and 11.

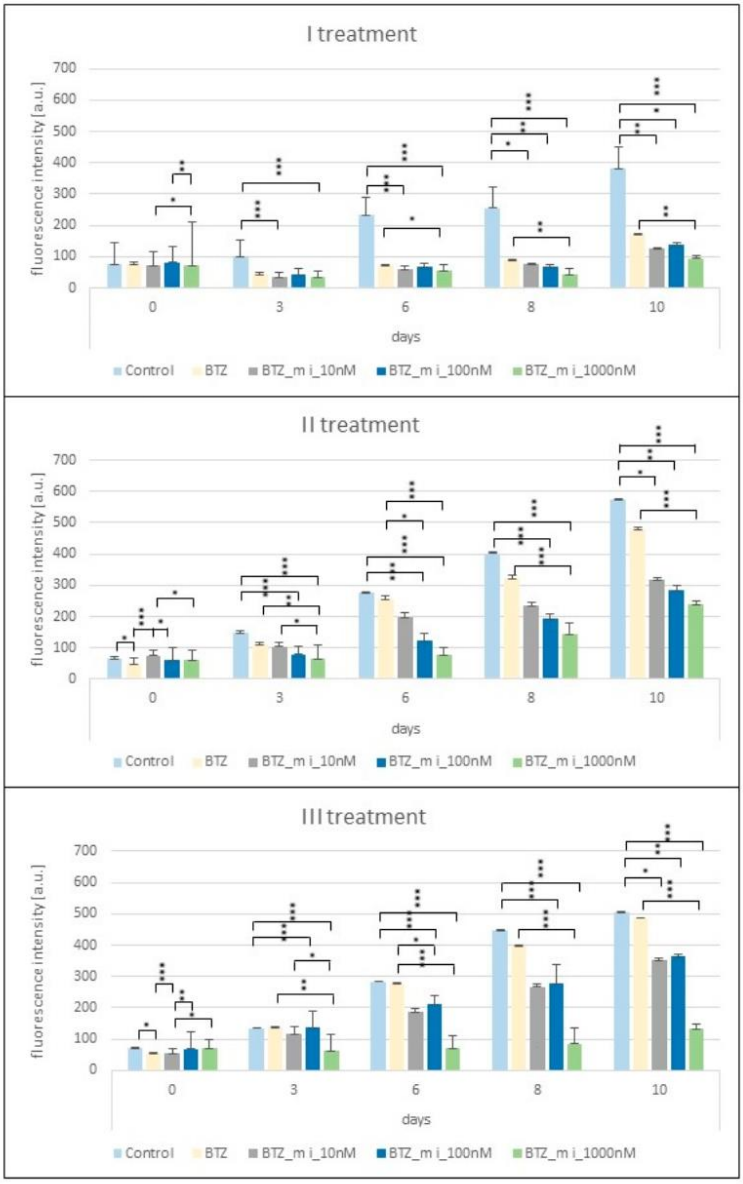


Figure 1. Graphs showing the level of proliferation of U266 cells after each treatment with BTZ and/or a methylation inhibitor (BTZ_m i). Data were not normally distributed; therefore, differences between the groups were analyzed with a Kruskal–Wallis test followed by a post-hoc Dunn test with Bonferroni correction for multiple testing. $p < 0.05$ was considered statistically significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

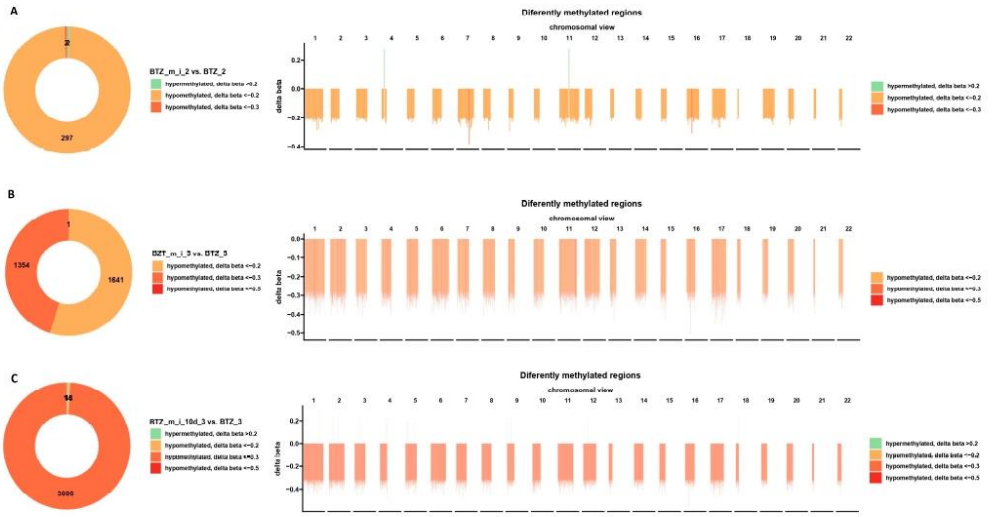


Figure 2. Graphs showing the DNA methylation profile of U266 myeloma cells after two (**A**) and three (**B**) simultaneous treatments with BTZ and a methylation inhibitor (BTZ_m_i) compared to cells treated with BTZ alone (BTZ). In addition, (**C**) shows the DNA methylation profile 10 days after the last treatment. Circular charts show differences in methylation levels in BTZ- and methylation-inhibitor-treated U266 cells relative to cells treated with BTZ ($p < 0.05$). In each section, the distribution of DNA methylation changes on individual chromosomes is presented (orange indicates hypomethylation, and green hypermethylation; $p < 0.05$). BTZ—bortezomib; BTZ_2—bortezomib, second treatment; BTZ_m_i—bortezomib and methylation inhibitor; BTZ_m_i_2—bortezomib and methylation inhibitor, second treatment; BTZ_3—bortezomib, third treatment; BTZ_m_i_3—bortezomib and methylation inhibitor, third treatment; BTZ_m_i_10d_3—bortezomib and methylation inhibitor 10 days after third treatment.

Moreover, bioinformatics analysis showed 2996 sites with altered methylation (only hypomethylated) in myeloma cells treated three times with BTZ and a methylation inhibitor compared to cells treated three times with BTZ alone (Figure 2B). The number of changes in the methylation level increased by almost 10-fold after the third treatment compared to the results obtained after the second treatment (second treatment: 301 changed sites vs. third treatment: 2996 changed sites).

Changes in methylation after double treatment of U266 cells with BTZ (BTZ_2) or BTZ and a methylation inhibitor (BTZ_m_i_2) in selected genes are shown in the heat map (Figure 3). The most interesting changes in methylation from the point of view of resistance development were observed in the following genes: FBXW7, ORAI3, and TBC1D16. It is also worth mentioning genes that regulate proliferation processes (ZDHHC5) and epigenetic modifications (KDM2B), as well as those that act as a transcription factor (COMMD3). The observed changes in these genes may significantly influence the development of resistance to BTZ.

Gene set enrichment analysis (GSEA) performed after double treatment of U266 cells with BTZ and a methylation inhibitor compared to cells treated twice with BTZ alone showed process changes correlated only with hypomethylation (Figure 4). GSEA allowed for the isolation of 20 statistically significant processes whose genes showed reduced methylation levels in cells treated with BTZ and a methylation inhibitor compared to cells treated with BTZ alone. The most interesting, statistically significant processes ($p < 0.05$) seem to be RNA splicing and epigenetic alterations, such as histone deacetylation, protein deacetylation, regulation of histone modification, and histone deacetylation, which are

important in considering the impact of epigenetic modifications on the development of BTZ resistance.

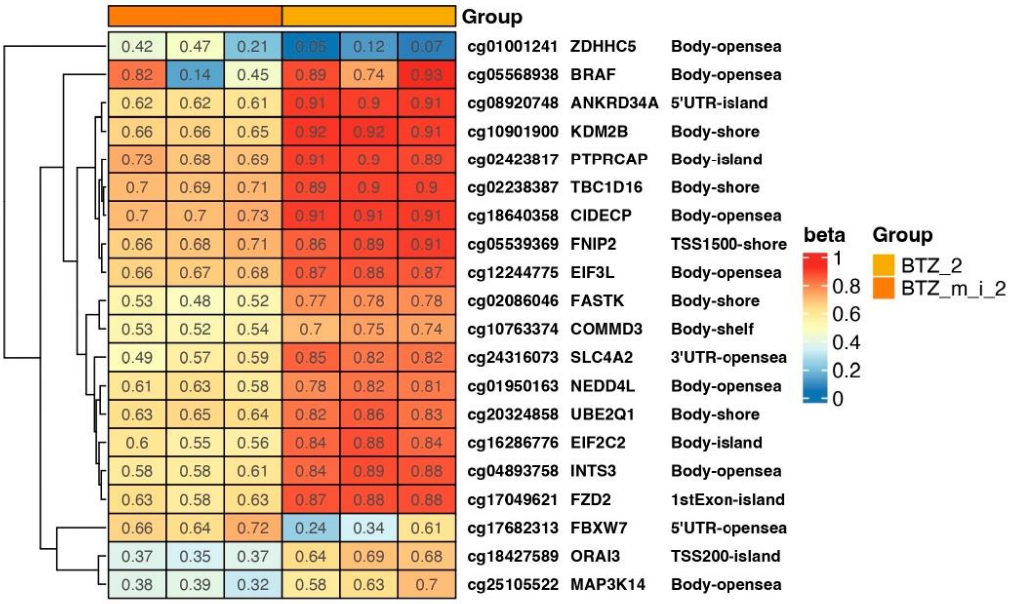


Figure 3. Heat map showing beta values representing the methylation level of selected genes in cells treated twice with BTZ (BTZ_2) and with BTZ and a methylation inhibitor (BTZ_m_i_2). Beta values of "1" indicate full methylation (red), and "0" indicates no methylation (blue) ($p < 0.05$). Gene symbols and methylation sites are marked on the heat map. The heatmap results were visualized using R library ComplexHeatmap. Island—CpG island; 5′UTR—5′ untranslated region; shore—the 2 kb sequences directly up- and downstream of CpG islands; shelf—the 2 kb sequences directly adjacent to the shore; opensea—outside of the shelf region.

2.4. Results of DNA Methylation Profile Analysis Obtained after Three Treatments

Bioinformatics analysis showed 2996 sites with altered methylation (only hypomethylated) in myeloma cells treated three times with BTZ and a methylation inhibitor compared to cells treated three times with BTZ alone (Figure 2B). The number of changes in the methylation level increased by almost 10-fold after the third treatment compared to the results obtained after the second treatment (second treatment: 301 changed sites vs. third treatment: 2996 changed sites).

Analysis of changes in methylation levels in individual genes after three treatments with BTZ and a methylation inhibitor relative to cells treated with BTZ alone showed hypomethylation of the following genes: MIR21, PRC1, AKAP13, and ORAI3 (Figure 5), which are directly related to the development of drug resistance.

GSEA performed after the third treatment of U266 cells with BTZ and a methylation inhibitor compared to cells treated three times with BTZ alone showed process changes correlated only with hypomethylation ($p < 0.05$) (Figure 6). The process of RNA splicing plays a very important role in the development of drug resistance. The formation of abnormal splice variants or splicing machinery disorders may cause the development of drug resistance and promote the development of cancer [27]. This process deepens with subsequent treatments (second-treatment NES = -1.78 $p = 3.4 \times 10^{-15}$; third-treatment NES = -2.37; $p = 8.2 \times 10^{-23}$) (Figures 4 and 6). Other processes that drew our attention

during the GSEA analysis concern DNA damage and repair processes. These processes participate in the development of resistance through the DNA modifications, which allow cancer cells to survive in environments with high levels of genotoxic stress provided by the therapy [28,29]. These biological processes were also identified 10 days after the last treatment, confirming both the importance of these changes in the process of BTZ resistance development and their persistence and transmission to daughter cells.

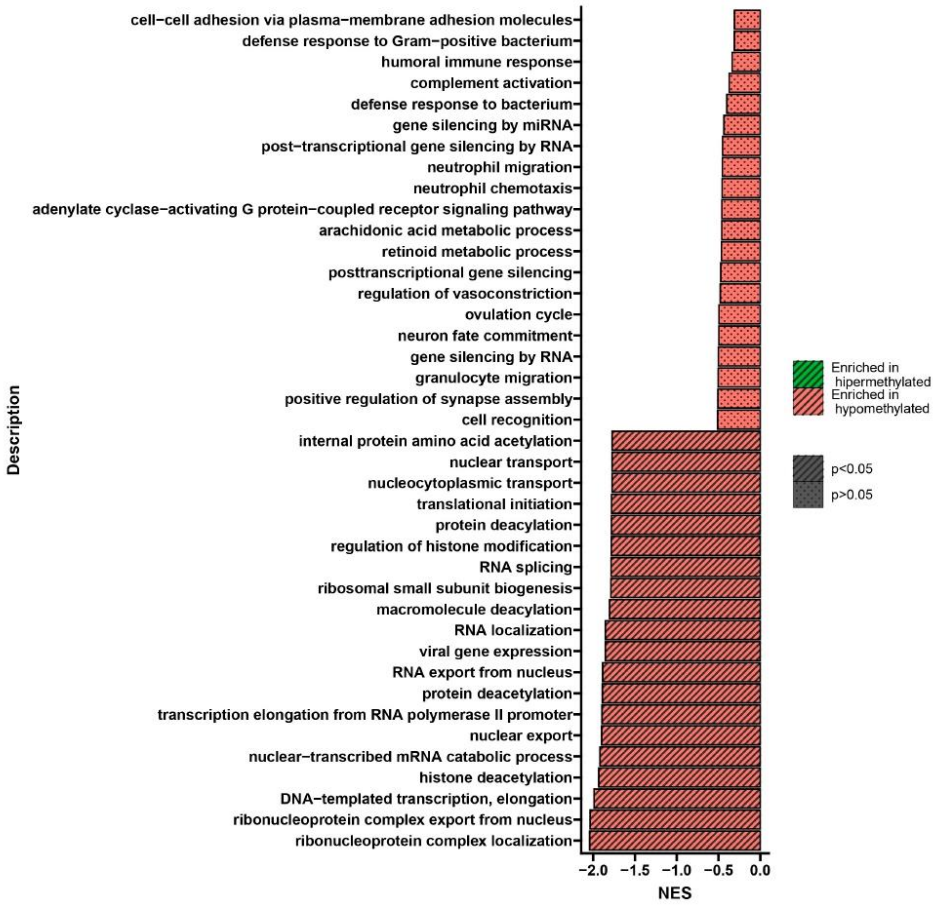


Figure 4. GSEA shows NES (normalized enrichment score) values indicating changes in DNA methylation in genes involved in the regulation of the listed biological processes in U266 cells treated twice with BTZ and a methylation inhibitor compared to cells treated twice with BTZ alone. Red indicates hypomethylation, and green indicates hypermethylation.

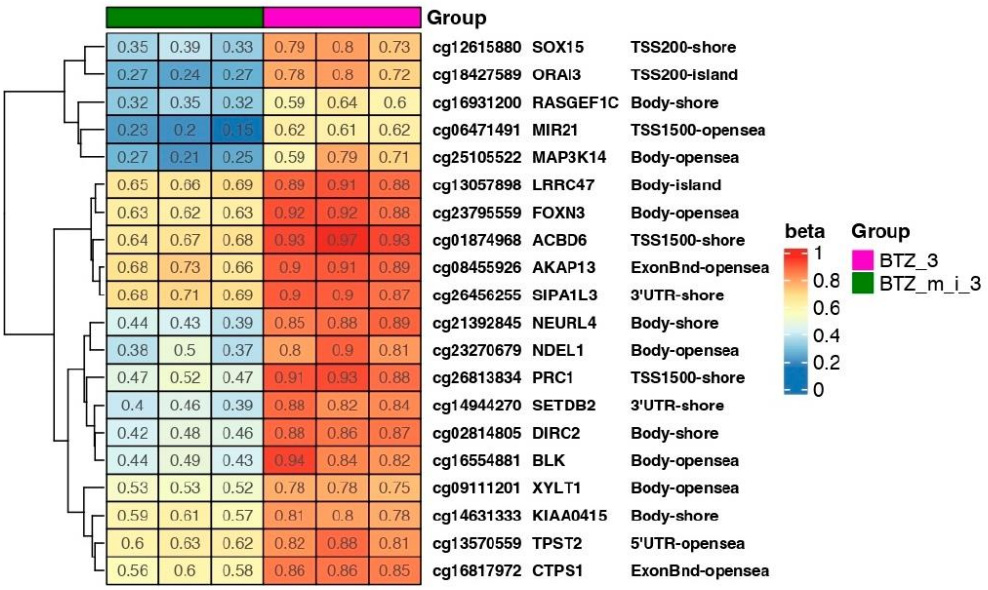


Figure 5. Heat map showing beta values representing the methylation levels of selected genes in cells treated three times with BTZ (BTZ_3) or BTZ and a methylation inhibitor (BTZ_m_i_3). Beta values of "1" indicate full methylation (red), and "0" indicates no methylation (blue) ($p < 0.05$). Gene symbols and methylation sites are marked on the heat map. The heatmap results were visualized using R library ComplexHeatmap). Island—CpG island; 5′UTR—5′ untranslated region; shore—the 2 kb sequences directly up- and downstream of CpG islands; shelf—the 2 kb sequences directly adjacent to the shore; opensea—outside of the shelf region.

2.5. Results of DNA Methylation Profile Analysis Obtained 10 Days after the Third Treatment

Three 24 h treatments of cells with BTZ and a methylation inhibitor induced permanent changes in a large number of genes, especially in the case of genes responsible for the development of resistance, such as FBXW7, ORAI3, MIR21, and PRC1 (Figures 7 and 8). Analysis of the data presented in Figure 7, which shows the beta values of the selected genes immediately after the third treatment and 10 days later, showed comparable levels of methylation at both time points. In addition, a bioinformatics analysis was performed comparing the results of the DNA methylation level immediately after three treatments (BTZ_m_i) vs. 10 days later (BTZ_m_i_10days). The analysis did not show significant changes in DNA methylation. This confirms that the induced changes in DNA methylation are permanent and transmitted to daughter cells despite the withdrawal of factors. There was no enrichment in methylation according to GSEA analysis (Figure 9).

Bioinformatics analysis conducted 10 days after the third treatment of cells with BTZ and a methylation inhibitor compared to cells treated with BTZ alone revealed 3023 (3009 hypomethylated and 14 hypermethylated) altered methylation sites (Figure 2C). These results are slightly different from those obtained immediately after the third treatment. Areas of hypermethylation were observed on chromosomes 1–5, 9, 12, and 18 (Figure 2C).

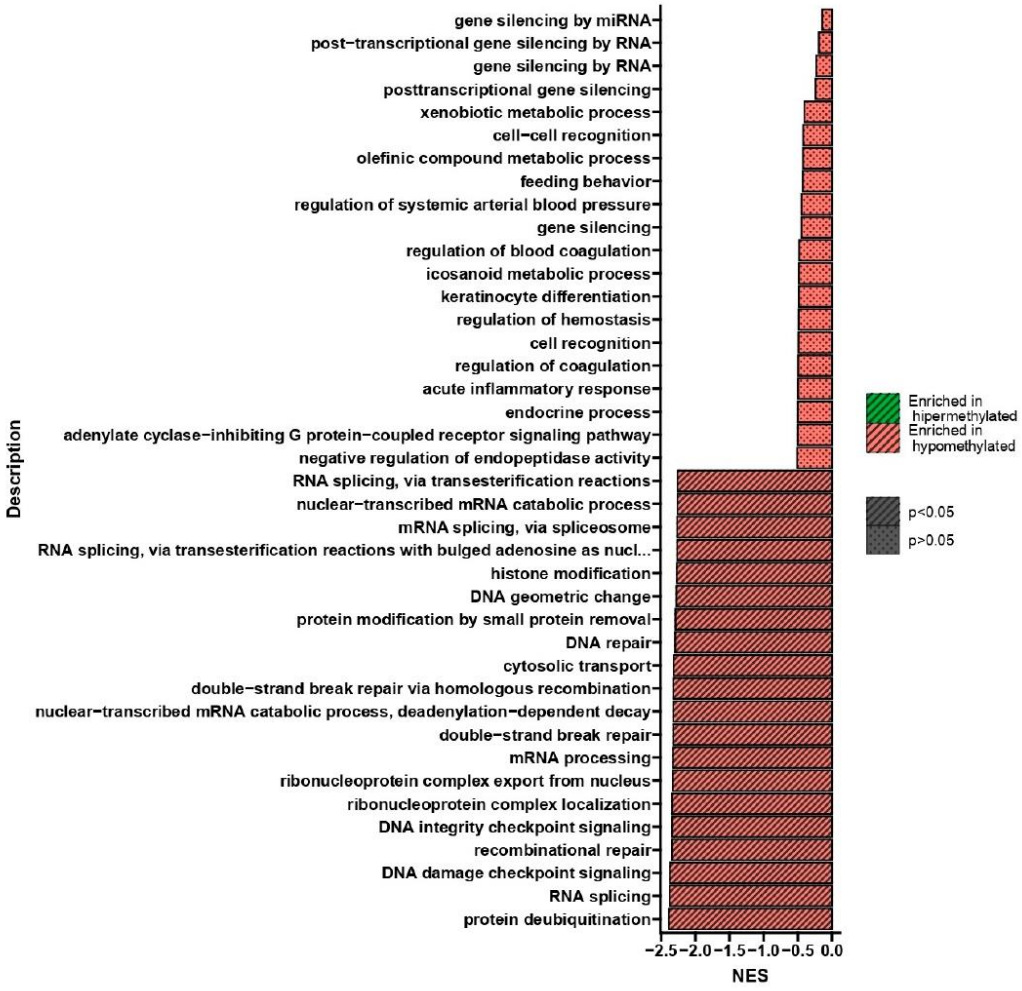


Figure 6. GSEA shows NES values indicating changes in DNA methylation in genes involved in the regulation of the listed biological processes in U266 cells treated three times with BTZ and a methylation inhibitor compared to cells treated three times with BTZ alone. Red indicates hypomethylation, and green indicates hypermethylation.

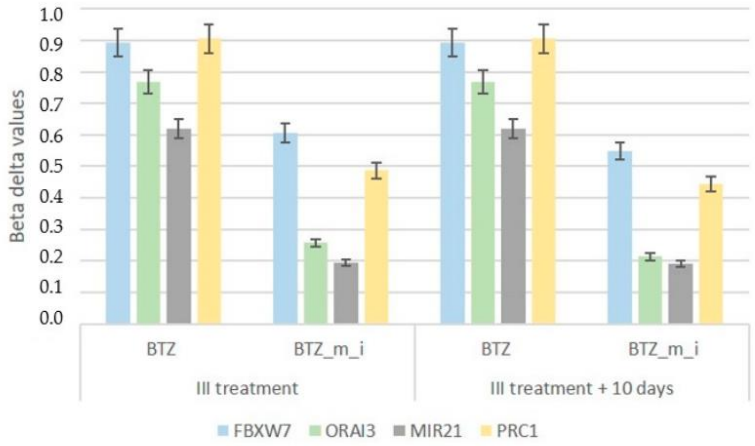


Figure 7. Beta delta values (mean $n = 3$; $p < 0.05$ for all results) for selected genes relevant to the development of BTZ resistance measured immediately and 10 days after the third treatment. BTZ—bortezomib; BTZ_m_i—bortezomib combined with a methylation inhibitor.

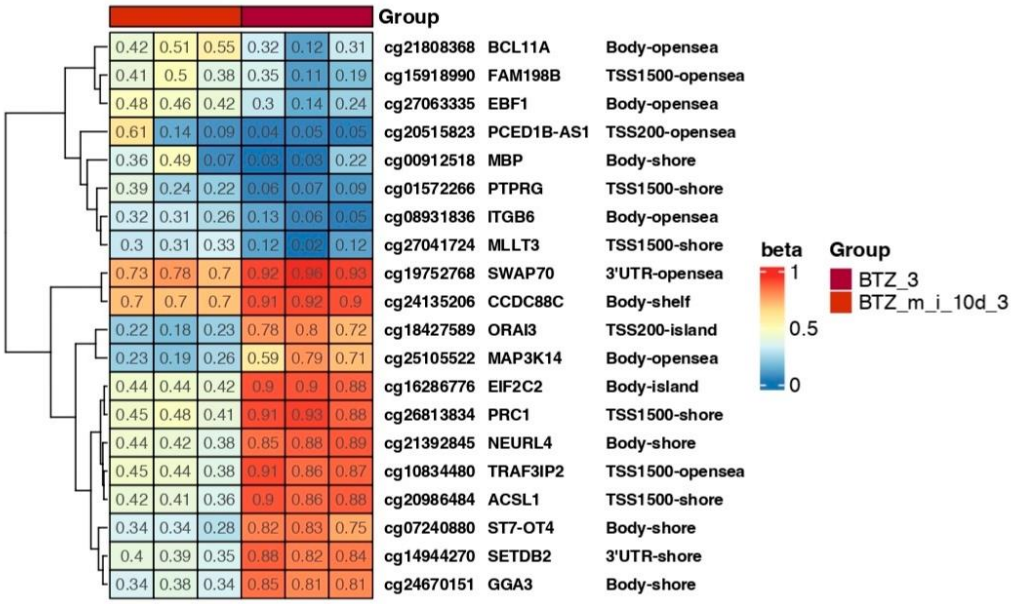


Figure 8. Heat map showing beta values representing the methylation level of selected genes in cells treated three times with BTZ (BTZ_3) or BTZ and a methylation inhibitor 10 days after the third treatment (BTZ_m_i_3). Beta values of "1" indicate full methylation (red), and "0" indicates no methylation (blue) ($p < 0.05$). Gene symbols and methylation sites are marked on the heat map. The heatmap results were visualized using R library ComplexHeatmap. Island—CpG island; 5′UTR—5′ untranslated region; shore—the 2 kb sequences directly up- and downstream of CpG islands; shelf—the 2 kb sequences directly adjacent to the shore; opensea—outside of the shelf region.

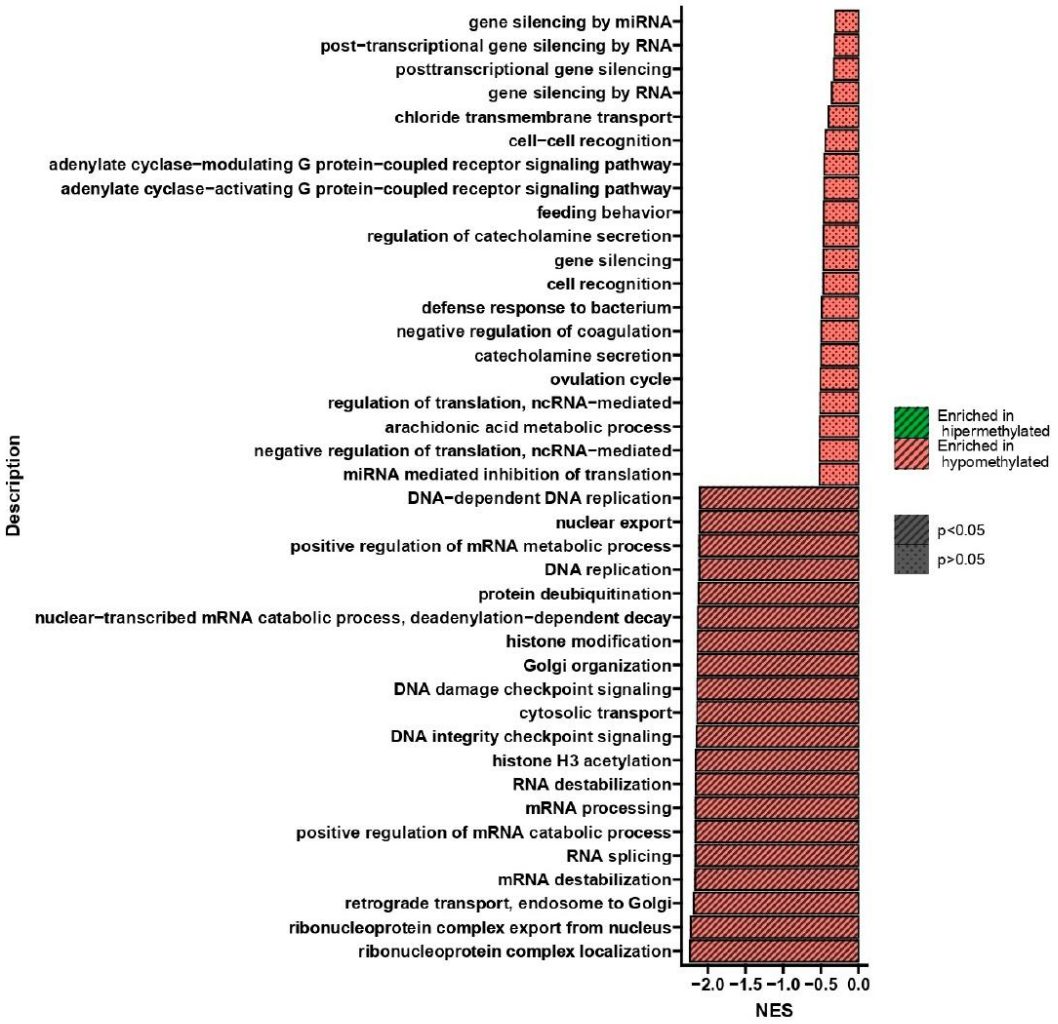


Figure 9. GSEA shows NES values indicating changes in DNA methylation in genes involved in the regulation of the listed biological processes in U266 cells 10 days after the third treatment of cells with both BTZ and a methylation inhibitor compared to cells treated three times with BTZ alone. Red indicates hypomethylation, and green indicates hypermethylation.

3. Discussion

Although MM remains an incurable disease, the clinical outcomes of MM patients have improved significantly over time with the development and implementation of various chemotherapy regimens. BTZ is a proteasome inhibitor that is an essential component of various anti-MM treatment regimens. Initially, it was successfully applied in a monotherapy [29,30]. Currently, BTZ is administered in combination with other molecules, e.g., monoclonal antibodies [31,32] or immunomodulatory drugs such as thalidomide [33], lenalidomide [34], and pomalidomide [35]. Moreover, there are treatment regimens consisting of more than three drugs, and often, BTZ is one of the basic ingredients, as in the D-VTd

protocol (daratumumab, bortezomib, thalidomide, and dexamethasone) [36]. As mentioned above, BTZ is widely used to treat MM. It can be hypothesized that almost every MM patient will be exposed to BTZ during treatment. Exposure to BTZ exerts environmental pressure on malignant plasma cells, which, in turn, may ultimately result in the selection BTZ-resistant clones. Although the exact basis of BTZ resistance is multifactorial and has not been fully elucidated, there are several mechanisms mediating this treatment-hindering phenomenon. BTZ is a proteasome inhibitor that selectively and reversibly blocks its chymotryptic site [30,37]. Therefore, upon exposure to BTZ, drug-induced environmental pressure causes MM cells to develop proteasome alterations that may impede the anti-MM activity of BTZ. Indeed, such a hypothesis was demonstrated to drive BTZ resistance in different models [38,39]. Another mechanism contributing to BTZ resistance is related to the bone marrow microenvironment and the interplay between proinflammatory macrophages and MM cells [40].

There is a shortage of studies investigating the role of epigenetic alterations in the pathophysiology of acquired BTZ resistance. Therefore, we previously investigated the molecular background of this phenomenon in various models. For instance, we demonstrated that changes in the methylome of neuroblastoma cells contribute to resistance to this compound. We performed repeatedly treated SH-SY5Y neuroblastoma cells with BTZ until resistance was acquired. Our results showed that BTZ induces methylation changes affecting the proliferative potential of neuroblastoma cells [22]. Using a similar methodology, we also investigated the mechanisms underlying BTZ resistance in MM. We established the BTZ-resistant U266 MM cell line by repeated coincubation with BTZ. Among others, we showed that SNORD-family genes were upregulated compared to control cells, suggesting the involvement of epigenetic mechanisms [23]. In another study, treatment of BTZ-resistant mantle cell lymphoma cells with BTZ and DNA methyltransferase inhibitor decitabine (DAC) resulted in the death of around 80% of cells. It should be noted that both BTZ and DAC monotherapy killed approximately 10–25% of BTZ-resistant mantle cell lymphoma cells [41]. Other studies have highlighted the role of long non-coding RNA in conferring BTZ resistance in MM [42,43].

Because SNORDs are involved in mediating epigenetic changes [44], we hypothesized that epigenetic alterations play a role in the development of BTZ resistance and, secondly, that epigenetic drugs such as 5-Aza-2′-deoxycytidine may potentially be beneficial in BTZ-resistant MM.

In the present study, based on our previous findings [22,23], we hypothesized that alterations in the methylation profile may mediate BTZ resistance and that epigenetic agents such as 5-Aza-2′-deoxycytidine may act synergistically with BTZ, similarly to VD and VK. We deepened our analysis and investigated the effect of a methylation inhibitor on the proliferative potential of MM cells and the development of BTZ resistance. In this paper, we demonstrated that alterations in the methylation profile are associated with BTZ resistance. First, our results revealed that the addition of methylation inhibitor 5-Aza-2′-deoxycytidine to BTZ-resistant MM cells led to a reduction in the proliferation of the BTZ-resistant phenotype, resulting in the restoration of sensitivity to BTZ. Moreover, a comparison of the global DNA methylation profile between the second and third treatments showed a continuous decrease in methylation. What should be emphasized is that no hypermethylation sites were found after the third treatment (BTZ_m i vs. BTZ_3). It can therefore be concluded that a decrease in methylation results in the restoration of chemosensitivity. Liu et al. demonstrated that BTZ induced global hypomethylation in acute myeloid leukemia cells both in vitro and in vivo [45]. Hence, it can be hypothesized that a good response to treatment is primarily associated with loss of methylation. Increased methylation may contribute to the development of a BTZ-resistant phenotype. The addition of a methylation inhibitor may act synergistically or, when added to resistant MM cells, restore sensitivity to BTZ. Heatmap analysis of selected genes after the second and third treatments revealed significant differences in methylation levels between BTZ_2 and BTZ_m_2 and between BTZ_3 (resistant cells) and BTZ_m_3. More precisely, the develop-

ment of BTZ resistance is associated with an increase in methylation, while drug sensitivity is associated with hypomethylation. Similar phenomena have been observed by other researchers. For instance, in an in vitro study, Hu and colleagues demonstrated that CD9 downregulation by methylation decreased BTZ sensitivity in U266 MM cells. Moreover, they demonstrated that a methylation inhibitor, namely 5-Aza-2-deoxycytidine, upregulated CD9 and raised sensitivity to BTZ [46]. Due to the complex nature of interactions in the human body, the results obtained in in vitro studies provide only first insights and may not be entirely conclusive in terms of translation to clinical conditions. De Larrea and coworkers investigated the relationship between overall survival (OS), progression-free survival (PFS), and both global and gene-specific methylation profiles in MM patients treated with BTZ-based chemotherapy regimens. A proportion of 62% of the analyzed patients responded to treatment (complete remission, 6.7%; partial response, 44%; minimal response, 10.7%). Globally, they observed low methylation status across the entire cohort. Nevertheless, patients with more than 3.95% of total DNA methylated achieved better OS than patients with more unmethylated DNA (median of 30 versus 15 months; $p = 0.004$). On the other hand, they demonstrated that hypomethylation of the *NFKB1* and *CXCR4* genes was associated with a better response to treatment [47].

In addition to the trend that global DNA hypomethylation confers BTZ sensitivity, our heat maps we demonstrate the methylation status of selected genes, as well as various processes, according to GSEA analysis. We identified several genes that differed in methylation status between MM cells treated with BTZ alone and those incubated additionally with a methylation inhibitor. For instance, after second passage, we identified differences in methylation levels in the island area of the *ORAI3* gene, the overexpression of which has been linked to chemotherapy resistance in breast cancer [48]. Furthermore, the 5′UTR-opensea region of the *FBXW7* gene was markedly less methylated in BTZ-treated MM cells. This gene and, more specifically, its downregulation have also been shown to play a role in mediating drug resistance and chemotherapy response in cancers [49–52]. After the third passage, we once again identified hypomethylation of the *ORAI3* gene in MM cells incubated with BTZ and a methylation inhibitor (BTZ-sensitive cells) and hypermethylation in BTZ-resistant MM cells. Moreover, we identified differences in the methylation status of the *MIR21* gene, which is linked to chemotherapy resistance in various cancers, such as ovarian cancer [53] and renal carcinoma [54]. In addition, the *PRC1* gene was demonstrated to be associated with drug resistance and poor clinical outcomes in various malignancies [55–57].

GSEA analysis showed statistically significant changes in several key processes involved in the development of resistance. The most important seems to be RNA splicing. The influence of RNA splicing on the development of drug resistance has been demonstrated in many oncological diseases. In some cases of chronic myeloid leukemia (CML), expression of high levels of alternatively spliced BCR-ABL mRNA with a 35 bp insertion (35INS) between exons 8 and 9 of the ABL kinase domain has been observed. This insertion shifts the frame, leading to the addition of 10 residues and the truncation of 653 residues due to early termination. These changes provide resistance to imatinib, which depends on the level of expression [58]. In breast and ovarian cancer cells, a mutation was induced in exon 11 of BRCA1, expressing the BRCA1-Δ11q splice variant lacking most of exon 11. The introduction of the mutation resulted in a frameshift to exon 11. The nascent BRCA1-Δ11q protein was able to promote partial resistance to the PARP inhibitor (PARPi) and cisplatin compared to full-length BRCA1 both in vitro and in vivo [59].

We identified differences in the methylation status of various genes that have been demonstrated to play an important role in mediating chemotherapy resistance. However, it should be noted that the vast majority of them were hyper- or hypomethylated in regions other than the promoter. Therefore, one may presume that their methylation status does not necessarily accurately reflect expression. To draw more firm conclusions, expression should be assessed at the RNA and protein levels.

4. Materials and Methods

4.1. Cell Culture and Course of the Experiment

The U266 human multiple myeloma cell line (ATCC, Manassas, VA, USA) was used in this experiment. U266 cells were cultured using RPMI-1640 medium (ATCC, Manassas, VA, USA, cat no. 30-2001), which contained 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate and was supplemented with 15% fetal bovine serum. The medium was changed every three days.

U266 cells were treated with BTZ at 2.75 nM (Cell Signaling Technology, Danvers, MA, USA) and methylation inhibitor 5-Aza-2′-deoxycytidine at 1 µM (Sigma Aldrich, St. Louis, MO, USA) three times for 24 h at 10-day intervals. The experiment was carried out in three technical repetitions. The dose of BTZ and a protocol for obtaining a resistant cell line were established in our previous article [23] and caused the death of over 50% of cells after the first treatment. The third treatment resulted in a fully BTZ-resistant cell phenotype. For this experiment, the concentration of 5-Aza-2′-deoxycytidine was determined by applying three experimental doses (10 nM, 100 nM, and 1000 nM) and performing a proliferation assay (results are presented in Section 2.1). DNA was isolated from the cells, and a proliferation assay was performed after each treatment, with some cells were left for further proliferation in medium without BTZ and 5-Aza-2′-deoxycytidine. DNA was also isolated from cells left for 10 days in a growth medium (free of BTZ and methylation inhibitor) after the third treatment to verify whether the changes in the methylation profile caused by 24 h incubation were permanent and transferred to the daughter cells.

4.2. DNA Extraction and Bisulfite Conversion

A PureLink Genomic DNA Mini Kit (Thermo Fisher, Waltham, MA, USA) was used for DNA isolation. Extraction was performed according to the manufacturer's instructions. A Genomic DNA ScreenTape kit (Agilent Technologies, Santa Clara, CA, USA) was used to measure the concentration and quality of genetic material. The analysis was performed using TapeStation 4510 (Agilent Technologies, Santa Clara, CA, USA). For further procedures, samples showing DINs ≥ 9 were used, proving the good quality of the material. An EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA) was used for DNA conversion, with aims of deaminating unmethylated cytosines, resulting in uracil molecules in place of cytosines. Methylated cytosines do not react with sodium bisulfate and therefore remain unchanged after this reaction. All conversion procedures were carried out in accordance with the manufacturer's recommendations. An amount of 500 ng of DNA from each sample was used for conversion.

4.3. Methylation Arrays

Changes in the DNA methylation profile were detected using a human Infinium Methylation EPIC BeadChip kit (Illumina, San Diego, CA, USA). Each technical replicate in the experiment was analyzed ($n = 3$). The procedure was performed strictly according to the array manufacturer's instructions. A NextSeq550 instrument (Illumina, San Diego, CA, USA) was used to scan the arrays.

4.4. Dose Titration of 5-Aza-2′-Deoxycytidine

To determine the appropriate dose of methylation inhibitor, U266 cell proliferation analysis was performed at three doses of 5-Aza-2′-deoxycytidine (10 nM, 100 nM, and 1000 nM). Proliferation analysis was performed using an Alamar Blue mitochondrial dye conversion assay kit (Thermo Fisher, Waltham, MA, USA). The assay was performed in 96-well plates (1×10^4 U266 cells/well). Each measurement was carried out in 8 wells. Proliferation-level analysis was performed after each of three 24 h treatments of cells with BTZ (control cell) (2.75 nM) or BTZ (2.75 nM) and methylation inhibitor (10 nM, 100 nM, and 1000 nM). After each treatment of U266 cells, proliferation analysis was performed 5 times (1, 3, 6, 8, and 10 days after administration).

4.5. Bioinformatics Analysis of Genome-Wide Methylation

Bioinformatics analyses were performed in the R programming environment using appropriate Bioconductor libraries. We screened for methylation changes using an Illumina Infinium Methylation EPIC Beadchip array. The array evaluated the methylation status of more than 850,000 CpG loci. Analyses were carried out in the R programming environment with the relevant Bioconductor libraries. The "ChAMP" pipeline was used to process the raw microarray data files (.idat) with the default processing options [60,61]. Probes meeting any of the following criteria were removed from the final dataset: detection p-value > 0.01, <3 beads in at least 5% of samples per probe, non-CpG probes, SNP-related probes, multihit probes, and probes located on chromosomes X and Y. Quality control and normalization of the obtained results were then performed. Batch effects and other unwanted variation were removed by the "sva" Bioconductor library [62]. A linear model from the "limma" package was employed to compute the adjusted p value and beta values (methylation scores for each CpG based on the fluorescent intensity, which varied between 0 (unmethylated) and 1 (completely methylated)) [63]. For each CpG probe, the delta beta value corresponding to differential methylation in compared groups was also calculated. A methylation difference with adjusted $p < 0.05$ and $|\text{delta beta}| > 0.2$ was considered statistically significant. The results were visualized using the following R libraries: "ggplot2", "ggprism", "ComplexHeatmap", and "ggpubr" [62,63].

Significantly hypermethylated and hypomethylated probes associated with specific genes were analyzed separately for functional annotation and clustering using the DAVID bioinformatics tool [64,65]. The ENTREZIDs of the methylated genes were matched with relevant GO terms, and significantly enriched gene ontological (GO) terms were further selected from BP DIRECT's GO database. Ontological groups with a corrected p value of less than 0.05 (after Benjamini–Hochberg correction) were visualized as bubble plots.

Gene set enrichment analysis (GSEA) was performed using the "clusterProfiler" library [27] to determine the level of depletion or enrichment in GO terms by calculating a normalized enrichment score (NES) with a corresponding p value. Delta beta values were sorted and used as an argument for the "gseGO" function. Enrichment of gene sets was performed for the GO category of "biological process," assuming that the minimum size of each gene set for analysis was 50, with a p-value cutoff of 0.05. The ten ontology groups with the highest enrichment scores (highest NES values) and those with the most depleted enrichment scores (lowest NES values) were visualized as a bar chart. Enrichment charts for the five most enriched and depleted GO terms were also presented.

5. Conclusions

Changes in DNA methylation influence the development of the BTZ resistance phenotype, and an attempt to limit them by using a methylation inhibitor resulted in a significant reduction in the development of this phenotype. The conducted study may indicate the direction of further clinical trials aimed at modifying existing treatment regimens or developing new ones by including a methylation inhibitor, which can significantly reduce/eliminate the common problem of developing resistance to chemotherapy in patients with MM.

6. Study Limitations

Our study revealed interesting results that may provide a molecular basis for future clinical trials and contribute to improving the clinical outcomes of MM patients. Nevertheless, it has some drawbacks. First, this was an in vitro study. Laboratory conditions do not always accurately reflect the complexity of interactions occurring in the human organism. Therefore, a similar experiment should be performed in an animal model and, ultimately, in a clinical trial. The second disadvantage is that this study was conducted on a single cell line. Repeating the experiment in different MM cell lines and obtaining similar results would strengthen our conclusions and provide stronger evidence to support our hypothesis.

Author Contributions: Conceptualization, B.M.; methodology, K.Ł.; formal analysis, K.Ł. and P.K.; investigation, K.Ł. and P.K.; writing—original draft preparation, K.Ł., P.K. and K.R.; writing—review and editing, B.M. and B.B.; visualization, K.Ł.; supervision, B.M.; funding acquisition, B.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was founded by the Minister of Science and Higher Education under the name "Regional Initiative of Excellence" in 2019–2022 (project number 002/RID/2018/19).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available from the corresponding author upon reasonable request.

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

References

1. Huang, J.; Chan, S.C.; Lok, V.; Zhang, L.; Lucero-Prisno, D.E.; Xu, W.; Zheng, Z.J.; Elcarte, E.; Withers, M.; Wong, M.C.S.; et al. The epidemiological landscape of multiple myeloma: A global cancer registry estimate of disease burden, risk factors, and temporal trends. *Lancet Haematol.* **2022**, *9*, e670–e677. [CrossRef]
2. Diamond, E.; Lahoud, O.B.; Landau, H. Managing multiple myeloma in elderly patients. *Leuk. Lymphoma* **2018**, *59*, 1300–1311. [CrossRef]
3. Jurczyszyn, A.; Suska, A. Multiple Myeloma. In *Reference Module in Biomedical Sciences*; Elsevier: Amsterdam, The Netherlands, 2019; p. B9780128012383114126.
4. Rajkumar, S.V. Multiple myeloma: 2020 update on diagnosis, risk-stratification and management. *Am. J. Hematol.* **2020**, *95*, 548–567. [CrossRef]
5. Medical Masterclass Contributors; Firth, J. Haematology: Multiple myeloma. *Clin. Med.* **2019**, *19*, 58–60.
6. Dhodapkar, M.V. MGUS to myeloma: A mysterious gammopathy of underexplored significance. *Blood* **2016**, *128*, 2599–2606. [CrossRef]
7. Kyle, R.A.; Therneau, T.M.; Rajkumar, S.V.; Offord, J.R.; Larson, D.R.; Plevak, M.F.; Melton 3rd, L.J. A Long-Term Study of Prognosis in Monoclonal Gammopathy of Undetermined Significance. *N. Engl. J. Med.* **2002**, *346*, 564–569. [CrossRef]
8. Kyle, R.A.; Remstein, E.D.; Therneau, T.M.; Dispenzieri, A.; Kurtin, P.J.; Hodnefield, J.M.; Larson, D.R.; Plevak, M.F.; Jelinek, D.F.; Fonseca, R.; et al. Clinical Course and Prognosis of Smoldering (Asymptomatic) Multiple Myeloma. *N. Engl. J. Med.* **2007**, *356*, 2582–2590. [CrossRef]
9. Rajkumar, S.V.; Kumar, S. Multiple Myeloma: Diagnosis and Treatment. *Mayo Clin. Proc.* **2016**, *91*, 101–119. [CrossRef]
10. Rajkumar, S.V.; Kumar, S. Multiple myeloma current treatment algorithms. *Blood Cancer J.* **2020**, *10*, 94. [CrossRef]
11. Koeppen, S. Treatment of Multiple Myeloma: Thalidomide-, Bortezomib-, and Lenalidomide-Induced Peripheral Neuropathy. *Oncol. Res. Treat.* **2014**, *37*, 506–513. [CrossRef]
12. Richardson, P.G.; Sonneveld, P.; Schuster, M.; Irwin, D.; Stadtmauer, E.; Facon, T.; Harousseau, J.-L.; Ben-Yehuda, D.; Lonial, S.; Goldschmidt, H.; et al. Extended follow-up of a phase 3 trial in relapsed multiple myeloma: Final time-to-event results of the APEX trial. *Blood* **2007**, *110*, 3557–3560. [CrossRef]
13. Soave, C.L.; Guerin, T.; Liu, J.; Dou, Q.P. Targeting the ubiquitin-proteasome system for cancer treatment: Discovering novel inhibitors from nature and drug repurposing. *Cancer Metastasis Rev.* **2017**, *36*, 717–736. [CrossRef]
14. Haeri, M.; Knox, B.E. Endoplasmic Reticulum Stress and Unfolded Protein Response Pathways: Potential for Treating Age-related Retinal Degeneration. *J. Ophthalmic Vis. Res.* **2012**, *7*, 45–59. [PubMed]
15. Adams, J.; Palombella, V.J.; Sausville, E.A.; Johnson, J.; Destree, A.; Lazarus, D.D.; Maas, J.; Pien, C.S.; Prakash, S.; Elliott, P.J. Proteasome inhibitors: A novel class of potent and effective antitumor agents. *Cancer Res.* **1999**, *59*, 2615–2622. [PubMed]
16. Handy, D.E.; Castro, R.; Loscalzo, J. Epigenetic Modifications: Basic Mechanisms and Role in Cardiovascular Disease. *Circulation* **2011**, *123*, 2145–2156. [CrossRef]
17. Holoch, D.; Moazed, D. RNA-mediated epigenetic regulation of gene expression. *Nat. Rev. Genet.* **2015**, *16*, 71–84. [CrossRef] [PubMed]
18. Zhang, P.; Torres, K.; Liu, X.; Liu, C.; Pollock, R.E. An Overview of Chromatin-Regulating Proteins in Cells. *Curr. Protein Pept. Sci.* **2016**, *17*, 401–410. [CrossRef]
19. Palomeras, S.; Diaz-Lagares, Á.; Viñas, G.; Setien, F.; Ferreira, H.J.; Oliveras, G.; Crujeiras, A.B.; Hernández, A.; Lum, D.H.; Welm, A.L.; et al. Epigenetic silencing of TGFBI confers resistance to trastuzumab in human breast cancer. *Breast Cancer Res.* **2019**, *21*, 79. [CrossRef]
20. Haertle, L.; Barrio, S.; Munawar, U.; Han, S.; Zhou, X.; Vogt, C.; Fernández, R.A.; Bittrich, M.; Ruiz-Heredia, Y.; Da Viá, M.; et al. Cereblon enhancer methylation and IMiD resistance in multiple myeloma. *Blood* **2021**, *138*, 1721–1726. [CrossRef]
21. Che, F.; Chen, J.; Dai, J.; Liu, X. Inhibition of Multiple Myeloma Using 5-Aza-2′-Deoxycytidine and Bortezomib-Loaded Self-Assembling Nanoparticles. *Cancer Manag. Res.* **2020**, *12*, 6969–6976. [CrossRef]

22. Łuczkowska, K.; Sokolowska, K.E.; Taryma-Lesniak, O.; Pastuszak, K.; Supernat, A.; Bybjerg-Grauholm, J.; Hansen, L.L.; Paczkowska, E.; Wojdacz, T.K.; Machaliński, B. Bortezomib induces methylation changes in neuroblastoma cells that appear to play a significant role in resistance development to this compound. *Sci. Rep.* **2021**, *11*, 9846. [CrossRef]
23. Łuczkowska, K.; Kulig, P.; Baumert, B.; Machaliński, B. The Evidence That 25(OH)D3 and VK2 MK-7 Vitamins Influence the Proliferative Potential and Gene Expression Profiles of Multiple Myeloma Cells and the Development of Resistance to Bortezomib. *Nutrients* **2022**, *14*, 5190. [CrossRef]
24. Moore, L.D.; Le, T.; Fan, G. DNA Methylation and Its Basic Function. *Neuropsychopharmacology* **2013**, *38*, 23–38. [CrossRef]
25. Romero-Garcia, S.; Prado-Garcia, H.; Carlos-Reyes, A. Role of DNA Methylation in the Resistance to Therapy in Solid Tumors. *Front. Oncol.* **2020**, *10*, 1152. [CrossRef]
26. Cao, Y.; Qiu, G.-Q.; Wu, H.-Q.; Wang, Z.-L.; Lin, Y.; Wu, W.; Xie, X.-B.; Gu, W.-Y. Decitabine enhances bortezomib treatment in RPMI 8226 multiple myeloma cells. *Mol. Med. Rep.* **2016**, *14*, 3469–3475. [CrossRef]
27. Wu, T.; Hu, E.; Xu, S.; Chen, M.; Guo, P.; Dai, Z.; Feng, T.; Zhou, L.; Tang, W.; Zhan, L.; et al. clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. *Innovation* **2021**, *2*, 100141. [CrossRef]
28. Wang, B.-D.; Lee, N.H. Aberrant RNA Splicing in Cancer and Drug Resistance. *Cancers* **2018**, *10*, 458. [CrossRef]
29. Salehan, M.R.; Morse, H.R. DNA damage repair and tolerance: A role in chemotherapeutic drug resistance. *Br. J. Biomed. Sci.* **2013**, *70*, 31–40. [CrossRef]
30. Richardson, P.G.; Barlogie, B.; Berenson, J.; Singhal, S.; Jagannath, S.; Irwin, D.; Rajkumar, S.V.; Srkalovic, G.; Alsina, M.; Alexanian, R.; et al. A Phase 2 Study of Bortezomib in Relapsed, Refractory Myeloma. *N. Engl. J. Med.* **2003**, *348*, 2609–2617. [CrossRef]
31. Palumbo, A.; Chanan-Khan, A.; Weisel, K.; Nooka, A.K.; Masszi, T.; Beksac, M.; Spicka, I.; Hungria, V.; Munder, M.; Mateos, M.V.; et al. Daratumumab, Bortezomib, and Dexamethasone for Multiple Myeloma. *N. Engl. J. Med.* **2016**, *375*, 754–766. [CrossRef]
32. Mateos, M.-V.; Sonneveld, P.; Hungria, V.; Nooka, A.K.; Estell, J.A.; Barreto, W.; Corradini, P.; Min, C.K.; Medvedova, E.; Weisel, K.; et al. Daratumumab, Bortezomib, and Dexamethasone Versus Bortezomib and Dexamethasone in Patients With Previously Treated Multiple Myeloma: Three-year Follow-up of CASTOR. *Clin. Lymphoma Myeloma Leuk.* **2020**, *20*, 509–518. [CrossRef]
33. Rosiñol, L.; Oriol, A.; Teruel, A.I.; Hernández, D.; López-Jiménez, J.; De La Rubia, J.; Granell, M.; Besalduch, J.; Palomera, L.; González, Y.; et al. Superiority of bortezomib, thalidomide, and dexamethasone (VTD) as induction pretransplantation therapy in multiple myeloma: A randomized phase 3 PETHEMA/GEM study. *Blood* **2012**, *120*, 1589–1596. [CrossRef]
34. Rosiñol, L.; Oriol, A.; Rios, R.; Sureda, A.; Blanchard, M.J.; Hernández, M.T.; Martínez-Martínez, R.; Moraleda, J.M.; Jarque, I.; Bargay, J.; et al. Bortezomib, lenalidomide, and dexamethasone as induction therapy prior to autologous transplant in multiple myeloma. *Blood* **2019**, *134*, 1337–1345. [CrossRef]
35. Richardson, P.G.; Oriol, A.; Beksac, M.; Liberati, A.M.; Galli, M.; Schjesvold, F.; Lindsay, J.; Weisel, K.; White, D.; Facon, T.; et al. Pomalidomide, bortezomib, and dexamethasone for patients with relapsed or refractory multiple myeloma previously treated with lenalidomide (OPTIMISMM): A randomised, open-label, phase 3 trial. *Lancet Oncol.* **2019**, *20*, 781–794. [CrossRef] [PubMed]
36. Moreau, P.; Attal, M.; Hulin, C.; Arnulf, B.; Belhadj, K.; Benboubker, L.; Béné, M.C.; Broijl, A.; Caillon, H.; Caillot, D.; et al. Bortezomib, thalidomide, and dexamethasone with or without daratumumab before and after autologous stem-cell transplantation for newly diagnosed multiple myeloma (CASSIOPEIA): A randomised, open-label, phase 3 study. *Lancet* **2019**, *394*, 29–38. [CrossRef]
37. Richardson, P.G.; Mitsiades, C.; Hideshima, T.; Anderson, K.C. Bortezomib: Proteasome Inhibition as an Effective Anticancer Therapy. *Annu. Rev. Med.* **2006**, *57*, 33–47. [CrossRef]
38. Oerlemans, R.; Franke, N.E.; Assaraf, Y.G.; Cloos, J.; Van Zantwijk, I.; Berkers, C.R.; Scheffer, G.L.; Debipersad, K.; Vojtekova, K.; Lemos, C.; et al. Molecular basis of bortezomib resistance: Proteasome subunit β5 (PSMB5) gene mutation and overexpression of PSMB5 protein. *Blood* **2008**, *112*, 2489–2499. [CrossRef] [PubMed]
39. Wu, Y.-X.; Yang, J.-H.; Saitsu, H. Bortezomib-resistance is associated with increased levels of proteasome subunits and apoptosis-avoidance. *Oncotarget* **2016**, *7*, 77622–77634. [CrossRef] [PubMed]
40. Beyar-Katz, O.; Magidey, K.; Reiner-Benaim, A.; Barak, N.; Avivi, I.; Cohen, Y.; Timaner, M.; Avraham, S.; Hayun, M.; Lavi, N.; et al. Proinflammatory Macrophages Promote Multiple Myeloma Resistance to Bortezomib Therapy. *Mol. Cancer Res.* **2019**, *17*, 2331–2340. [CrossRef] [PubMed]
41. Leshchenko, V.V.; Kuo, P.-Y.; Jiang, Z.; Weniger, M.A.; Overbey, J.; Dunleavy, K.; Wilson, W.H.; Wiestner, A.; Parekh, S. Harnessing Noxa demethylation to overcome Bortezomib resistance in mantle cell lymphoma. *Oncotarget* **2015**, *6*, 27332–27342. [CrossRef] [PubMed]
42. Yang, L.-H.; Du, P.; Liu, W.; An, L.-K.; Li, J.; Zhu, W.-Y.; Yuan, S.; Wang, L.; Zang, L. LncRNA ANRIL promotes multiple myeloma progression and bortezomib resistance by EZH2-mediated epigenetically silencing of PTEN. *Neoplasma* **2021**, *68*, 788–797. [CrossRef] [PubMed]
43. Tang, J.; Chen, Q.; Li, Q.; He, Y.; Xiao, D. Exosomal mRNAs and lncRNAs involved in multiple myeloma resistance to bortezomib. *Cell Biol. Int.* **2021**, *45*, 965–975. [CrossRef] [PubMed]
44. Watkins, N.J.; Bohnsack, M.T. The box C/D and H/ACA snoRNPs: Key players in the modification, processing and the dynamic folding of ribosomal RNA. *WIREs RNA* **2012**, *3*, 397–414. [CrossRef] [PubMed]

45. Liu, S.; Liu, Z.; Xie, Z.; Pang, J.; Yu, J.; Lehmann, E.; Huynh, L.; Vukosavljevic, T.; Takeki, M.; Klisovic, R.B.; et al. Bortezomib induces DNA hypomethylation and silenced gene transcription by interfering with Sp1/NF-κB–dependent DNA methyltransferase activity in acute myeloid leukemia. *Blood* **2008**, *111*, 2364–2373. [CrossRef] [PubMed]
46. Hu, X.; Xuan, H.; Du, H.; Jiang, H.; Huang, J. Down-Regulation of CD9 by Methylation Decreased Bortezomib Sensitivity in Multiple Myeloma. Agoulnik IU, editor. *PLoS ONE* **2014**, *9*, e95765.
47. Fernández De Larrea, C.; Martín-Antonio, B.; Cibeira, M.T.; Navarro, A.; Tovar, N.; Díaz, T.; Rosiñol, L.; Monzó, M.; Urbano-Ispizua, A.; Bladé, J. Impact of global and gene-specific DNA methylation pattern in relapsed multiple myeloma patients treated with bortezomib. *Leuk. Res.* **2013**, *37*, 641–646. [CrossRef] [PubMed]
48. Hasna, J.; Hague, F.; Rodat-Despoix, L.; Geerts, D.; Leroy, C.; Tulasne, D.; Ouadid-Ahidouch, H.; Kischel, P. Orai3 calcium channel and resistance to chemotherapy in breast cancer cells: The p53 connection. *Cell Death Differ.* **2018**, *25*, 693–707. [CrossRef]
49. Fan, J.; Bellon, M.; Ju, M.; Zhao, L.; Wei, M.; Fu, L.; Nicot, C. Clinical significance of FBXW7 loss of function in human cancers. *Mol. Cancer* **2022**, *21*, 87. [CrossRef]
50. Chen, S.; Lin, J.; Zhao, J.; Lin, Q.; Liu, J.; Wang, Q.; Mui, R.; Ma, L. FBXW7 attenuates tumor drug resistance and enhances the efficacy of immunotherapy. *Front. Oncol.* **2023**, *13*, 1147239. [CrossRef]
51. Mun, G.-I.; Choi, E.; Lee, Y.; Lee, Y.-S. Decreased expression of FBXW7 by ERK1/2 activation in drug-resistant cancer cells confers transcriptional activation of MDR1 by suppression of ubiquitin degradation of HSF1. *Cell Death Dis.* **2020**, *11*, 395. [CrossRef]
52. Wang, Z.; Fukushima, H.; Gao, D.; Inuzuka, H.; Wan, L.; Lau, A.W.; Liu, P.; Wei, W. The two faces of FBW7 in cancer drug resistance. *BioEssays* **2011**, *33*, 851–859. [CrossRef] [PubMed]
53. Sheng, S.; Su, W.; Mao, D.; Li, C.; Hu, X.; Deng, W.; Yao, Y.; Ji, Y. MicroRNA-21 induces cisplatin resistance in head and neck squamous cell carcinoma. *PLoS ONE* **2022**, *17*, e0267017. [CrossRef] [PubMed]
54. Gaudelot, K.; Gibier, J.-B.; Pottier, N.; Hémon, B.; Van Seuningen, I.; Glowacki, F.; Leroy, X.; Cauffiez, C.; Gnemmi, V.; Aubert, S.; et al. Targeting miR-21 decreases expression of multi-drug resistant genes and promotes chemosensitivity of renal carcinoma. *Tumor Biol.* **2017**, *39*, 101042831770737. [CrossRef]
55. Bu, H.; Li, Y.; Jin, C.; Yu, H.; Wang, X.; Chen, J.; Wang, Y.; Ma, Y.; Zhang, Y.; Kong, B. Overexpression of PRC1 indicates a poor prognosis in ovarian cancer. *Int. J. Oncol.* **2020**, *56*, 685–696. [CrossRef] [PubMed]
56. Parreno, V.; Martinez, A.-M.; Cavalli, G. Mechanisms of Polycomb group protein function in cancer. *Cell Res.* **2022**, *32*, 231–253. [CrossRef] [PubMed]
57. Liang, Z.; Li, X.; Chen, J.; Cai, H.; Zhang, L.; Li, C.; Tong, J.; Hu, W. PRC1 promotes cell proliferation and cell cycle progression by regulating p21/p27-pRB family molecules and FAK-paxillin pathway in non-small cell lung cancer. *Transl. Cancer Res.* **2019**, *8*, 2059–2072. [CrossRef]
58. Lee, T.-S.; Ma, W.; Zhang, X.; Giles, F.; Cortes, J.; Kantarjian, H.; Albitar, M. BCR-ABL alternative splicing as a common mechanism for imatinib resistance: Evidence from molecular dynamics simulations. *Mol. Cancer Ther.* **2008**, *7*, 3834–3841. [CrossRef]
59. Wang, Y.; Bernhardy, A.J.; Cruz, C.; Krais, J.J.; Nacson, J.; Nicolas, E.; Peri, S.; van der Gulden, H.; van der Heijden, I.; O'Brien, S.W.; et al. The BRCA1-Δ11q Alternative Splice Isoform Bypasses Germline Mutations and Promotes Therapeutic Resistance to PARP Inhibition and Cisplatin. *Cancer Res.* **2016**, *76*, 2778–2790. [CrossRef]
60. Tian, Y.; Morris, T.J.; Webster, A.P.; Yang, Z.; Beck, S.; Feber, A.; Teschendorff, A.E. ChAMP: Updated methylation analysis pipeline for Illumina BeadChips. *Bioinformatics* **2017**, *33*, 3982–3984. [CrossRef]
61. Morris, T.J.; Butcher, L.M.; Feber, A.; Teschendorff, A.E.; Chakravarthy, A.R.; Wojdacz, T.K.; Beck, S. ChAMP: 450k Chip Analysis Methylation Pipeline. *Bioinformatics* **2014**, *30*, 428–430. [CrossRef]
62. Leek, J.T.; Evan Johnson, W.; Parker, H.S.; Fertig, E.J.; Jaffe, A.E.; Zhang, Y.; Storey, J.D.; Collado Torres, L. *Sva: Surrogate Variable Analysis*; CRAN: Vienna, Austria, 2021.
63. Ritchie, M.E.; Phipson, B.; Wu, D.; Hu, Y.; Law, C.W.; Shi, W.; Smyth, G.K. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* **2015**, *43*, e47. [CrossRef] [PubMed]
64. Dennis, G.; Sherman, B.T.; Hosack, D.A.; Yang, J.; Gao, W.; Lane, H.C.; Lempicki, R.A. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol.* **2003**, *4*, P3. [CrossRef]
65. Fresno, C.; Fernández, E.A. RDAVIDWebService: A versatile R interface to DAVID. *Bioinformatics* **2013**, *29*, 2810–2811. [CrossRef] [PubMed]

nutrients

MDPI

Article

Vitamin D and K Supplementation Is Associated with Changes in the Methylation Profile of U266-Multiple Myeloma Cells, Influencing the Proliferative Potential and Resistance to Bortezomib

Karolina Łuczkowska [1,*,†], Piotr Kulig [1,†], Bartłomiej Baumert [2,*] and Bogusław Machaliński [1,2]

[1] Department of General Pathology, Pomeranian Medical University, 70-111 Szczecin, Poland; piotrkulig@interia.eu (P.K.); boguslaw.machalinski@pum.edu.pl (B.M.)
[2] Department of Hematology and Transplantology, Pomeranian Medical University, 71-252 Szczecin, Poland
* Correspondence: karolina.luczkowska@pum.edu.pl (K.Ł.); bbaumert@pum.edu.pl (B.B.); Tel.: +48-914661546 (K.Ł.); +48-914250428 (B.B.); Fax: +48-914661548 (K.Ł.); +48-914253357 (B.B.)
† These authors contributed equally to this work.

Abstract: Multiple myeloma (MM) is a plasma cell malignancy that, despite recent advances in therapy, continues to pose a major challenge to hematologists. Currently, different classes of drugs are applied to treat MM, among others, proteasome inhibitors, immunomodulatory drugs, and monoclonal antibodies. Most of them participate in an interplay with the immune system, hijacking its effector functions and redirecting them to anti-MM activity. Therefore, adjuvant therapies boosting the immune system may be potentially beneficial in MM therapy. Vitamin D (VD) and vitamin K (VK) have multiple so called "non-classical" actions. They exhibit various anti-inflammatory and anti-cancer properties. In this paper, we investigated the influence of VD and VK on epigenetic alterations associated with the proliferative potential of MM cells and the development of BTZ resistance. Our results showed that the development of BTZ resistance is associated with a global decrease in DNA methylation. On the contrary, both control MM cells and BTZ-resistant MM cells exposed to VD alone and to the combination of VD and VK exhibit a global increase in methylation. In conclusion, VD and VK in vitro have the potential to induce epigenetic changes that reduce the proliferative potential of plasma cells and may at least partially prevent the development of resistance to BTZ. However, further ex vivo and in vivo studies are needed to confirm the results and introduce new supplementation recommendations as part of adjuvant therapy.

Keywords: multiple myeloma; vitamin D; bortezomib; drug resistance

Citation: Łuczkowska, K.; Kulig, P.; Baumert, B.; Machaliński, B. Vitamin D and K Supplementation Is Associated with Changes in the Methylation Profile of U266-Multiple Myeloma Cells, Influencing the Proliferative Potential and Resistance to Bortezomib. *Nutrients* **2024**, *16*, 142. https://doi.org/10.3390/nu16010142

Received: 30 November 2023
Revised: 28 December 2023
Accepted: 28 December 2023
Published: 31 December 2023

1. Introduction

Multiple myeloma (MM) is an incurable plasma cell malignancy; nevertheless, the implementation of novel therapies has significantly improved patient outcomes. One of the turning points in MM therapy was the implementation of bortezomib (BTZ), a potent anti-MM drug that could be included in numerous chemotherapy regimens. It acts as a proteasome inhibitor. More precisely, BTZ reversibly blocks the 26S proteasome unit. Blocking this molecular pathway inhibits the degradation and thus the turnover and metabolism of proteins, events that are essential for cell proliferation and survival, ultimately leading to growth inhibition and apoptosis [1]. In addition to the main mechanism of action, there are also less-known downstream mechanisms that interfere with cellular metabolism. It has been shown that BTZ can induce epigenetic changes in cells exposed to the drug. Importantly, epigenetic alterations appear to be responsible for some adverse effects and may also contribute to the occurrence of BTZ resistance. For instance, BTZ can induce complex epigenetic alterations in nerve cells, including changes in methylation profile as well as in miRNAs expression, which subsequently regulate the expression of their target genes. All these changes were demonstrated to contribute to the development of BTZ-induced

peripheral neuropathy in MM patients [2]. Moreover, in the neuroblastoma cell line, BTZ has the propensity to induce aberrant expression of miRNAs and alter miRNA–mRNA interactions in nerve cells, enhancing apoptosis and neuronal death as well as inhibiting neurogenesis [3]. In addition, global changes in the methylation profile were associated with the development of BTZ resistance in a human neuroblastoma cell line [4].

Vitamin D (VD) is a prohormone that, after hydroxylation in the liver and kidneys, acts through the vitamin D receptor (VDR). VDR is located in the nucleus and regulates the expression of multiple genes when activated [5]. It is very well-known that VD actions are tightly bound to calcium metabolism and bone health [6]. However, the VDR is located not only in tissues related to calcium metabolism and bone health, such as the intestines, bones, and kidneys, but also in numerous other cell types. The location of VDR implies that VD has under its influence multiple processes in many different tissues. In addition to the main mechanism of action via VDR, there are also membrane receptors responsible for non-genomic mechanisms. Their activation results in the activation of various downstream activities such as the opening of ion channels and subsequent generation of signaling molecules and second messengers [7]. Several studies suggest that VD itself has no significant potential to directly induce epigenetic changes [8,9]. Nevertheless, it was demonstrated by Chauss and colleagues that VD can exert epigenetic changes in helper T cells [10]. In addition, our team demonstrated the upregulation of genes from the SNORD family, which is associated with, among others, methylation, in the U266 MM cell line treated with different combinations of BTZ, VD, and vitamin K (VK) [11]. Therefore, we decided to investigate epigenetic changes and their role in the development of BTZ resistance. VD research is currently experiencing its renaissance.

There is a focus on its so-called non-classical actions, i.e., not directly related to bone health, phosphate, and calcium metabolism. Its influence on both the adaptive and innate immune response is particularly important [12]. Modulation of the immune system results in plenty of anti-cancer entities. VD has been shown to induce differentiation and decrease the proliferation of malignant cells [5]. Its anti-tumor activity was demonstrated for solid tumors, for instance, by enhancing the response to chemotherapy [13], as well as hematological malignancies, including MM [14,15]. The exact role of VD in MM has been reviewed by our team elsewhere [16]. On top of VD, VK (particularly the K2 MK7 isoform) was demonstrated to exhibit various anti-cancer effects [17], which were also apparent in hematological malignancies [18,19] including MM [20]. Although, as mentioned above, VK possesses anti-cancer properties, these are not as prominent as in VD. In fact, VK is rarely supplemented alone in different settings than coagulation abnormalities due to VK deficiency or VK antagonists. Nevertheless, there is evidence that VD and VK act synergistically, especially in terms of bone and cardiovascular health. Moreover, VK prevents calcification [21–24]. This provides the rationale for supplementation with both vitamins together, rather than considering VK as an independent anti-MM agent. Therefore, it may be hypothesized that those vitamins may enhance their biological effects when administered together in MM. If their efficacy was confirmed in clinical trials, these vitamins could in the future be recommended for MM patients as an adjuvant therapy. We recently demonstrated that VD and VK have the potential to affect the proliferative potential of MM cells in vitro. Moreover, we found that one of the underlying mechanisms is mediated by genes belonging to the SNORD family, which are associated with epigenetic alterations [11]. After establishing that VD and VK treatment could induce epigenetic modifications in MM cells, we decided to further explore this area.

In our previous paper, we thoroughly described the proliferation analysis. Briefly, after the first treatment with BTZ, a reduction in proliferation of 70.06% was noted compared to control cells ($p < 0.0001$). After the second treatment with BTZ, there was a decrease in proliferation by 24.12% ($p < 0.0001$). The third treatment resulted in the development of a BTZ-resistant phenotype. The addition of 25(OH)D3 resulted in a decrease in proliferation by more than 20%. Similar effects were observed when VK was added. In non-BTZ-resistant cells, vitamins exhibited synergic effects with BTZ. What is particularly important is that

vitamins reduced the proliferation of BTZ-resistant U266 MM cells. To see a detailed analysis, please refer to our previous paper [11].

The aim of this study was to further investigate the role of VD and VK2 MK7 in MM. We wanted to assess the effect of VD and VK on epigenetic alterations associated with the proliferative potential of MM cells and the development of BTZ resistance. Furthermore, we intend to provide a molecular background for subsequent clinical trials. The results are presented by dividing them into three sections. The first shows the influence of methylation on the development of BTZ resistance, the second shows the influence of VD and VK on the alteration of the methylation profile in cancer cells not resistant to BTZ, and the third presents the methylation profile after the action of VD and VK on cancer cells with a BTZ-resistant phenotype.

2. Materials and Methods

2.1. Cell Culture and the Course of the Experiment

The human multiple myeloma cell line U266 (ATCC, Manassas, VA, USA) was used in this study. U266 cells were cultured according to the manufacturer's instructions with RPMI-1640 medium (ATCC, Manassas, VA, USA, cat no. 30-2001) modified to contain 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate. A complete growth medium was prepared by adding fetal bovine serum to the basal medium to a final concentration of 15%. The medium was changed every three days. U266 cells were treated three times with BTZ at 2.75 nM (Cell Signaling Technology, Danvers, MA, USA) and/or 25(OH)D3 (VD) (10^{-6} M) (Sigma-Aldrich, Saint Louis, MO, USA), and/or K2MK7 (VK) (10^{-5} M) (Sigma-Aldrich, Saint Louis, MO, USA) for 24 h with 10-day intervals between treatments. The dose of BTZ was established in our previous articles [11,25] and causes over 50% of cell death after the first treatment. The third treatment results in a BTZ-resistant cell phenotype. In addition, the effectiveness of the BTZ-resistant cell line derivation scheme was confirmed on the SH-SY5Y neuroblastoma cell line in our previous article [4]. Vitamin concentrations were established in our previous article [11]. DNA was isolated from the cells after each treatment and portions of the cells were left to proliferate further in a medium without BTZ and vitamins. In the experiment, four study groups (D—treated with vitamin 25(OH)D3; BTZ—treated with bortezomib; DK—treated simultaneously with vitamin 25(OH)D3 and K2MK7; BTZ_DK—treated simultaneously with bortezomib, vitamin 25(OH)D3, and K2MK7); and a control group were distinguished.

2.2. DNA Extraction and Bisulfate Conversion

DNA was isolated from three separate cell incubations for all groups. PureLink Genomic DNA Mini Kit (Thermo Fisher, Waltham, MA, USA) was used for DNA isolation following the manufacturer's instructions. The concentration and quality of the isolated DNA were measured using TapeStation 4510 (Agilent Technologies, Santa Clara, CA, USA) and the Genomic DNA ScreenTape kit (Agilent Technologies, Santa Clara, CA, USA). All samples showed DINs $\geq$ 9, which confirms the high quality of the genetic material. The bisulfate conversion was performed using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. In total, 500 ng of DNA from each sample was used for conversion.

2.3. Methylation Arrays

An Infinium MethylationEPIC v2.0 BeadChip Kit, human (Illumina, San Diego, CA, USA) was used to analyze the methylation profile. Methylation arrays were performed in triplicate for each group included in the experiment (n = 3). Methylation arrays were made strictly in accordance with the manufacturer's instructions. In short, after bisulfate conversion, the genetic material was amplified for 24 h. The DNA was then fragmented and precipitated, allowing the material to hybridize with the BeadChips array. Hybridization was carried out for 20 h at 48 °C. The next steps were to wash out non-hybridized and

non-specifically hybridized DNA from the BeadChips, add labeled nucleotides to extend the primers hybridized with the sample, and stain them. The NextSeq550 instrument (Illumina, San Diego, CA, USA) was used to scan the arrays.

2.4. Bioinformatics Analysis of Genome-Wide Methylation

Screening for methylation changes was performed using the Illumina Infinium Methylation EPIC Beadchip array (Illumina, San Diego, CA, USA). The analyses were carried out in the R programming environment with the relevant Bioconductor libraries. We presented a detailed description of bioinformatics analyses in our previous article [25].

2.5. Gene Expression Analysis by qRT-PCR

The qRT-PCR method was used to analyze the expression of selected genes (*ARHGAP26*, *MYH10*, *PMP2*, *RFX8*, *BAMBI*, *CLEC12b*). The main criterion for selecting the above genes was their significant variability in the methylation level between the studied groups and their possible involvement in the discussed processes. Primers were designed using the BLAST program (Table 1). The qRT-PCR method consists of two stages. The first one requires performing the mRNA reverse transcription process (0.1 µg) using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA), and the second one is a qPCR reaction using the SYBR Green PCR Master Mix kit (Bio-Rad, Hercules, CA, USA). The GAPDH gene was used as an endogenous control gene. qPCR reactions were performed on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Inc., Hercules, CA, USA).

Table 1. Primer sequences used in the qRT-PCR reactions.

Gene	Primer Sequences
ARHGAP26	F 5′-CCTTAGGGGCAGAGTTGCTC-3′ R 5′-CAGCCCCGATCTGTTCCTTT-3′
MYH10	F 5′-CCAAACGTCAGGGAGCATCT-3′ R 5′-GGTGGCTCATGAAGACCAGA-3′
PMP2	F 5′-AAGCTCTGGGTGTGGGGTTA-3′ R 5′-TGCAGGGTTACGATGCTCTT-3′
RFX8	F 5′-AGCAGCTCCATGTCACACAG-3′ R 5′-TCCTTGACTTGCTTGAGCGT-3′
BAMBI	F 5′-GGTGCAGGAGCTGACTTCTT-3′ R 5′-ATTCCAGCTCCCTTGGATGC-3′
CLEC12b	F 5′-AAAAGAGGGCATCCAGCTCC-3′ R 5′-GCCCAGTTGCTGGGATAAGT-3′

3. Results

3.1. DNA Methylation Changes

Analysis of global changes in the methylation profile was performed after each treatment in each group. However, the article presents the results obtained after the last, third, treatment. After the first treatment with BTZ and/or vitamins, we did not obtain any significant changes in methylation. After the second treatment, there were slight changes in the groups: BTZ_DK_2 vs. BTZ_2 (21 genes with altered methylation); D_2 vs. Control_2 (128 genes with altered methylation); DK_2 vs. Control_2 (150 genes with altered methylation). The results of the bioinformatics analysis after the second treatment are included in the Supplementary Material (Figures S1–S3).

3.1.1. DNA Methylation Changes Associated with the Development of Resistance of U266 Myeloma Cells to BTZ

First, we investigated the epigenetic mechanisms involved in the development of BTZ resistance. We compared control MM cells with BTZ-resistant plasma cells and performed

a bioinformatics analysis that revealed epigenetic alterations and mechanisms underlying the development of resistance to BTZ.

Bioinformatics analysis showed 413 sites with altered methylation in cells treated three times with BTZ compared to untreated control cells (Figure 1A). Delta beta values were calculated according to the normalized ratios of probe fluorescence intensity between methylated and unmethylated signals (value 0 = fully unmethylated, 1 = fully methylated).

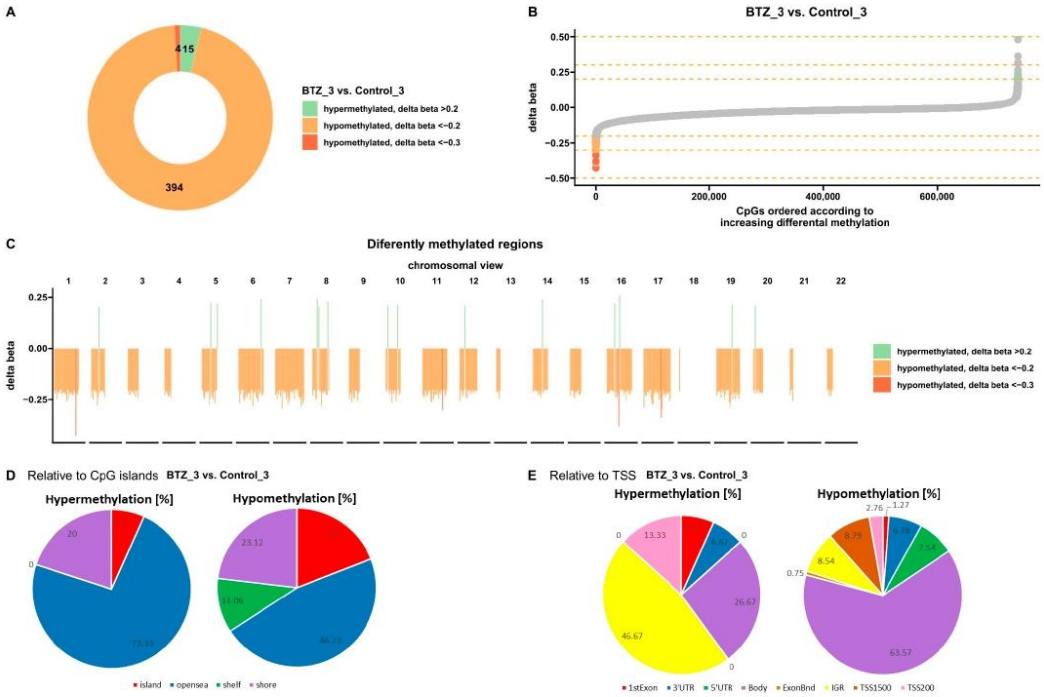


Figure 1. Methylation profile after three consecutive BTZ treatments of U266 myeloma cells. (**A**,**B**) charts show differences in methylation levels in BTZ-treated U266 cells relative to untreated control cells ($p < 0.05$). Orange represents hypomethylation and green represents hypermethylation (**C**) View of changes in the level of methylation in individual chromosomes. Orange represents hypomethylation and green represents hypermethylation ($p < 0.05$). (**D**) Classification of differentially methylated sites in the genome according to their location relative to the CpG islands ($p < 0.05$). (**E**) Classification of differentially methylated sites in the genome according to their location relative to the transcription start site (TSS) ($p < 0.05$). (D—treated with vitamin 25(OH)D3; BTZ—treated with bortezomib; DK—treated simultaneously with vitamin 25(OH)D3 and K2MK7; BTZ_DK—treated simultaneously with bortezomib, vitamin 25(OH)D3, and K2MK7; Control—control group; 3—third incubation).

The analysis identified 398 hypomethylated and 15 hypermethylated sites (Figure 1A,B). In addition, these differences are shown on each chromosome (Figure 1C). Hypermethylated sites are marked in green and hypomethylated sites in orange. Further analysis allowed the classification of the altered sites according to the location of the CpG islands (regions with a high concentration of phosphate-linked cytosine–guanine pairs found in many gene promoters) (Figure 1D) and the transcription start site (TSS) (Figure 1E). Changes in methylation levels were observed in the following regions of the genome: open sea (isolated CpG sites in the genome that do not have a specific designation), shelf (regions 2–4 kb from CpG islands),

shore (regions 0–2 kb from CpG islands). Most of the altered sites, both hypo- and hypermethylated, were found in the open sea. Overall, 19.1% of hypomethylated sites and 6.67% of hypermethylated sites were observed in CpG islands. In addition, no hypermethylated shelf changes were observed. An increased level of methylation in relation to TSS was mainly observed in the IGR (intergenic region) and decreased in the body. More importantly, no hypermethylation was observed in TSS1500. In contrast, hypomethylation sites were observed in both TSS1500 and TSS200 (Figure 1E).

Methylation levels in selected genes in control cells and cells treated three times with BTZ are shown in Figure 2 as a heatmap. The most important observations are the hypomethylation of the *FBXL6*, *CLRN3*, and *PMP2* genes in BTZ-treated cells relative to control cells. Their high expression is associated with tumor progression and poor prognosis in oncological patients [26]. At the same time, hypermethylation of the VPS53 gene was observed (Figure 2), which enhances the process of apoptosis and autophagy.

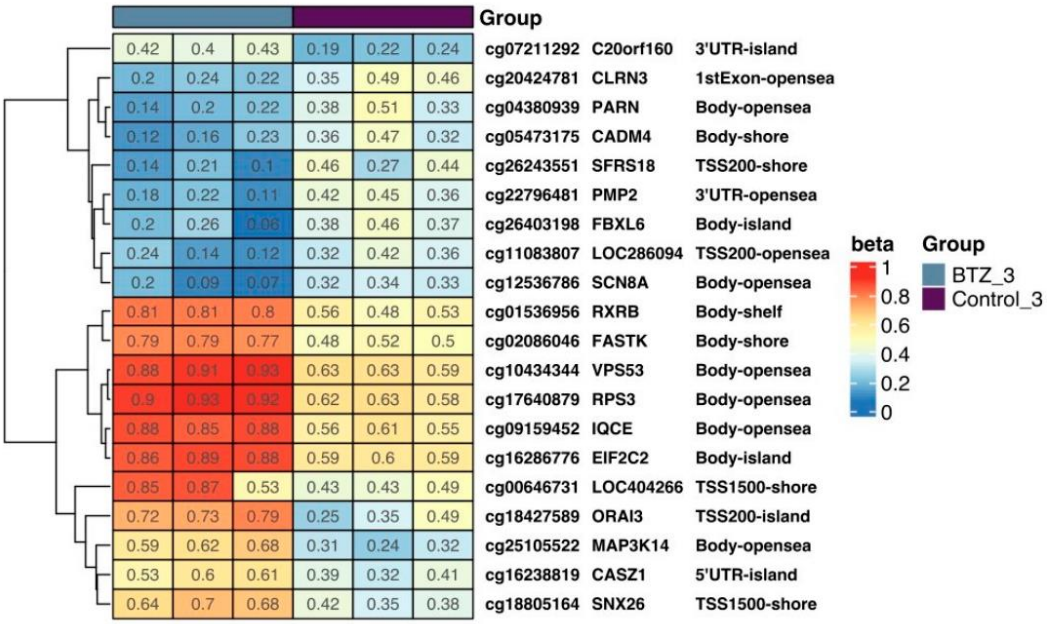


Figure 2. The heat map shows the methylation level of selected genes in control cells and cells treated three times with BTZ. Methylation results are presented as beta values where 1 means full methylation (red) and 0 means no methylation (blue) ($p < 0.05$). In addition, gene symbols and methylation locations are marked on the heat map. Heatmap results were visualized using the R library ComplexHeatmap, version 2.13.1.

Gene Set Enrichment Analysis (*GSEA*)

GSEA is a bioinformatics tool that enables the isolation of groups of genes related to a specific biological process or signaling pathway. Figure 3 shows the processes whose genes altered methylation levels in cells treated three times with BTZ compared to control cells. Only processes whose genes were hypomethylated were identified ($p < 0.05$). The most important processes seem to be the regulation of histone methylation, histone modification, protein deacylation, regulation of telomere maintenance, DNA-templated transcription, and elongation.

The development of BTZ resistance is associated with alterations in epigenetic mechanisms. We demonstrated above that BTZ-resistant plasma cells exhibit a different methyla-

tion profile than control MM cells. Taken together, the development of BTZ resistance is associated with the global hypomethylation of MM cells.

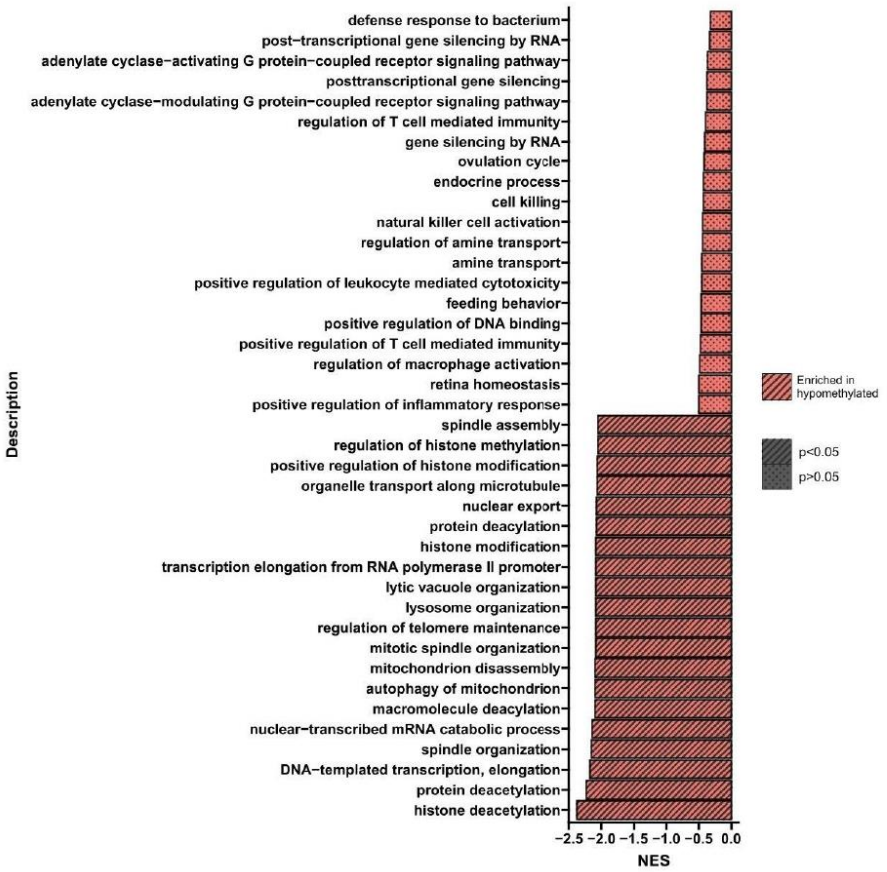


Figure 3. GSEA shows biological processes whose genes showed a change in methylation levels in U266 cells treated three times with BTZ compared to control cells. Red indicates hypomethylation and green indicates hypermethylation. NES—normalized enrichment score.

3.1.2. Effect of VD on DNA Methylation Changes in U266 Myeloma Cells

Subsequently, we intended to explore if VD has the proclivity to induce epigenetic changes in control MM cells. Therefore, we investigated changes in the global methylation profile in control MM cells. In addition, we analyzed how exposure to VD affected various cellular processes and identified the genes whose methylation profile was most altered.

Bioinformatics analysis showed 950 sites with altered methylation in cells treated three times with VD compared to untreated control cells (Figure 4A). The analysis identified 53 hypomethylated sites and 897 hypermethylated sites (Figure 4A,B). In addition, these differences are shown on each chromosome (Figure 4C). Hypermethylated sites are marked in green and hypomethylated in orange. Further analysis allowed the classification of altered sites according to the location of the CpG islands (Figure 4D) and the transcription start site (TSS) (Figure 4E).

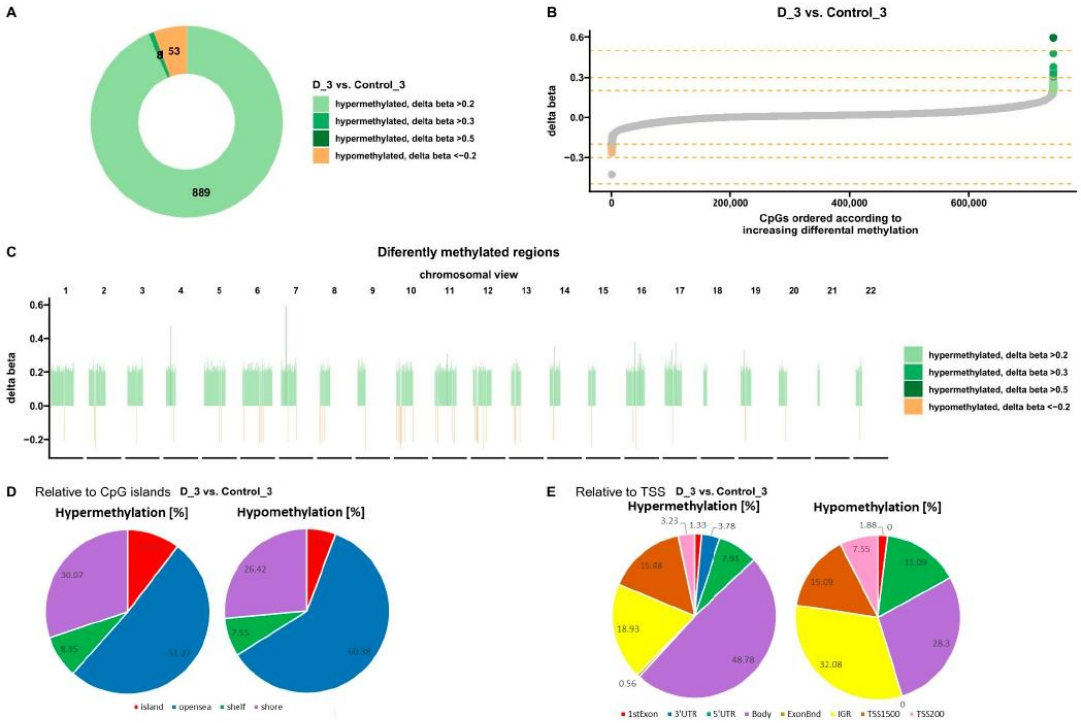


Figure 4. Methylation profile after three VD treatments of U266 myeloma cells. (**A**,**B**) Charts show differences in methylation levels in U266 cells treated with VD relative to untreated control cells ($p < 0.05$). Orange represents hypomethylation and green represents hypermethylation (**C**) View of changes in the level of methylation in individual chromosomes. Orange represents hypomethylation and green represents hypermethylation ($p < 0.05$). (**D**) Classification of differentially methylated sites in the genome according to their location relative to the CpG islands ($p < 0.05$). (**E**) Classification of differentially methylated sites in the genome according to their location relative to the transcription start site (TSS) ($p < 0.05$). (D—treated with vitamin 25(OH)D3; BTZ—treated with bortezomib; DK—treated simultaneously with vitamin 25(OH)D3 and K2MK7; BTZ_DK—treated simultaneously with bortezomib, vitamin 25(OH)D3, and K2MK7; Control—control group; 3—third incubation).

Most altered sites, both hypo- and hypermethylated, were found in the open sea. In total, 5.66% of hypomethylated sites and 10.36% of hypermethylated sites were observed in CpG islands. Both increases and decreases in methylation relative to TSS were mainly observed in the IGR and body (the entire gene from the transcription start site to the end of the transcript) (Figure 4E). More importantly, no hypomethylation was observed in the 3′UTR, a regulatory region that can affect the expression of many genes.

Methylation levels in selected genes in control cells and triple-VD-treated cells are shown in Figure 5 as a heatmap. The most important observations are the hypomethylation of the *CLEC12B* and *BAMBI* genes, which are responsible for inhibiting the proliferation of cancer cells. The study also observed an increase in the methylation levels of genes (NTN1, MYH10) involved in tumor development, progression, proliferation, and migration.

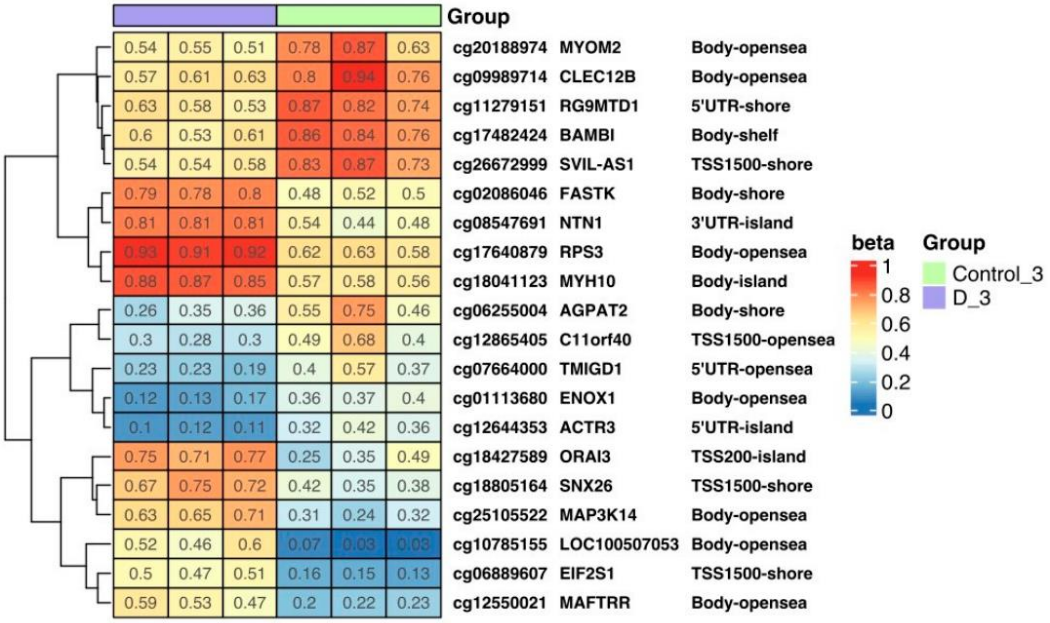


Figure 5. The heat map shows the methylation level of selected genes in control cells and triple-VD-treated cells. Methylation results are presented as beta values where 1 means full methylation (red) and 0 means no methylation (blue) ($p < 0.05$). In addition, gene symbols and methylation locations are marked on the heat map. (D—treated with vitamin 25(OH)D3; BTZ—treated with bortezomib; DK—treated simultaneously with vitamin 25(OH)D3 and K2MK7; BTZ_DK—treated simultaneously with bortezomib, vitamin 25(OH)D3, and K2MK7; Control—control group; 3—third incubation). Heatmap results were visualized using the R library ComplexHeatmap.

Gene Set Enrichment Analysis (*GSEA*)

Figure 6 shows the processes whose genes altered methylation levels in triple-VD-treated cells compared to control cells. Hypo- and hypermethylated genes were identified and assigned to the specific processes they regulate ($p < 0.05$). The most important hypermethylated gene processes are histone methylation, mRNA destabilization, and protein methylation.

VD significantly affects the methylation profile in MM cells. Both hypo- and hypermethylation were observed. More precisely, we identified genes in which various regions were predominantly hypermethylated, yet in some of them, hypomethylation was also observed. Similarly, in multiple cellular processes, we revealed hyper- and hypomethylation. Nevertheless, in summary, co-culturing with VD is associated with a global increase in methylation.

3.1.3. Effect of VD and VK on DNA Methylation Changes in U266 Myeloma Cells

Next, we evaluated the impact of the combination of VD and VK on the methylation profile in control MM cells.

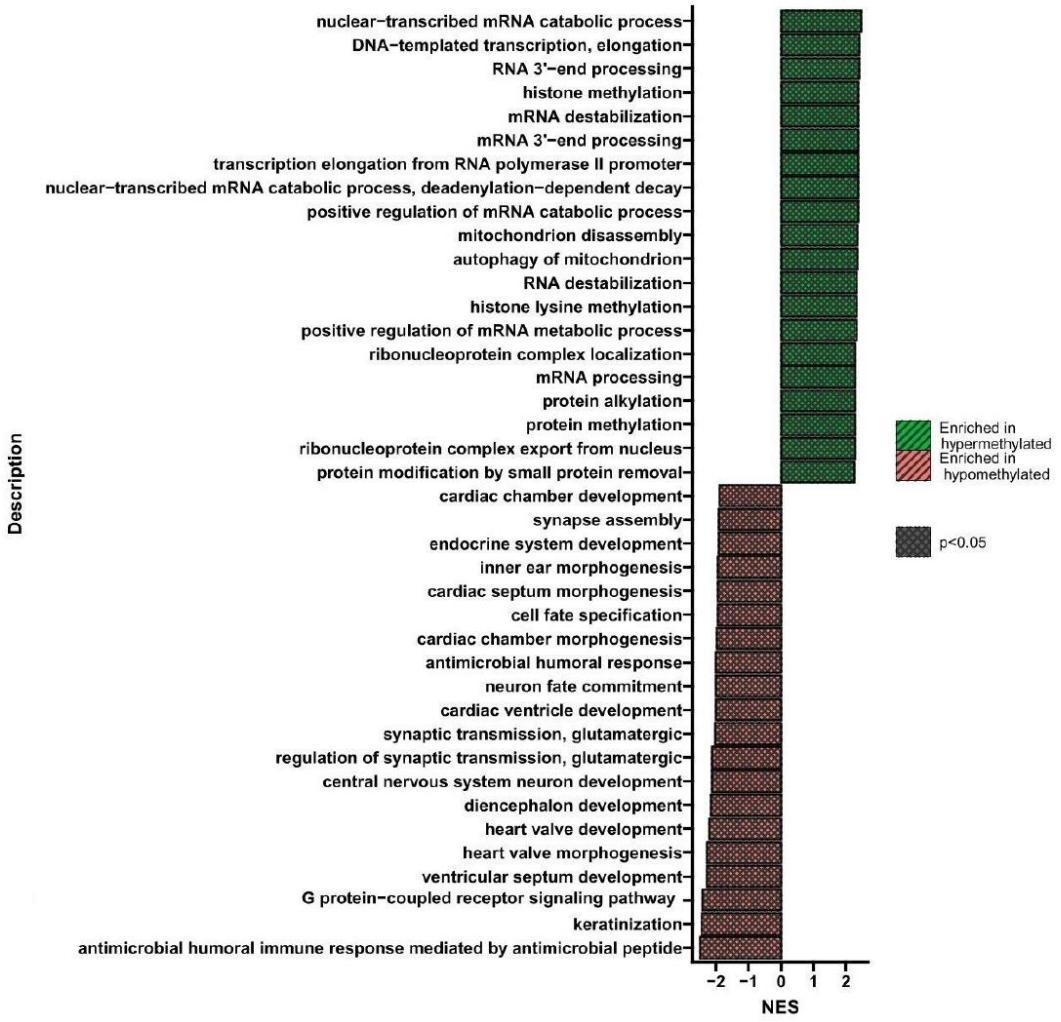


Figure 6. GSEA shows biological processes whose genes showed a change in methylation levels in U266 cells treated three times with VD compared to control cells. Red indicates hypomethylation and green indicates hypermethylation. NES—normalized enrichment score.

Bioinformatics analysis showed 805 sites with altered methylation in cells treated three times with VD and VK compared to untreated control cells (Figure 7A). The analysis identified 36 hypomethylated sites and 769 hypermethylated sites (Figure 7A,B). In addition, these differences are shown on each chromosome (Figure 7C). Hypermethylated sites are marked in green and hypomethylated in orange. Further analysis allowed the classification of the altered sites according to the location of the CpG islands (Figure 7D) and the transcription start site (TSS) (Figure 7E). Most altered sites, both hypo- and hypermethylated, were found in the open sea. In addition, 8.33% of hypomethylated sites and 12.48% of hypermethylated sites were observed in CpG islands. Regarding TSS, an increase in the

level of methylation was observed mainly in the body, and a decrease in IGR (Figure 7E). More importantly, no hypomethylation was observed in the 3′UTR.

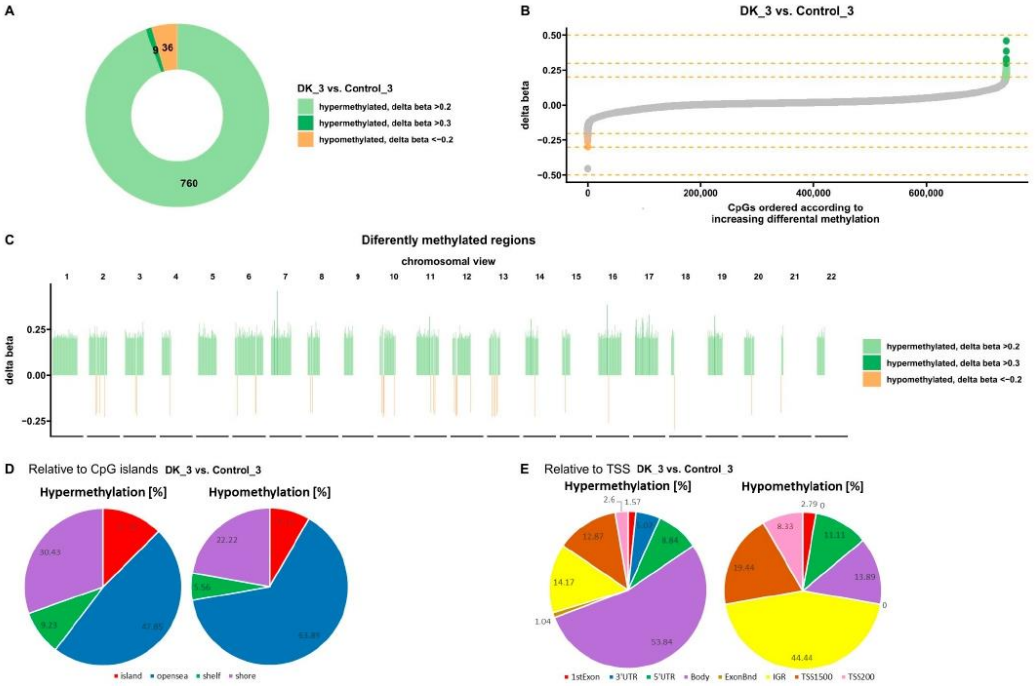


Figure 7. Methylation profile after three VD and VK treatments of U266 myeloma cells. (**A**,**B**) Charts show differences in methylation levels in U266 cells treated with VD and VK compared to untreated control cells ($p < 0.05$). Orange represents hypomethylation and green represents hypermethylation (**C**) View of changes in the level of methylation in individual chromosomes. Orange represents hypomethylation and green represents hypermethylation ($p < 0.05$). (**D**) Classification of differentially methylated sites in the genome according to their location relative to the CpG islands ($p < 0.05$). (**E**) Classification of differentially methylated sites in the genome according to their location relative to the transcription start site (TSS) ($p < 0.05$). (D—treated with vitamin 25(OH)D3; BTZ—treated with bortezomib; DK—treated simultaneously with vitamin 25(OH)D3 and K2MK7; BTZ_DK—treated simultaneously with bortezomib, vitamin 25(OH)D3, and K2MK7; Control—control group; 3—third incubation).

Methylation levels in selected genes in control cells and triple-VD- and -VK-treated cells are shown in Figure 8 as a heatmap. The most important finding is the hypomethylation of the *RFX8* gene in cells treated three times with VD and VK. This gene is thought to be involved in the regulation of transcription by RNA polymerase II and thus, in the regulation of various processes [27]. Moreover, hypermethylation of the *NTN 1* gene was noted, whose epigenetic regulation was associated with the development of colorectal cancer [28].

Figure 9 shows the processes whose genes had altered methylation levels in triple-VD- and -VK-treated cells compared to control cells. Hypo- and hypermethylated genes have been identified and assigned to the individual processes they regulate ($p < 0.05$). Hypermethylated gene processes are RNA localization, DNA-templated transcription, elongation, and telomere organization.

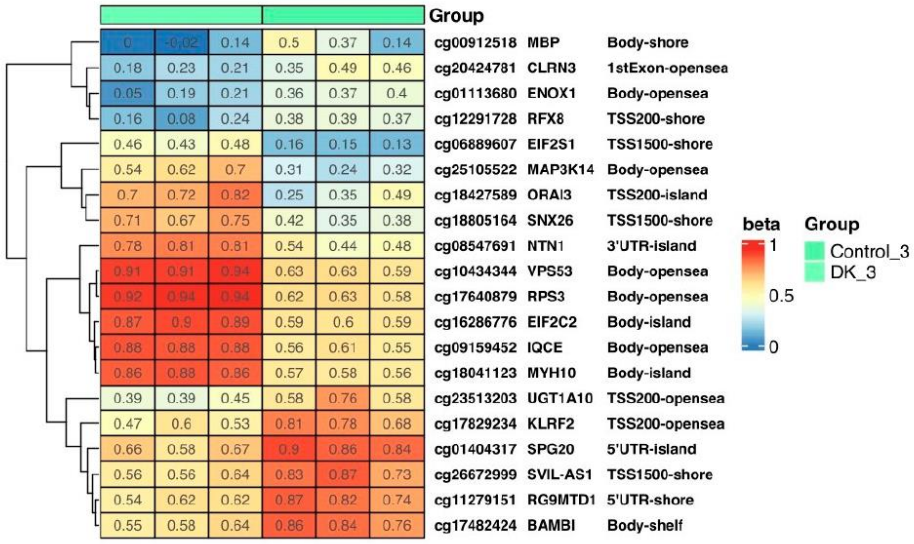


Figure 8. The heat map shows the methylation level of selected genes in control cells and triple-VD- and -VK-treated cells. Methylation results are presented as beta values where 1 means full methylation (red) and 0 means no methylation (blue) ($p < 0.05$). In addition, gene symbols and methylation locations are marked on the heat map. Heatmap results were visualized using the R library ComplexHeatmap.

MM cells exposed to the combination of VD and VK exhibited a similar methylation profile to control cells co-cultured with VD alone. Similarly, we identified genes in which various regions were predominantly hypermethylated; however, hypomethylation was also observed in some of them. Moreover, we detected both hyper- and hypomethylation in various cellular processes. In conclusion, similarly to VD alone, the combination of VD and VK is associated with a global increase in methylation.

3.1.4. DNA Methylation Changes Induced by VD and VK in U266 Myeloma Cells in a BTZ-Resistant Phenotype

We established that VD alone and in combination with VK has the potential to induce epigenetic alterations in control MM cells. Thus, we hypothesized that these vitamins may also affect the methylation profile in MM cells exhibiting a BTZ-resistant phenotype. Therefore, we conducted a similar analysis to explore the impact of both VD and VK on BTZ-resistant MM cells.

Bioinformatics analysis showed 121 sites with altered methylation in cells treated three times simultaneously with BTZ, VD, and VK compared to cells treated three times with BTZ alone (Figure 10A). The analysis identified 27 hypomethylated sites and 94 hypermethylated sites (Figure 10A,B). In addition, these differences are shown on each chromosome (Figure 10C). Hypermethylated sites are marked in green and hypomethylated in orange. Further analysis allowed the classification of the altered sites according to the location of the CpG islands (Figure 10D) and the transcription start site (TSS) (Figure 10E). Most altered sites, both hypo- and hypermethylated, were found in the open sea. Overall, 18.52% of hypomethylated sites and 5.32% of hypermethylated sites were observed in CpG islands. Anincreased level of methylation in relation to TSS was mainly observed in the IGR and decreased in the body. More importantly, no hypomethylation was observed in the 3′UTR (Figure 10E).

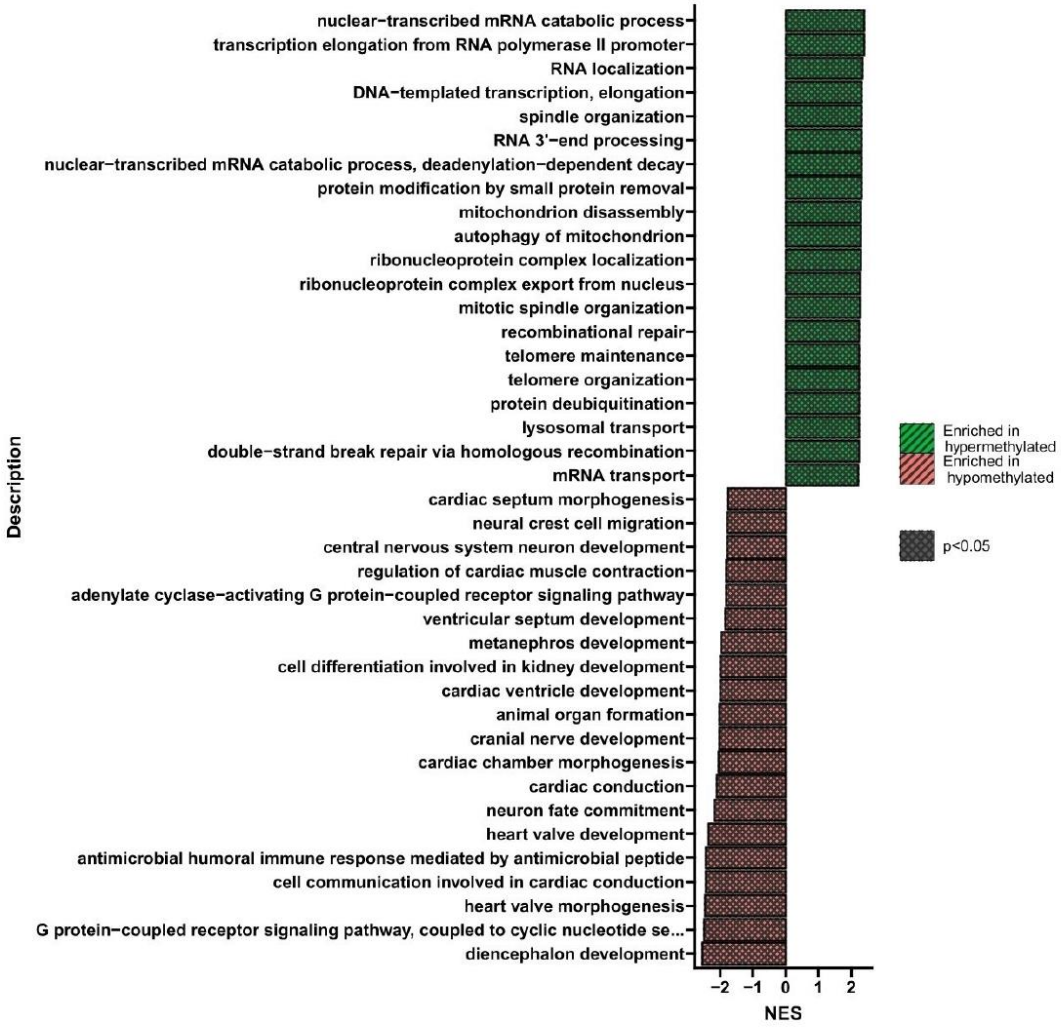


Figure 9. GSEA shows the biological processes whose genes showed a change in methylation levels in U266 cells treated three times with VD and VK compared to control cells. Red indicates hypomethylation and green indicates hypermethylation. NES—normalized enrichment score.

Methylation levels in selected genes in cells treated three times with BTZ, VD, and VK (BTZ_DK_3) and in cells treated with BTZ alone cells are shown in Figure 11 as a heatmap. The most important observation is the hypermethylation of the *ARHGAP26* gene in cells treated three times with BTZ, which is involved in tumorigenesis and progression of human cancers [29].

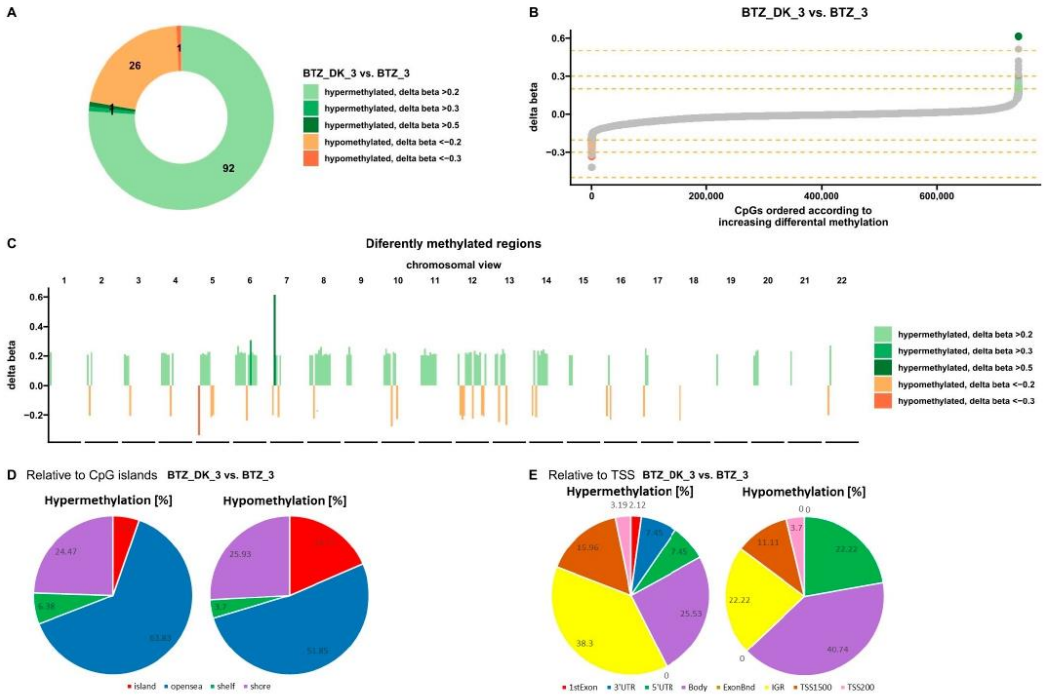


Figure 10. Methylation profile after three subsequent BTZ, VD, and VK treatments of U266 myeloma cells. (**A**,**B**) Charts show differences in methylation levels in U266 cells treated with BTZ, VD, and VK relative to cells treated with BTZ alone ($p < 0.05$). Orange represents hypomethylation and green represents hypermethylation (**C**) View of changes in the level of methylation in individual chromosomes. Orange represents hypomethylation and green represents hypermethylation ($p < 0.05$). (**D**) Classification of differentially methylated sites in the genome according to their location relative to the CpG islands ($p < 0.05$). (**E**) Classification of differentially methylated sites in the genome according to their location relative to the transcription start site (TSS) ($p < 0.05$). (D—treated with vitamin 25(OH)D3; BTZ—treated with bortezomib; DK—treated simultaneously with vitamin 25(OH)D3 and K2MK7; BTZ_DK—treated simultaneously with bortezomib, vitamin 25(OH)D3, and K2MK7; Control—control group; 3—third incubation).

Figure 12A shows the processes whose genes altered methylation levels in triple-BTZ-treated cells compared to cells treated three times with BTZ, VD, and VK. Hypo- and hypermethylated genes have been identified and assigned to the individual processes they regulate ($p < 0.05$). The most important hypomethylated gene processes are cell–cell fusion and cell–substrate adhesion and hypermethylated are RNA 3′-end processing, mRNA 3′-end processing, positive regulation of DNA biosynthetic process, and regulation of cell aging.

Figure 12B shows global changes in methylation in control cells in comparison with BTZ_3, D_3, and DK_3 cells as well as in BTZ_3 cells compared with BTZ_DK_3 cells. (D—treated with vitamin 25(OH)D3; BTZ—treated with bortezomib; DK—treated simultaneously with vitamin 25(OH)D3 and K2MK7; BDK—treated simultaneously with bortezomib, vitamin 25(OH)D3, and K2MK7; Control—control group; 3—third incubation) ($p < 0.05$).

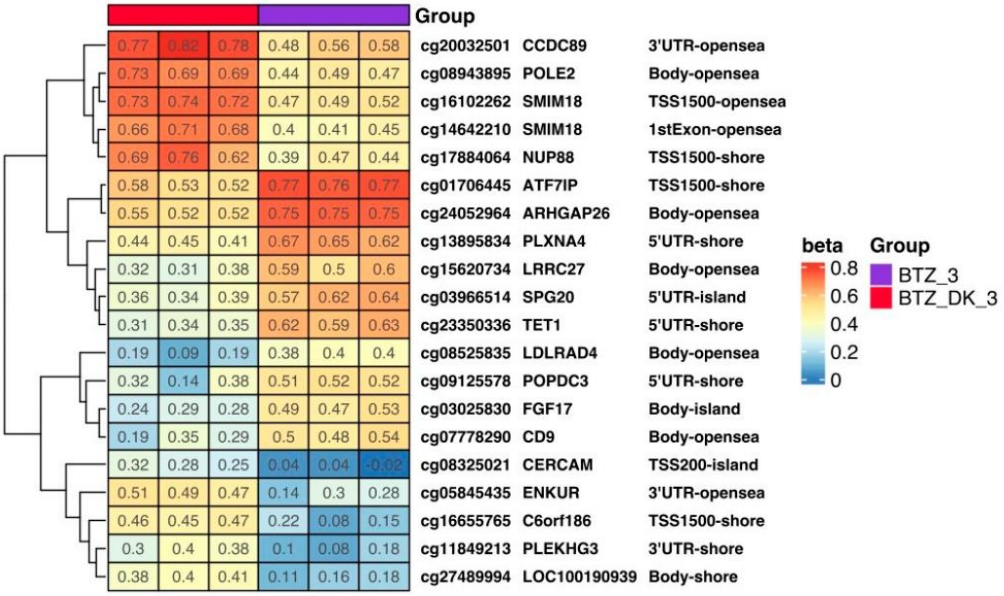


Figure 11. The heat map shows the methylation level of selected genes in cells treated three times with BTZ alone (BTZ_3) and with BTZ, VD, and VK (BTZ_DK_3). Methylation results are presented as beta values, where 1 means full methylation (red) and 0 means no methylation (blue) ($p < 0.05$). In addition, gene symbols and methylation locations are marked on the heat map. (D—treated with vitamin 25(OH)D3; BTZ—treated with bortezomib; DK—treated simultaneously with vitamin 25(OH)D3 and K2MK7; BTZ_DK—treated simultaneously with bortezomib, vitamin 25(OH)D3, and K2MK7; Control—control group; 3—third incubation). Heatmap results were visualized using the R library ComplexHeatmap.

The combination of VD and VK is associated with a global increase in methylation in BTZ-resistant MM cells. However, it should be noted that this effect is not as prominent as in control MM cells. Nevertheless, BTZ-resistant cells, when co-cultured with VD alone or with VD and VK, at least temporarily regained sensitivity to BTZ, which is a promising result and may provide a background for future studies.

3.2. Expression of Selected Genes

We selected genes (*ARHGAP26*, *MYH10*, *PMP2*, *RFX8*, *BAMBI*, *CLEC12b*) with significant changes in methylation level to validate their expression using qRT-PCR in U266 myeloma cells (Table 2). The selected genes confirmed the actual impact of the methylation level on gene expression (according to the principle of the higher the methylation level, the lower the gene expression and vice versa). From the point of view of the effect of vitamins D and K on the U266 myeloma line, not resistant to BTZ, an important observation is an almost 60% decrease in the expression of the MYH10 gene after three exposures to vitamin D in relation to the control cells and a 73% increase in the expression of the CLEC12b gene. Additionally, after three exposures to both vitamins, a 70% increase in RFX8 gene expression was observed compared to control cells.

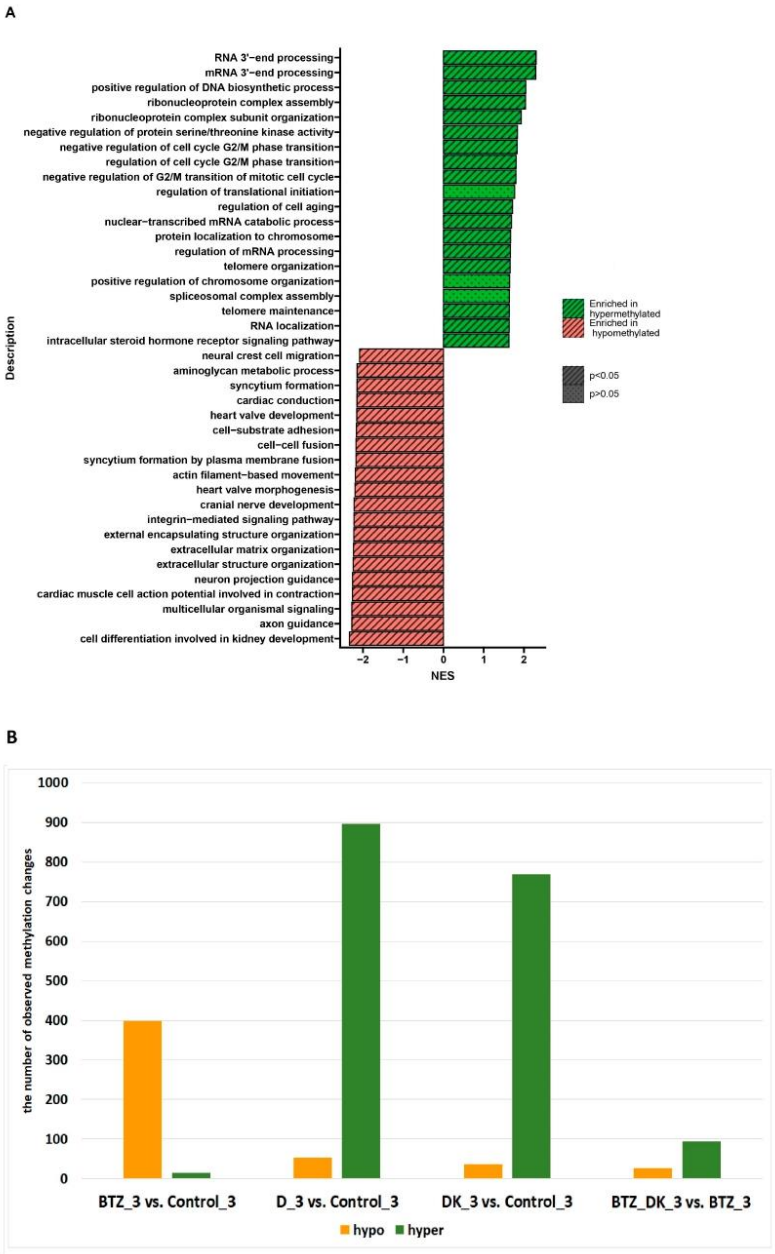


Figure 12. (**A**) GSEA shows biological processes whose genes showed a change in methylation levels in U266 cells treated three times with BTZ, VD, and VK compared to cells treated three times with BTZ alone. Red indicates hypomethylation and green indicates hypermethylation. NES—normalized enrichment score. (**B**) number of observed methylation changes. Orange indicates hypomethylation, green indicates hypermethylation.

Table 2. Real-time quantitation of selected genes in the U266 cell line.

Gene	Control_3 Mean ± SD; 95% CI	BTZ_3 Mean ± SD; 95% CI	D_3 Mean ± SD; 95% CI	DK_3 Mean ± SD; 95% CI	BTZ_DK_3 Mean ± SD; 95% CI
ARHGAP26	1.00 ± 0.08; 0.75–1.25	0.78 ± 0.09; 0.50–1.06	1.16 ± 0.01; 1.01–1.31	1.34 ± 0.11; 1.00–1.68	1.69 ± 0.24; 0.94–2.43
MYH10	0.21 ± 0.03; −0.28–0.71	0.06 ± 0.01; 0.01–0.10	0.09 ± 0.02; 0.03–0.16	0.18 ± 0.07; −0.06–0.42	0.19 ± 0.04; 0.06–0.32
PMP2	0.05 ± 0.02; −0.01–0.12	0.11 ± 0.04; −0.02–0.23	0.36 ± 0.24; −0.39–1.11	0.41 ± 0.03; 0.30–0.52	0.44 ± 0.17; −0.09–0.98
RFX8	0.23 ± 0.04; 0.10–0.35	0.13 ± 0.03; 0.02–0.25	0.40 ± 0.12; 0.02–0.78	0.73 ± 0.16; 0.21–1.25	0.33 ± 0.13; −0.09–0.75
BAMBI	11.50 ± 1.94; 5.57–17.42	11.55 ± 1.41; 7.26–15.84	15.46 ± 1.37; 10.98–19.95	29.10 ± 7.02; 7.73–50.46	20.58 ± 4.66; 6.39–34.77
CLEC12b	0.16 ± 0.09; −0.15–0.46	0.10 ± 0.01; 0.04–0.15	0.22 ± 0.07; −0.02–0.45	0.38 ± 0.1; 0.06–0.69	0.35 ± 0.07; 0.12–0.58

SD—standard deviation, 95% CI—95% confidence interval of the mean (D—treated with vitamin 25(OH)D3; BTZ—treated with bortezomib; DK—treated simultaneously with vitamin 25(OH)D3 and K2MK7; BTZ_DK—treated simultaneously with bortezomib, vitamin 25(OH)D3, and K2MK7; Control—control group; 3—third incubation).

However, the most important result is a significant 54% increase in ARHGAP26 gene expression in the BTZ-resistant U266 myeloma cells that were administered vitamin D and K three times compared to BTZ-resistant cells. This result confirms that the lower level of methylation of this gene in BTZ_DK_3 cells (beta value = 0.52 ± 0.01) compared to BTZ_3 (beta value = 0.75 ± 0.00) correlates with a higher level of its expression.

4. Discussion

Multiple myeloma remains an incurable disease in most cases. However, the implementation of novel therapies has significantly improved patient outcomes. The underlying background of contemporary treatment strategies relies, among others, upon the modulation of the immune system and enhancing its activity against malignant plasma cells. Thalidomide and its derivatives, named lenalidomide and pomalidomide, belong to the class of immunomodulatory drugs (IMiDs). Their pleiotropic mechanisms of actions reach far beyond the simple degradation of Ikaros and Aiolos, two transcription factors essential for B cells, including plasma cells [30,31]. Another class of drugs that modulate the immune system are monoclonal antibodies. Daratumumab and isatuximab are anti-CD38 monoclonal antibodies that have significantly improved clinical outcomes for patients with MM [32,33]. BTZ and carfilzomib belong to a proteasome inhibitor drug class and block the 26S proteasome unit. Blocking this molecular pathway inhibits the degradation and thus, the turnover and metabolism of proteins that are essential for cell proliferation and survival, ultimately leading to growth inhibition and apoptosis [1,34]. Therefore, a BTZ-based regimen can be considered an anti-plasma cell therapy. A combination of drugs modulating the immune system and "anti-plasma cell" drugs such as BTZ turned out to be a highly effective treatment for MM [35–38]. Considering the above, the implementation of complementary, adjuvant therapies enhancing the activity of the immune system appears to be a reasonable strategy in the treatment of MM.

VD and VK, with particular emphasis on VD, exhibit multiple so-called "non-classical actions" that are peculiarly associated with the modulation of the immune system [39]. In addition to healthy cells, the presence of VDR has been demonstrated in malignant cells [40]. Therefore, it can be hypothesized that VD may potentially affect them and thus, interfere with cancer growth and progression. Indeed, there is ample evidence that malignant growth is prone to VD-related suppression. Fife at al. investigated the effects of VD on the MDA-MB-435 human breast cancer cell line, the LNCaP human prostate cancer cell line, and the U2OS human osteosarcoma cell line. The study demonstrated that VD inhibits proliferation and induces apoptosis in all three tested cell lines [41].

VD anti-cancer activity is not limited to solid tumors but also affects hematological malignancies. Moreover, what is particularly important, VD affects MM cells, exerting its tumoricidal properties. Although most of the studies published to date have been conducted in vitro on MM cell lines, they have yielded promising and encouraging results. Moreover, human studies already conducted confirm the benefits of implementation of VD in the treatment of MM. For instance, according to Busch et al., maintaining a proper VD

concentration in MM patients seems to be of paramount importance, especially alongside IMiD-based treatment regimen. They demonstrated in vitro that VD is a key molecule for restoring and maintaining the effector functions of myeloma-associated macrophages and that VD supplementation in combination with IMiDs can enhance the therapeutic efficacy of anti-CD38 antibodies. However, clinical effectiveness needs to be verified in clinical studies [15]. On top of vitamin D itself, its analogs, such as EB1089, also exhibit anti-MM activity. Most importantly, EB1089 has reduced hypercalcemic effects. Despite the promising results of in vitro studies, VD analogs should be further investigated in clinical trials [42–44]. Maintaining proper VD status in MM patients appears to be particularly important. For instance, there is an association between low serum VD status and the prevalence of peripheral neuropathy in the population of MM patients [45]. In addition, according to a study by Wang and colleagues, patients with VD deficiency who were treated with either BTZ or lenalidomide were significantly more likely to develop severe peripheral neuropathy of both motor and sensory types [46]. Furthermore, in a study by Eicher et al., patients with MM without VD deficit who underwent high-dose chemotherapy followed by autologous hematopoietic stem cell transplantation have been shown to have lower overall mortality and longer overall survival and progression-free survival [47]. Nevertheless, it should be noted that studies revealing that low VD status is associated with poorer prognosis in MM or in increase in complications such as peripheral neuropathy, or between appropriate serum VD concertation and good clinical outcome, are correlational studies without any intervention (such as VD supplementation). Therefore, clinical trials are needed to ultimately prove the causative relationship between VD status and better clinical outcomes in MM. Interventional studies and clinical trials should be conducted to further explore this area. Particularly interesting would be a study exploring the relationship between VD serum concentration and gene expression profile, similarly to an interesting study conducted by Donati et al. They demonstrated that VD deficiency indeed affects gene expression and activates stress-protective and pro-survival pathways mediated by NF-κB in classical Hodgkin's lymphoma [48].

We also investigated the potential role of VD in MM. We have demonstrated that 25(OH)D3 has the potential to inhibit the proliferative potential of MM cells. Furthermore, we have shown that 25(OH)D3 can overcome resistance to BTZ in vitro and that its anti-MM properties are at least partially controlled by epigenetic alterations [11].

We have herein deepened our research and thoroughly investigated the epigenetic mechanisms underlying the anti-MM activity of 25(OH)D3 and its role in the development of BTZ resistance. In this study, we showed that the development of BTZ resistance is associated with global changes in methylation. More precisely, MM cells with a BTZ-resistant phenotype were hypomethylated, mainly in the open sea, compared to control cells. In particular, hypomethylation of the *FBXL6*, *CLRN3*, and *PMP2* genes was observed in BTZ-resistant cells compared to control cells. Their high expression is associated with tumor progression and poor clinical outcomes in oncology patients [26,49]. Since these genes have been found to be hypomethylated, it can be hypothesized that given biological processes controlled by them tend to be more active in comparison to control cells and in this way contribute to the resistance development. In addition, GSEA analysis revealed only processes that were hypomethylated. The most significant were histone methylation, histone modification, and protein diacylation, as well as regulation of telomere maintenance. These results suggest that epigenetic mechanisms are associated with BTZ resistance. Globally, resistance to BTZ is associated with hypomethylation. The role of epigenetic mechanisms in tumor progression and the development of drug resistance has been studied in various malignancies. For instance, hypomethylation in prostate cancer is associated with poorer clinical outcomes and more aggressive metastatic disease [50]. Moreover, according to a systematic review by Zelic et al., global DNA methylation levels were consistently lower in prostate cancer tissue compared to healthy prostate tissue. At the same time, methylation levels were lower in more aggressive tumors [51]. Alterations in methylation have also been studied in MM. The results of the study conducted by Sieve and colleagues

revealed that global loss of methylation may be a hallmark of progressive disease with poorer clinical outcomes. They found that an extremely high overlap of hypomethylated genes was associated with poorer survival. Interestingly, most of the hypomethylated genes were outside the CpG islands [52]. Moreover, it has been shown that global changes in methylation can play a significant role in myelomagenesis and promote the transition from monoclonal gammopathy of unknown significance (MGUS) to fully symptomatic MM [53]. We have demonstrated in our previous study that incubation with VD or with both VD and VK has the potential to reduce the proliferation of U266 MM cells, including cells exhibiting a BTZ-resistant phenotype [11]. Consistent with our results in this study, the reduced proliferative potential is also reflected in the altered methylation profile. MM cells incubated with VD or with both VD and VK are globally hypermethylated. Consistently with the overall methylation profile, we observed hypermethylation of genes involved in cancer development and progression. For instance, in control U266 MM cells incubated with VD only, an increase in the methylation levels of the *NTN1* and *MYH10* genes was observed. These genes are involved in the development, progression, and proliferation of tumors in a variety of cancers, including hematological malignancies. For instance, Huang et al. demonstrated that *NTN1* expression was upregulated in B-ALL patients with high and intermediate risk. Therefore, *NTN1* can be considered an oncogene [54]. On the other hand, *MYH10* acts as a tumor suppressor and its downregulation is associated with poor outcomes in hepatocellular carcinoma [55]. The exact role of these genes in MM requires further studies. Similarly, in BTZ-resistant cells, we observed hypermethylation in the *ARHGAP26* gene, which is involved in tumorigenesis and progression of human cancers. However, in various types of cancer, its expression changes significantly [29]. For instance, Qian et al. demonstrated that *ARHGAP26* hypermethylation resulted in reduced gene expression, which may be an early event in the pathogenesis of AML. Similar associations were observed by Bojesen and colleagues [56,57]. On the other hand, Li et al. showed an increase in its expression in prostate cancer [58]. Our observations of the global methylation profile in BTZ-resistant MM cells and VD-incubated control cells are consistent with the trend shown by other researchers that hypomethylation is associated with cancer development and progression [59–61]. On the contrary, it can be hypothesized that hypermethylation suggests a decreased proliferative potential of malignant cells and a better response to therapy. The results of our preclinical study in an in vitro model revealed intriguing relationships and associations that should be further investigated and provided the background for subsequent clinical trials in MM.

5. Conclusions

VD and VK have the potential to induce epigenetic changes in MM cells. Moreover, these alterations are associated with the proliferative potential of plasma cells as well as the development of resistance to BTZ. Globally, hypermethylation is correlated with decreased proliferative potential of malignant plasma cells, and hypomethylation with BTZ resistance. There is an urgent need to conduct in vivo studies in clinical trials to determine whether similar relationships will also occur in patients with multiple myeloma. If the results turn out to be consistent, it will be necessary to introduce new supplementation recommendations as part of adjuvant therapy.

6. Study Limitations

Although our study provided novel insights into the role of VD and VK2 MK7 in MM and shed more light on the regulation of epigenetic mechanisms, it has several drawbacks. First, our study was conducted on only one MM cell line. Second, our study provided preliminary results with the intention to shed more light on the pathophysiology of BTZ-resistance and potential easy-to-implement and cost-effective adjuvant therapies. Nevertheless, it should be emphasized that the obtained results require further validation in more complex preclinical models, e.g., using different MM cell lines, especially those obtained from MM patients and other second-generation proteasome inhibitors (PIs) such

as carfilzomib and ixazomib. Ultimately, the results obtained should be evaluated in an animal model, and finally, in a clinical trial.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu16010142/s1, Figure S1: Methylation profile after second subsequent BTZ, VD, and VK treatments of U266 myeloma cells; Figure S2: Methylation profile after second VD treatments of U266 myeloma cells.; Figure S3: Methylation profile after second VD and VK treatments of U266 myeloma cells.

Author Contributions: Conceptualization, B.M.; methodology, K.Ł.; formal analysis, K.Ł., B.B. and P.K; investigation, K.Ł. and P.K; writing—original draft preparation, K.Ł and P.K.; writing—review and editing, B.B. and B.M.; visualization, K.Ł.; supervision, B.M.; funding acquisition, K.Ł. and B.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Science Centre grant Miniatura 2021/05/X/NZ5/00614 to K.Ł. and Minister of Science and Higher Education under the name "Regional Initiative of Excellence" in 2019–2022, project number 002/RID/2018/19, the amount of funding: PLN 12 000 000.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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

References

1. Richardson, P.G.; Mitsiades, C.; Hideshima, T.; Anderson, K.C. Bortezomib: Proteasome Inhibition as an Effective Anticancer Therapy. *Annu. Rev. Med.* **2006**, *57*, 33–47. [CrossRef] [PubMed]
2. Łuczkowska, K.; Rogińska, D.; Kulig, P.; Bielikowicz, A.; Baumert, B.; Machaliński, B. Bortezomib-Induced Epigenetic Alterations in Nerve Cells: Focus on the Mechanisms Contributing to the Peripheral Neuropathy Development. *Int. J. Mol. Sci.* **2022**, *23*, 2431. [CrossRef] [PubMed]
3. Łuczkowska, K.; Rogińska, D.; Ulańczyk, Z.; Paczkowska, E.; Schmidt, C.A.; Machaliński, B. Molecular Mechanisms of Bortezomib Action: Novel Evidence for the miRNA–mRNA Interaction Involvement. *Int. J. Mol. Sci.* **2020**, *21*, 350. [CrossRef] [PubMed]
4. Łuczkowska, K.; Sokolowska, K.E.; Taryma-Lesniak, O.; Pastuszak, K.; Supernat, A.; Bybjerg-Grauholm, J.; Hansen, L.L.; Paczkowska, E.; Wojdacz, T.K.; Machaliński, B. Bortezomib induces methylation changes in neuroblastoma cells that appear to play a significant role in resistance development to this compound. *Sci. Rep.* **2021**, *11*, 9846. [CrossRef] [PubMed]
5. Jeon, S.-M.; Shin, E.-A. Exploring vitamin D metabolism and function in cancer. *Exp. Mol. Med.* **2018**, *50*, 1–14. [CrossRef]
6. Christakos, S.; Dhawan, P.; Verstuyf, A.; Verlinden, L.; Carmeliet, G. Vitamin D: Metabolism, Molecular Mechanism of Action, and Pleiotropic Effects. *Physiol. Rev.* **2016**, *96*, 365–408. [CrossRef]
7. Hii, C.S.; Ferrante, A. The Non-Genomic Actions of Vitamin D. *Nutrients* **2016**, *8*, 135. [CrossRef]
8. Cruz, M.A.D.; Ulfenborg, B.; Blomstrand, P.; Faresjö, M.; Ståhl, F.; Karlsson, S. Characterization of methylation patterns associated with lifestyle factors and vitamin D supplementation in a healthy elderly cohort from Southwest Sweden. *Sci. Rep.* **2022**, *12*, 12670. [CrossRef]
9. Valencia, R.A.C.; Martino, D.J.; Saffery, R.; Ellis, J.A. In vitro exposure of human blood mononuclear cells to active vitamin D does not induce substantial change to DNA methylation on a genome-scale. *J. Steroid Biochem. Mol. Biol.* **2014**, *141*, 144–149. [CrossRef]
10. Chauss, D.; Freiwald, T.; McGregor, R.; Yan, B.; Wang, L.; Nova-Lamperti, E.; Kumar, D.; Zhang, Z.; Teague, H.; West, E.E.; et al. Autocrine vitamin D signaling switches off pro-inflammatory programs of TH1 cells. *Nat. Immunol.* **2021**, *23*, 62–74. [CrossRef]
11. Łuczkowska, K.; Kulig, P.; Baumert, B.; Machaliński, B. The Evidence That 25(OH)D3 and VK2 MK-7 Vitamins Influence the Proliferative Potential and Gene Expression Profiles of Multiple Myeloma Cells and the Development of Resistance to Bortezomib. *Nutrients* **2022**, *14*, 5190. [CrossRef] [PubMed]
12. Veldman, C.M.; Cantorna, M.T.; DeLuca, H.F. Expression of 1,25-Dihydroxyvitamin D3 Receptor in the Immune System. *Arch. Biochem. Biophys.* **2000**, *374*, 334–338. [CrossRef] [PubMed]
13. Bunch, B.L.; Ma, Y.; Attwood, K.; Amable, L.; Luo, W.; Morrison, C.; Guru, K.A.; Woloszynska-Read, A.; Hershberger, P.A.; Trump, D.L. Vitamin D _3 enhances the response to cisplatin in bladder cancer through VDR and TA p73 signaling crosstalk. *Cancer Med.* **2019**, *8*, 2449–2461. [CrossRef] [PubMed]
14. Ozdemir, F.; Esen, N.; Ovali, E.; Tekelioglu, Y.; Yilmaz, M.; Aydin, F.; Kavgaci, H.; Boruban, C. Effects of Dexamethasone, All-Trans Retinoic Acid, Vitamin D_3 and Interferon-α on FO Myeloma Cells. *Chemotherapy* **2004**, *50*, 190–193. [CrossRef] [PubMed]

15. Busch, L.; Mougiakakos, D.; Büttner-Herold, M.; Müller, M.J.; Volmer, D.A.; Bach, C.; Fabri, M.; Bittenbring, J.T.; Neumann, F.; Boxhammer, R.; et al. Lenalidomide enhances MOR202-dependent macrophage-mediated effector functions via the vitamin D pathway. *Leukemia* **2018**, *32*, 2445–2458. [CrossRef] [PubMed]
16. Kulig, P.; Łuczkowska, K.; Bielikowicz, A.; Zdrojewska, D.; Baumert, B.; Machaliński, B. Vitamin D as a Potential Player in Immunologic Control over Multiple Myeloma Cells: Implications for Adjuvant Therapies. *Nutrients* **2022**, *14*, 1802. [CrossRef] [PubMed]
17. Duan, F.; Mei, C.; Yang, L.; Zheng, J.; Lu, H.; Xia, Y.; Hsu, S.; Liang, H.; Hong, L. Vitamin K2 promotes PI3K/AKT/HIF-1α-mediated glycolysis that leads to AMPK-dependent autophagic cell death in bladder cancer cells. *Sci. Rep.* **2020**, *10*, 7714. [CrossRef]
18. Xu, W.; Wu, H.; Chen, S.; Wang, X.; Tanaka, S.; Sugiyama, K.; Yamada, H.; Hirano, T. Cytotoxic effects of vitamins K1, K2, and K3 against human T lymphoblastoid leukemia cells through apoptosis induction and cell cycle arrest. *Chem. Biol. Drug Des.* **2020**, *96*, 1134–1147. [CrossRef]
19. Yokoyama, T.; Miyazawa, K.; Naito, M.; Toyotake, J.; Tauchi, T.; Itoh, M.; Yuo, A.; Hayashi, Y.; Georgescu, M.-M.; Kondo, Y.; et al. Vitamin K2 induces autophagy and apoptosis simultaneously in leukemia cells. *Autophagy* **2008**, *4*, 629–640. [CrossRef]
20. Tsujioka, T.; Miura, Y.; Otsuki, T.; Nishimura, Y.; Hyodoh, F.; Wada, H.; Sugihara, T. The mechanisms of vitamin K2-induced apoptosis of myeloma cells. *Haematologica* **2006**, *91*, 613–619.
21. Matsunaga, S.; Ito, H.; Sakou, T. The effect of vitamin K and D supplementation on ovariectomy-induced bone loss. *Calcif. Tissue Int.* **1999**, *65*, 285–289. [CrossRef] [PubMed]
22. Ushiroyama, T.; Ikeda, A.; Ueki, M. Effect of continuous combined therapy with vitamin K2 and vitamin D3 on bone mineral density and coagulofibrinolysis function in postmenopausal women. *Maturitas* **2002**, *41*, 211–221. [CrossRef] [PubMed]
23. Braam, L.A.J.L.M.; Hoeks, A.P.G.; Brouns, F.; Hamulyák, K.; Gerichhausen, M.J.; Vermeer, C. Beneficial effects of vitamins D and K on the elastic properties of the vessel wall in postmenopausal women: A follow-up study. *Thromb Haemost.* **2004**, *91*, 373–380. [CrossRef] [PubMed]
24. Kurnatowska, I.; Grzelak, P.; Masajtis-Zagajewska, A.; Kaczmarska, M.; Stefańczyk, L.; Vermeer, C.; Maresz, K.; Nowicki, M. Effect of vitamin K2 on progression of atherosclerosis and vascular calcification in nondialyzed patients with chronic kidney disease stages 3–5. *Pol. Arch. Intern. Med.* **2015**, *125*, 631–640. [CrossRef] [PubMed]
25. Luczkowska, K.; Kulig, P.; Rusińska, K.; Baumert, B.; Machaliński, B. 5-Aza-2′-Deoxycytidine Alters the Methylation Profile of Bortezomib-Resistant U266 Multiple Myeloma Cells and Affects Their Proliferative Potential. *Int. J. Mol. Sci.* **2023**, *24*, 16780. [CrossRef] [PubMed]
26. Li, Y.; Cui, K.; Zhang, Q.; Li, X.; Lin, X.; Tang, Y.; Prochownik, E.V.; Li, Y. FBXL6 degrades phosphorylated p53 to promote tumor growth. *Cell Death Differ.* **2021**, *28*, 2112–2125. [CrossRef] [PubMed]
27. Sugiaman-Trapman, D.; Vitezic, M.; Jouhilahti, E.-M.; Mathelier, A.; Lauter, G.; Misra, S.; Daub, C.O.; Kere, J.; Swoboda, P. Characterization of the human RFX transcription factor family by regulatory and target gene analysis. *BMC Genom.* **2018**, *19*, 181. [CrossRef]
28. Nakayama, H.; Ohnuki, H.; Nakahara, M.; Nishida-Fukuda, H.; Sakaue, T.; Fukuda, S.; Higashiyama, S.; Doi, Y.; Mitsuyoshi, M.; Okimoto, T.; et al. Inactivation of axon guidance molecule netrin-1 in human colorectal cancer by an epigenetic mechanism. *Biochem. Biophys. Res. Commun.* **2022**, *611*, 146–150. [CrossRef]
29. Zhang, L.; Zhou, A.; Zhu, S.; Min, L.; Liu, S.; Li, P.; Zhang, S. The role of GTPase-activating protein ARHGAP26 in human cancers. *Mol. Cell. Biochem.* **2021**, *477*, 319–326. [CrossRef]
30. Kulig, P.; Milczarek, S.; Bakinowska, E.; Szalewska, L.; Baumert, B.; Machaliński, B. Lenalidomide in Multiple Myeloma: Review of Resistance Mechanisms, Current Treatment Strategies and Future Perspectives. *Cancers* **2023**, *15*, 963. [CrossRef]
31. Davies, F.; Baz, R. Lenalidomide mode of action: Linking bench and clinical findings. *Blood Rev.* **2010**, *24*, S13–S19. [CrossRef] [PubMed]
32. Mikhael, J.; Belhadj-Merzoug, K.; Hulin, C.; Vincent, L.; Moreau, P.; Gasparetto, C.; Pour, L.; Spicka, I.; Vij, R.; Zonder, J.; et al. A phase 2 study of isatuximab monotherapy in patients with multiple myeloma who are refractory to daratumumab. *Blood Cancer J.* **2021**, *11*, 89. [CrossRef] [PubMed]
33. Dima, D.; Dower, J.; Comenzo, R.L.; Varga, C. Evaluating Daratumumab in the Treatment of Multiple Myeloma: Safety, Efficacy and Place in Therapy. *Cancer Manag. Res.* **2020**, *12*, 7891–7903. [CrossRef] [PubMed]
34. Siegel, D.S.; Wang, M.; Martin, T.G.; Infante, J.R.; Kaufman, J.L.; Ranjangam, K.; Huang, M.; Bilotti, E.; Vij, R. A Phase 2 Study of Prolonged Carfilzomib Therapy in Patients with Multiple Myeloma Previously Enrolled in Carfilzomib Clinical Trials. *Blood* **2012**, *120*, 2962. [CrossRef]
35. Palumbo, A.; Chanan-Khan, A.; Weisel, K.; Nooka, A.K.; Masszi, T.; Beksac, M.; Spicka, I.; Hungria, V.; Munder, M.; Mateos, M.V.; et al. Daratumumab, Bortezomib, and Dexamethasone for Multiple Myeloma. *N. Engl. J. Med.* **2016**, *375*, 754–766. [CrossRef] [PubMed]
36. Moreau, P.; Attal, M.; Hulin, C.; Arnulf, B.; Belhadj, K.; Benboubker, L.; Béné, M.C.; Broijl, A.; Caillon, H.; Caillot, D.; et al. Bortezomib, thalidomide, and dexamethasone with or without daratumumab before and after autologous stem-cell transplantation for newly diagnosed multiple myeloma (CASSIOPEIA): A randomised, open-label, phase 3 study. *Lancet* **2019**, *394*, 29–38. [CrossRef] [PubMed]

37. Voorhees, P.M.; Kaufman, J.L.; Laubach, J.P.; Sborov, D.W.; Reeves, B.; Rodriguez, C.; Chari, A.; Silbermann, R.; Costa, L.J.; Anderson, L.D., Jr.; et al. Daratumumab, lenalidomide, bortezomib, and dexamethasone for transplant-eligible newly diagnosed multiple myeloma: The GRIFFIN trial. *Blood* **2020**, *136*, 936–945. [CrossRef]
38. Facon, T.; Kumar, S.; Plesner, T.; Orlowski, R.Z.; Moreau, P.; Bahlis, N.; Basu, S.; Nahi, H.; Hulin, C.; Quach, H.; et al. Daratumumab plus Lenalidomide and Dexamethasone for Untreated Myeloma. *N. Engl. J. Med.* **2019**, *380*, 2104–2115. [CrossRef]
39. Baeke, F.; Takiishi, T.; Korf, H.; Gysemans, C.; Mathieu, C. Vitamin D: Modulator of the immune system. *Curr. Opin. Pharmacol.* **2010**, *10*, 482–496. [CrossRef]
40. Campbell, M.J.; Trump, D.L. Vitamin D Receptor Signaling and Cancer. *Endocrinol. Metab. Clin. N. Am.* **2017**, *46*, 1009–1038. [CrossRef]
41. Fife, R.; Sledge, G.; Proctor, C. Effects of vitamin D3 on proliferation of cancer cells in vitro. *Cancer Lett.* **1997**, *120*, 65–69. [CrossRef] [PubMed]
42. Park, W.H.; Seol, J.G.; Kim, E.S.; Binderup, L.; Koeffler, H.P.; Kim, B.K.; Lee, Y.Y. The induction of apoptosis by a combined 1,25(OH)2D3 analog, EB1089 and TGF-β1 in NCI-H929 multiple myeloma cells. *Int. J. Oncol.* **2002**, *20*, 533–542. [CrossRef] [PubMed]
43. Park, W.H.; Seol, J.G.; Kim, E.S.; Jung, C.W.; Lee, C.C.; Binderup, L.; Koeffler, H.; Kim, B.K.; Lee, Y.Y. Cell Cycle Arrest Induced by the Vitamin D3 Analog EB1089 in NCI-H929 Myeloma Cells Is Associated with Induction of the Cyclin-Dependent Kinase Inhibitor p27. *Exp. Cell Res.* **2000**, *254*, 279–286. [CrossRef] [PubMed]
44. Park, W.H.; Seol, J.G.; Kim, E.S.; Hyun, J.M.; Jung, C.W.; Lee, C.C.; Binderup, L.; Koeffler, H.P.; Kim, B.K.; Lee, Y.Y. Induction of apoptosis by vitamin D _3 analogue EB1089 in NCI-H929 myeloma cells via activation of caspase 3 and p38 MAP kinase: Induction of Apoptosis by EB1089 in NCI-H929 Cells. *Br. J. Haematol.* **2000**, *109*, 576–583. [CrossRef] [PubMed]
45. Oortgiesen, B.E.; Kroes, J.A.; Scholtens, P.; Hoogland, J.; de Keijzer, P.D.; Siemes, C.; Jansman, F.G.A.; Kibbelaar, R.E.; Veeger, N.J.G.M.; Hoogendoorn, M.; et al. High prevalence of peripheral neuropathy in multiple myeloma patients and the impact of vitamin D levels, a cross-sectional study. *Support. Care Cancer* **2021**, *30*, 271–278. [CrossRef] [PubMed]
46. Wang, J.; Udd, K.A.; Vidisheva, A.; Swift, R.A.; Spektor, T.M.; Bravin, E.; Ibrahim, E.; Treisman, J.; Masri, M.; Berenson, J.R. Low serum vitamin D occurs commonly among multiple myeloma patients treated with bortezomib and/or thalidomide and is associated with severe neuropathy. *Support. Care Cancer* **2016**, *24*, 3105–3110. [CrossRef] [PubMed]
47. Eicher, F.; Mansouri Taleghani, B.; Schild, C.; Bacher, U.; Pabst, T. Reduced survival after autologous stem cell transplantation in myeloma and lymphoma patients with low vitamin D serum levels. *Hematol. Oncol.* **2020**, *38*, 523–530. [CrossRef]
48. Donati, B.; Ferrari, A.; Ruffini, A.; Manzotti, G.; Fragliasso, V.; Merli, F.; Zanelli, M.; Valli, R.; Luminari, S.; Ciarrocchi, A. Gene expression profile unveils diverse biological effect of serum vitamin D in Hodgkin's and diffuse large B-cell lymphoma. *Hematol. Oncol.* **2020**, *39*, 205–214. [CrossRef]
49. Graf, S.A.; Heppt, M.V.; Wessely, A.; Krebs, S.; Kammerbauer, C.; Hornig, E.; Strieder, A.; Blum, H.; Bosserhoff, A.; Berking, C. The myelin protein PMP2 is regulated by SOX10 and drives melanoma cell invasion. *Pigment. Cell Melanoma Res.* **2019**, *32*, 424–434. [CrossRef]
50. Yegnasubramanian, S.; Haffner, M.C.; Zhang, Y.; Gurel, B.; Cornish, T.C.; Wu, Z.; Irizarry, R.A.; Morgan, J.; Hicks, J.; DeWeese, T.L.; et al. DNA Hypomethylation Arises Later in Prostate Cancer Progression than CpG Island Hypermethylation and Contributes to Metastatic Tumor Heterogeneity. *Cancer Res* **2008**, *68*, 8954–8967. [CrossRef]
51. Zelic, R.; Fiano, V.; Grasso, C.; Zugna, D.; Pettersson, A.A.; Gillio-Tos, A.A.; Merletti, F.; Richiardi, L. Global DNA hypomethylation in prostate cancer development and progression: A systematic review. *Prostate Cancer Prostatic Dis.* **2014**, *18*, 1–12. [CrossRef] [PubMed]
52. Sive, J.I.; Feber, A.; Smith, D.; Quinn, J.; Beck, S.; Yong, K. Global hypomethylation in myeloma is associated with poor prognosis. *Br. J. Haematol.* **2015**, *172*, 473–475. [CrossRef] [PubMed]
53. Heuck, C.J.; Mehta, J.; Bhagat, T.; Gundabolu, K.; Yu, Y.; Khan, S.; Chrysofakis, G.; Schinke, C.; Tariman, J.; Vickrey, E.; et al. Myeloma Is Characterized by Stage-Specific Alterations in DNA Methylation That Occur Early during Myelomagenesis. *J. Immunol.* **2013**, *190*, 2966–2975. [CrossRef] [PubMed]
54. Huang, L.; An, X.; Zhu, Y.; Zhang, K.; Xiao, L.; Yao, X.; Zeng, X.; Liang, S.; Yu, J. Netrin-1 induces the anti-apoptotic and pro-survival effects of B-ALL cells through the Unc5b-MAPK axis. *Cell Commun. Signal.* **2022**, *20*, 122. [CrossRef] [PubMed]
55. Jin, Q.; Cheng, M.; Xia, X.; Han, Y.; Zhang, J.; Cao, P.; Zhou, G. Down-regulation of *MYH10* driven by chromosome 17p13.1 deletion promotes hepatocellular carcinoma metastasis through activation of the EGFR pathway. *J. Cell. Mol. Med.* **2021**, *25*, 11142–11156. [CrossRef] [PubMed]
56. Qian, J.; Qian, Z.; Lin, J.; Yao, D.-M.; Chen, Q.; Li, Y.; Ji, R.-B.; Yang, J.; Xiao, G.-F.; Wang, Y.-L. Abnormal methylation of GRAF promoter Chinese patients with acute myeloid leukemia. *Leuk. Res.* **2011**, *35*, 783–786. [CrossRef] [PubMed]
57. Bojesen, S.E.; Ammerpohl, O.; Weinhäusl, A.; Haas, O.A.; Mettal, H.; Bohle, R.M.; Borkhardt, A.; Fuchs, U. Characterisation of the GRAF gene promoter and its methylation in patients with acute myeloid leukaemia and myelodysplastic syndrome. *Br. J. Cancer* **2006**, *94*, 323–332. [CrossRef] [PubMed]
58. Li, J.; Xu, Y.-H.; Lu, Y.; Ma, X.-P.; Chen, P.; Luo, S.-W.; Jia, Z.-G.; Liu, Y.; Guo, Y. Identifying Differentially Expressed Genes and Small Molecule Drugs for Prostate Cancer by a Bioinformatics Strategy. *Asian Pac. J. Cancer Prev.* **2013**, *14*, 5281–5286. [CrossRef]

59. Debernardi, C.; Libera, L.; Berrino, E.; Sahnane, N.; Chiaravalli, A.M.; Laudi, C.; Berselli, M.; Sapino, A.; Sessa, F.; Venesio, T. Evaluation of global and intragenic hypomethylation in colorectal adenomas improves patient stratification and colorectal cancer risk prediction. *Clin. Epigenetics* **2021**, *13*, 154. [CrossRef]
60. Kushwaha, G.; Dozmorov, M.; Wren, J.D.; Qiu, J.; Shi, H.; Xu, D. Hypomethylation coordinates antagonistically with hypermethylation in cancer development: A case study of leukemia. *Hum. Genom.* **2016**, *10*, 83–102. [CrossRef]
61. Sheaffer, K.L.; Elliott, E.N.; Kaestner, K.H. DNA Hypomethylation Contributes to Genomic Instability and Intestinal Cancer Initiation. *Cancer Prev. Res.* **2016**, *9*, 534–546. [CrossRef] [PubMed]

Załącznik nr 4 – Oświadczenia współautorów

Szczecin, 16.01.2024

dr hab. n. med. Karolina Łuczkowska
Zakład Patologii Ogólnej
Pomorskiego Uniwersytetu Medycznego

Oświadczam, że w publikacji naukowej Łuczkowska K*, Kulig P*, Rusińska K, Baumert B, Machaliński B. 5-Aza-2′-Deoxycytidine Alters the Methylation Profile of Bortezomib-Resistant U266 Multiple Myeloma Cells and Affects Their Proliferative Potential. *International Journal of Molecular Sciences*. 2023; 24(23):16780. https://doi.org/10.3390/ijms242316780 mój udział polegał na opracowaniu metodologii, uczestniczeniu w analizie formalnej, pisaniu manuskryptu oraz przygotowaniu rycin.

Karolina Łuczkowska

Oświadczam, że w publikacji naukowej Łuczkowska K*, Kulig P*, Baumert B, Machaliński B. Vitamin D and K Supplementation Is Associated with Changes in the Methylation Profile of U266-Multiple Myeloma Cells, Influencing the Proliferative Potential and Resistance to Bortezomib. *Nutrients*. 2024; 16(1):142. https://doi.org/10.3390/nu16010142 mój udział polegał na opracowaniu metodologii, uczestniczeniu w analizie formalnej, pisaniu manuskryptu, przygotowaniu rycin oraz pozyskaniu funduszy.

Karolina Łuczkowska

Oświadczam, że w publikacji naukowej Kulig P, Łuczkowska K, Bakinowska E, Baumert B, Machaliński B. Epigenetic Alterations as Vital Aspects of Bortezomib Molecular Action. *Cancers*. 2024; 16(1):84. https://doi.org/10.3390/cancers16010084 mój wkład polegał na korekcie manuskryptu.

Karolina Łuczkowska

Szczecin, 16.01.2024

Estera Bakinowska
Zakład Patologii Ogólnej
Pomorskiego Uniwersytetu Medycznego

Oświadczam, że w publikacji naukowej Kulig P, Łuczkowska K, Bakinowska E, Baumert B, Machaliński B. Epigenetic Alterations as Vital Aspects of Bortezomib Molecular Action. Cancers. 2024; 16(1):84. https://doi.org/10.3390/cancers16010084 mój wkład polegał przygotowaniu rycin.

Estera Bakinowska

Szczecin, 16.01.2024

Lek. Klaudia Rusińska
Zakład Patologii Ogólnej
Pomorskiego Uniwersytetu Medycznego

Oświadczam, że w publikacji naukowej Łuczkowska K*, Kulig P*, Rusińska K, Baumert B, Machaliński B. 5-Aza-2'-Deoxycytidine Alters the Methylation Profile of Bortezomib-Resistant U266 Multiple Myeloma Cells and Affects Their Proliferative Potential. *International Journal of Molecular Sciences.* 2023; 24(23):16780. https://doi.org/10.3390/ijms242316780 mój udział polegał na pomocy w pisaniu manuskryptu.

Klaudia Rusińska

Szczecin, 16.01.2024

dr hab. n. med. Bartłomiej Baumert
Klinika Hematologii i Transplantologii
Pomorskiego Uniwersytetu Medycznego

Oświadczam, że w publikacji naukowej Łuczkowska K*, Kulig P*, Rusińska K, Baumert B, Machaliński B. 5-Aza-2'-Deoxycytidine Alters the Methylation Profile of Bortezomib-Resistant U266 Multiple Myeloma Cells and Affects Their Proliferative Potential. *International Journal of Molecular Sciences.* 2023; 24(23):16780. https://doi.org/10.3390/ijms242316780 mój udział polegał na korekcie manuskryptu.

.....

Oświadczam, że w publikacji naukowej Łuczkowska K*, Kulig P*, Baumert B, Machaliński B. Vitamin D and K Supplementation Is Associated with Changes in the Methylation Profile of U266-Multiple Myeloma Cells, Influencing the Proliferative Potential and Resistance to Bortezomib. *Nutrients*. 2024; 16(1):142. https://doi.org/10.3390/nu16010142 mój wkład polegał na analizie formalnej oraz korekcie manuskryptu

.....

Oświadczam, że w publikacji naukowej Kulig P, Łuczkowska K, Bakinowska E, Baumert B, Machaliński B. Epigenetic Alterations as Vital Aspects of Bortezomib Molecular Action. *Cancers*. 2024; 16(1):84. https://doi.org/10.3390/cancers16010084 mój wkład polegał na korekcie manuskryptu.

.....

Szczecin, 16.01.2024

prof. dr hab. n. med. Bogusław Machaliński
Kierownik Zakładu Patologii Ogólnej
Pomorskiego Uniwersytetu Medycznego

Oświadczam, że w publikacji naukowej Łuczkowska K*, Kulig P*, Rusińska K, Baumert B, Machaliński B. 5-Aza-2'-Deoxycytidine Alters the Methylation Profile of Bortezomib-Resistant U266 Multiple Myeloma Cells and Affects Their Proliferative Potential. *International Journal of Molecular Sciences.* 2023; 24(23):16780. https://doi.org/10.3390/ijms242316780 mój udział polegał na konceptualizacji, nadzorze merytorycznym, korekcie manuskryptu oraz pozyskaniu finansowania.

.....

Oświadczam, że w publikacji naukowej Łuczkowska K*, Kulig P*, Baumert B, Machaliński B. Vitamin D and K Supplementation Is Associated with Changes in the Methylation Profile of U266-Multiple Myeloma Cells, Influencing the Proliferative Potential and Resistance to Bortezomib. *Nutrients*. 2024; 16(1):142. https://doi.org/10.3390/nu16010142 mój wkład polegał na konceptualizacji, nadzorze merytorycznym, korekcie manuskryptu oraz pozyskaniu finansowania.

.....

Oświadczam, że w publikacji naukowej Kulig P, Łuczkowska K, Bakinowska E, Baumert B, Machaliński B. Epigenetic Alterations as Vital Aspects of Bortezomib Molecular Action. *Cancers*. 2024; 16(1):84. https://doi.org/10.3390/cancers16010084 mój wkład polegał na nadzorze merytorycznym, korekcie manuskryptu oraz pozyskaniu finansowania.

.....

Szczecin, 16.01.2024

Lek. Piotr Kulig
Zakład Patologii Ogólnej
Pomorskiego Uniwersytetu Medycznego

Oświadczam, że w publikacji naukowej Łuczkowska K*, Kulig P*, Rusińska K, Baumert B, Machaliński B. 5-Aza-2'-Deoxycytidine Alters the Methylation Profile of Bortezomib-Resistant U266 Multiple Myeloma Cells and Affects Their Proliferative Potential. *International Journal of Molecular Sciences*. 2023; 24(23):16780. https://doi.org/10.3390/ijms242316780 mój udział polegał na analizie formalnej, pisaniu manuskryptu oraz przygotowaniu rycin.

Oświadczam, że w publikacji naukowej Łuczkowska K*, Kulig P*, Baumert B, Machaliński B. Vitamin D and K Supplementation Is Associated with Changes in the Methylation Profile of U266-Multiple Myeloma Cells, Influencing the Proliferative Potential and Resistance to Bortezomib. *Nutrients*. 2024; 16(1):142. https://doi.org/10.3390/nu16010142 mój udział polegał na analizie formalnej oraz pisaniu manuskryptu.

Oświadczam, że w publikacji naukowej Kulig P, Łuczkowska K, Bakinowska E, Baumert B, Machaliński B. Epigenetic Alterations as Vital Aspects of Bortezomib Molecular Action. *Cancers*. 2024; 16(1):84. https://doi.org/10.3390/cancers16010084 mój wkład polegał przeprowadzeniu przeglądu literatury oraz napisaniu manuskryptu.