Uniwersytet Medyczny w Białymstoku

Wydział Farmaceutyczny

z Oddziałem Medycyny Laboratoryjnej

Iwona Kwiatkowska

Ocena aktywności przeciwnowotworowej terapii skojarzonej z użyciem pochodnej 1,2,4- triazyny (MM-129) oraz inhibitora 2,3-dioksygenazy indolowej-1 (indoximodu)

Rozprawa doktorska w oparciu o cykl publikacji naukowych

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Promotor

Prof. dr hab. n. med. Dariusz Pawlak
Zakład Farmakodynamiki Uniwersytetu Medycznego w Białymstoku

Promotor pomocniczy

Dr hab. n. farm. Justyna Hermanowicz
Zakład Farmakodynamiki Uniwersytetu Medycznego w Białymstoku

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Rozdział 1. Wykaz publikacji będących podstawą rozprawy doktorskiej.

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Wykaz stosowanych skrótów:

1-L-MT	1-metyl-L-tryptofan
5-FU	5-fluorouracyl
AhR	ang. Aryl hydrocarbon receptor; Receptor węglowodorów aromatycznych
AKT	ang. Protein Kinase B; Kinaza białkowa B
BCA	ang. Bicinchoninic acid; Kwas bicynchonowy
BTK	ang. Bruton's Tyrosine Kinase; Kinaza tyrozynowa Brutona
CDK2	ang. Cyclin-dependent kinase 2; Kinaza zależna od cyklin 2
CRC	ang. Colorectal cancer; Rak jelita grubego
CTLA4	ang. Cytotoxic T-lymphocyte associated protein 4; Cytotoksyczne białko związane z limfocytami T 4
DCs	ang. Dendritic Cell; Komórki dendrytyczne
DLD-1	Linia komórkowa gruczolakoraka jelita grubego
DMSO	ang. Dimethyl sulfoxide; Dimetylosulfotlenek
DNA	Kwas deoksyrybonukleinowy
ECIS	ang. European Cancer Information System; Europejski System Informacji o Raku
EMT	ang. Epithelial–Mesenchymal Transition; Przejście epitelialno-mezenchymalne
FOXP3	ang. Forkhead box P3, Scurfin
GCN-2	ang. General control nonderepressible 2
HeLa	Linia komórkowa raka szyjki macicy
HT-29	Linia komórkowa gruczolakoraka jelita grubego
IDO1	ang. Indoleamine 2,3-dioxygenase-1; 2,3-dioksygenaza indolowa-1
IDO2	ang. Indoleamine 2,3-dioxygenase-2; 2,3-dioksygenaza indolowa-2
IFN-γ	Interferon gamma
ILT3	ang. Immunoglobulin-like transcript 3; Immunoglobulinopodobny transkrypt 3
ILT4	ang. Immunoglobulin-like transcript 4; Immunoglobulinopodobny transkrypt 4
IND	Indoximod
KP	ang. Kynurenine pathway; Szlak kinureninowy
Kyn	Kinurenina

Lag-3	ang. Lymphocyte activation gene 3; Gen aktywacji limfocytów 3
MM-129	Pirazolo[4,3-e]tetrazolo[4,5-b][1,2,4]triazynosulfonamid
MMP	ang. Mitochondrial Membrane Potential; Potencjał błony mitochondrialnej
mTOR	ang. Mammalian Target of Rapamycin; Ssaczy cel rapamycyny
MTT	ang. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Bromek 3-(4,5-dimetylo-2-tiazolilo)-2,5-difenylo-2H-tetrazoliowy
NK	ang. Natural killers
PD-1	ang. Programmed cell death protein 1; Receptor programowanej śmierci 1
PD-L1	ang. Programmed death-ligand 1; Ligand receptora programowanej śmierci 1
PI	ang. Propidium iodide; Jodek propidyny
PI3K	ang. Phosphoinositol-3-Kinase; Kinaza fosfoinozytolu 3
qRT-PCR	ang. Quantitative real-time Polymerase Chain Reaction; Ilościowa reakcja łańcuchowa polimerazy
RORC	ang. Retinoic acid-related orphan nuclear hormone receptor C; Jądrowy receptor kwasu retinowego związany z receptorem sierocym
TAMs	ang. Tumor associated macrophages; Makrofagi związane z nowotworem
TDO2	ang. Tryptophan 2,3-dioxygenase; 2,3-dioksygenaza tryptofanowa
Teff	Limfocyty T efektorowe
Th	Limfocyty T pomocnicze
Tim-3	ang. T cell immunoglobulin and mucin domain-containing protein 3; Immunoglobulina komórek T i białko 3 zawierające domenę mucyny
Treg	Limfocyty T regulatorowe
TRP	Tryptofan

Rozdział 2. Wprowadzenie

MM-129, czyli pochodna 1,2,4-triazyny swoją strukturą przypomina roskowitynę (RSC) - związek o potencjalnej aktywności przeciwnowotworowej [1–3]. W badaniach przedklinicznych opisano zdolność antyproliferacyjną oraz proapoptotyczną RSC wobec różnych komórek nowotworowych [4–8]. MM-129 łączy w sobie cechy klasycznego cytostatyku z nowoczesnymi związkami modulującymi szlaki immunosupresyjne. Analizy toksykologiczne pozwoliły zaklasyfikować MM-129 jako związek stosunkowo bezpieczny i dobrze tolerowany przez organizmy zwierząt w dawkach wykazujących aktywność przeciwnowotworową [9]. W testach z użyciem komórek ludzkiego gruczolakoraka jelita grubego wykazano, że MM-129 hamuje syntezę DNA i indukuje apoptozę w sposób zależny od kaspaz. Nasila także ekspresję białka p53, którego rola przeciwnowotworowa polega na zatrzymaniu cyklu komórkowego i wprowadzaniu zmutowanych komórek na szlak apoptozy [1]. Ponadto MM-129 zmniejsza ekspresję CDK2 prowadząc do zahamowania cyklu komórkowego, a w konsekwencji do ograniczenia podziałów komórkowych. Związek ten jest również inhibitorem szlaków sygnalizacyjnych zależnych od mTOR, AKT i BTK, czyli szlaków warunkujących przeżycie komórek [9,10]. Dzięki takiemu działaniu MM-129 osłabia podziały i żywotność komórek raka jelita grubego. Pochodna triazyny hamuje ekspresję PD-L1 w komórkach nowotworowych, dzięki czemu może przywracać funkcjonalność komórkom układu odpornościowego.

Odkrycie szczególnej roli układu odpornościowego w procesie nowotworowym wymusiło zmiany w rozumieniu jego patogenezy. Onkogeneza postrzegana była jako wieloetapowy i złożony proces zachodzący wyłącznie w komórkach nowotworowych. Nowe doniesienia naukowe wskazują, że powinna być ona rozumiana jako choroba tocząca się zarówno w komórkach zmutowanych jak i w ich mikrośrodowisku. Znaczną część mikrośrodowiska tworzą komórki immunologiczne i czynniki immunosupresyjne wydzielane przez komórki nowotworowe [11,12]. Wiedza ta dała podstawy do opracowania koncepcji immunoedycji zakładającej, że układ immunologiczny może zarówno hamować jak i nasilać rozwój komórek nowotworowych, a komórki odpornościowe i nowotworowe wchodzą ze sobą w interakcje wzajemnie się zwalczając. W prawidłowo funkcjonującym organizmie układ immunologiczny spełnia rolę supresora nowotworowego, jednak pod wpływem zachodzących zjawisk ulega upośledzeniu stając się jego promotorem. Immunoedycja obejmuje trzy fazy: eliminację, równowagę i ucieczkę [13]. W pierwszej z nich poprawnie funkcjonujące i wyspecjalizowane komórki immunologiczne, takie jak komórki dendrytyczne (DCs) i makrofagi prezentują antygeny nowotworowe limfocytom T prowadząc do ich aktywacji [14,15]. Aktywowane limfocyty T efektorowe wywołują działanie cytotoksyczne wobec komórek nowotworowych. Aby uniknąć apoptozy komórki nowotworowe wydzielają czynniki immunosupresyjne takie jak: cytotoksyczne białko związane z limfocytami T 4 (CTLA4), immunoglobulinę komórek T i białko 3 zawierające domenę mucyny (Tim-3), gen aktywacji limfocytów 3 (Lag-3), czy wspomniany powyżej ligand programowanej śmierci (PD-L1). Ich sekrecja prowadzi do zahamowania odpowiedzi immunologicznej [16–18]. W momencie znacznej utraty zdolności komórek odpornościowych do eliminowania komórek

nowotworowych, te drugie uciekają spod nadzoru immunologicznego (ang. immune escape) i zaczynają się intensywnie rozwijać.

Jednym z najlepiej poznanych mechanizmów zaangażowanych w ucieczkę spod nadzoru immunologicznego jest szlak PD-1/PD-L1. Nowotworowe białko PD-L1 wiąże się ze swoistym receptorem obecnym na komórkach odpornościowych (PD-1) prowadząc do zahamowania aktywacji limfocytów T i indukcji apoptozy [19,20]. Dane kliniczne wskazują, że wysoka ekspresja PD-L1 występuje w bardziej zaawansowanych stadiach nowotworów, koreluje z krótszym czasem przeżycia, gorszymi prognozami dla pacjentów, a także z obniżoną skutecznością leczenia [21–24]. Z tego względu szlak PD-1/PD-L1 stał się celem nowoczesnych terapii, bazujących na przywróceniu zdolności układu odpornościowego do wywoływania immunologicznej odpowiedzi przeciwnowotworowej. Ponieważ czynniki immunosupresyjne często ulegają koekspresji, zasadne stało się łączenie związków celujących w różne punkty kontroli immunologicznej [25–28]. Wykazano, że przeciwnowotworowe działanie inhibitorów PD-1/PD-L1 ulega nasileniu przy jednoczesnym hamowaniu szlaku kinureninowego [29].

Układ kinureninowy (KP) jest główną ścieżką metabolizmu tryptofanu (TRP), a jej elementy połączono z rozwojem immunosupresyjnego mikrośrodowiska guza. Rolę KP w onkogenezie opisano szczegółowo w artykule pt. *„Not Only Immune Escape-The Confusing Role of the TRP Metabolic Pathway in Carcinogenesis”,* opublikowanym na łamach czasopisma **Cancers**.

Tryptofan jest egzogennym aminokwasem niezbędnym do syntezy białek i utrzymania ich homeostazy [30]. Około 95% spożytego TRP ulega przemianom w szlaku kinureninowym, czyli w wieloetapowym procesie rozpoczynającym się od katalitycznego przekształcenia tego aminokwasu do N-formylokinureniny [31]. Białkami umożliwiającymi tę przemianę, a jednocześnie limitującymi jej szybkość są trzy enzymy: 2,3-dioksygenaza indolowa-1, 2,3-dioksygenaza indolowa-2 oraz 2,3-dioksygenaza tryptofanowa (IDO1, IDO2, TDO2). N-formylokinurenina przekształcana jest do kinureniny, z której tworzone są następujące związki: kwas kinurenowy, kwas antranilowy, 3-hydroksykinurenina. W obecności kinureninazy 3-hydroksykinurenina rozkładana jest do kwasu 3-hydroksyantranilowego, a następnie do acetylo-CoA, kwasu pikolinowego i kwasu chinolinowego. Z kwasu chinolinowego powstaje kwas nikotynowy, finalnie ulegający przemianie do dinukleotydu nikotynoamidoadeninowego (NAD+). NAD+ jest koenzymem zaangażowanym w reakcje redoks, które pozwalają utrzymać prawidłowy metabolizm energetyczny komórek. Wpływa on także na kluczowe funkcje warunkujące przeżycie komórek, moduluje szlaki sygnałowe, naprawę DNA, starzenie komórkowe, a także zapewnia prawidłowe funkcjonowanie komórek immunologicznych [32–34]. Poza regulowaniem procesów fizjologicznych, elementy KP coraz częściej wskazywane są jako czynniki zaangażowane w patogenezę licznych chorób o podłożu neurologicznym, kardiologicznym, endokrynologicznym, i immunologicznym oraz onkologicznym [35–39].

Znaczącą rolę pronowotworową przypisuje się enzymowi pierwszego etapu przemiany tryptofanu - 2,3-dioksygenazie indolowej-1 (IDO1). Gen IDO1 znajduje się w ludzkim chromosomie 8p22, a do jego konstytutywnej ekspresji dochodzi głównie w łożysku [40]. Znacznie częściej ekspresja IDO1 jest indukowana czynnikami prozapalnymi. Wykazano, że rejon promotorowy genu IDO1 zawiera elementy stymulowane przez interferon gamma (IFN-γ), co tłumaczy jego silny wpływ na ekspresję IDO1 [41]. Przewlekły stan zapalny i podwyższony poziom IFN-γ są cechami charakterystycznymi toczącego się procesu nowotworowego [42].

Większość efektów promujących rozwój komórek nowotworowych IDO1 wiązana jest z jej funkcją enzymatyczną, która polega na rozszczepieniu pierścienia tryptofanu poprzez jego utlenienie i przekształcenie do N-formylokinureniny [43]. Prowadzi to do wyczerpania TRP w mikrośrodowisku guza z następczą indukcją kinazy GCN-2 oraz inhibicją szlaku mTOR w komórkach immunologicznych [44]. Aktywacja GCN-2 hamuje proliferację efektorowych limfocytów T, uniemożliwia transdukcję sygnału ze swoistego receptora TCR, a także upośledza produkcję cytokin [45]. Finalnie limfocyty Teff tracą zdolność eliminowania komórek nowotworowych. Ponadto kinaza GCN-2 wpływa na funkcjonowanie komórek dendrytycznych wywołując w nich nadekspresję receptorów hamujących ILT3, ILT4. Prowadzi to do zaburzenia interakcji DCs z limfocytami T [46]. Zaobserwowano, że ekspresja ILT3, ILT4 powoduje różnicowanie limfocytów Teff w immunosupresyjne limfocyty Treg. Poza tym ekspresja omawianych receptorów wywołuje anergię, czyli utratę reaktywności limfocytów Teff [47], co jest uznanym procesem zaangażowanym w ucieczkę immunologiczną [48]. Opisane zjawiska mają implikacje kliniczne. Wzrost ekspresji receptorów ILT3 i ILT4 połączono z progresją nowotworową oraz gorszymi prognozami przeżycia pacjentów onkologicznych [49–51]. Podobne działanie immunosupresyjne takie jak zmniejszenie liczby limfocytów Teff i wzrost liczby limfocytów Treg w populacji ogólnej obserwuje się w wyniku zahamowania szlaku mTOR spowodowanego wyczerpaniem TRP w mikrośrodowisku guza. Niekorzystny stosunek limfocytów Teff do limfocytów Treg jest markerem prognostycznym gorszego przebiegu choroby nowotworowej [52,53].

Metabolity powstające w szlaku przemian tryptofanu również odgrywają istotną rolę w progresji nowotworowej. Kinurenina, główny metabolit TRP, jest ligandem receptora AhR - czynnika transkrypcyjnego. Interakcja Kyn/AhR promuje onkogenezę zarówno poprzez indukcję mechanizmów immunosupresyjnych, jak i przez bezpośredni wpływ na procesy toczące się w komórkach nowotworowych. Pobudzenie receptora AhR w limfocytach T skutkuje wzrostem ekspresji białka PD-1 wzmagając ich różnicowanie do immunosupresyjnej subpopulacji Treg. Wykazano, że hamowanie szlaku IDO/Kyn/AhR, nasilając odpowiedź limfocytów T, ogranicza rozwój raka jelita grubego [54]. Wzrost ekspresji białka PD-1 pod wpływem pochodnej tryptofanu wskazuje na złożoność interakcji pomiędzy czynnikami immunosupresyjnymi i podkreśla zasadność łącznego stosowania różnych związków celujących w punkty kontroli immunologicznej. Pod wpływem interakcji Kyn/AhR dochodzi do upośledzenia aktywności również innych komórek odpornościowych. Takenakya i wsp. wykazali, że ten mechanizm odpowiedzialny jest za różnicowanie makrofagów związanych z nowotworem (TAMs) w wysoce immunosupresyjną populację M2 [55]. Kinurenina poprzez

receptor AhR obniża funkcje lityczne komórek NK, a także ogranicza immunogenność komórek dendrytycznych [44,56]. Dodatkowo zaburzone zostają interakcje pomiędzy poszczególnymi typami komórek odpornościowych. Warto zaznaczyć, że aktywacja szlaku Kyn/AhR nasila ekspresję genów kodujących cytokiny prozapalne oraz INF-γ. Podtrzymywany w ten sposób stan zapalny nasila ekspresję IDO1 oraz metabolizm TRP, co dodatkowo wzmaga interakcję Kyn/AhR i promuje onkogenezę [44,57]. Jak wspomniano powyżej, kinurenina wpływa także bezpośrednio na komórki nowotworowe. Indukowana Kyn aktywacja receptora AhR nasila ich przeżycie w mechanizmie zależnym od aktywacji szlaków AKT i STAT3 [58]. Aktywność kinureniny połączono z zahamowaniem apoptozy komórek raka piersi [59]. Wykazano również, że interakcja Kyn/AhR zwiększa ekspresję metaloproteinaz, akwaporyn oraz markerów przejścia epitelialne-mezenchymalnego (EMT), co wzmaga inwazyjność komórek nowotworowych [60,61].

Najnowsze doniesienia wskazują, że funkcjonalną częścią IDO1 jest jej domena mała [62]. W przeciwieństwie do domeny dużej odpowiada ona za funkcje sygnalizacyjne białka, co sugeruje, że IDO1 może wpływać na procesy życiowe komórek niezależnie od metabolizmu TRP [41]. Efekty IDO1 jako białka biorącego udział w komunikacji wewnątrzkomórkowej są znacząco mniej poznane. Nieliczne badania wskazują na zaangażowanie IDO1 w programowaną śmierć komórki i jej rolę antyapoptotyczną zależną od hamowania cyklu komórkowego [63,64]. Ponadto wykazano, że nadekspresja IDO1 koreluje z regulacją w dół ekspresji E-kadheryny i nadekspresją N-kadheryny oraz wimentyny, czyli markerami przejścia epitelialno-mezenchymalnego. Jest to proces, dzięki któremu komórki nowotworowe zwiększają ruchliwość, a tym samym nabywają możliwość tworzenia przerzutów stając się bardziej inwazyjne. Tang i wsp. opisali ograniczenie zdolności migracyjnej komórek raka płuc po wyciszeniu IDO1 [65]. Nadekspresję omawianego białka połączono także z nasiloną angiogenezą, co dodatkowo podkreśla jego rolę jako czynnika kancerogennego [66]. Wysoki poziom IDO1 oraz zmniejszony stosunek TRP/Kyn skorelowano z wyższym stadium zaawansowania nowotworów oraz gorszymi rokowaniami przeżycia pacjentów [67–71]. Ze względu na znaczenie kliniczne białko IDO1, podobnie jak wcześniej wspomniany szlak PD-1/PD-L1, stało się celem nowoczesnych terapii onkologicznych.

Jednym ze związków wykazujących aktywność inhibicyjną wobec IDO1 jest indoximod (IND) - 1-metyl-D-tryptofan. IND hamuje metabolizm TRP, co skutkuje wzrostem jego stężenia w mikrośrodowisku guza. Zaobserwowano, że związek ten przywraca funkcjonalność kinazy mTOR limfocytom Teff, z jednoczesnym nasileniem ich proliferacji oraz spadkiem liczby limfocytów Treg [72]. Ponadto IND moduluje czynność receptora AhR w limfocytach T, co prowadzi do nasilenia transkrypcji czynnika RORC oraz zmniejszenia transkrypcji czynnika FOXP3. W rezultacie dochodzi do polaryzacji limfocytów T w kierunku antynowotworowej subpopulacji Th, z jednoczesnym spadkiem ich przekształcenia do immunosupresyjnych Treg [73]. Co więcej, w komórkach dendrytycznych interakcja IND/AhR ogranicza ekspresję IDO1, przywraca DCs możliwość prezentowania antygenów i aktywacji odpowiedzi immunologicznej. Słabo poznane jest natomiast działanie IND wywoływane bezpośrednio wobec komórek nowotworowych. Mimo korzystnych efektów wywieranych przez IND jego stosowanie w monoterapii nie przynosi zadowalających skutków

terapeutycznych w onkologii. Z tego względu poszukiwane są nowe połączenia związków, które zapewnią optymalne rezultaty lecznicze.

Poszukiwania efektywnej terapii skojarzonej koncentrują się na łączeniu związków bezpośrednio celujących w procesy zachodzące w komórkach nowotworowych z cząsteczkami modulującymi szlaki immunologiczne. Zaobserwowano, że połączenie IND z chemioterapeutykami uszkadzającymi DNA lub inhibitorami szlaku PD-1/PD-L1 zwiększa skuteczność leczenia i ogranicza rozwój komórek nowotworowych [74].

Obecnie leczenie nowotworu jelita grubego (CRC) opiera się na stosowaniu środków klasycznej chemioterapii, radioterapii, a także interwencji chirurgicznej. Ze względu na niewystarczającą skuteczność kliniczną, a także działania niepożądane znacząco obniżające jakość życia pacjentów, poszukuje się nowych rozwiązań terapeutycznych. Komórki raka jelita grubego charakteryzują się nadekspresją IDO1, co sprawia, że inhibitory tego białka są związkami o potencjalnym znaczeniu klinicznym [75]. Próby wykorzystania inhibitorów IDO1 w monoterapii raka jelita grubego [76,77], pomimo ich dobrej tolerancji, ciągle nie przynoszą zadawalających efektów [78]. Z tego względu niezbędne jest poszukiwanie takich kombinacji związków, które pozwolą na skuteczną eliminację komórek nowotworowych.

Rozdział 3. Cel pracy

MM-129 jest pochodną 1,2,4-triazyny, która łączy cechy klasycznego cytostatyku, a jednocześnie obniża ekspresję czynnika PD-L1 [1]. Wykazano, że związek ten nasila ekspresję białka p53, obniża ekspresję CDK2, aktywuje kaspazy i hamuje kluczowe szlaki sygnalizacji wewnątrzkomórkowej (mTOR, AKT, BTK) w komórkach raka jelita grubego. Skutkuje to istotnym efektem antyproliferacyjnym i proapoptotycznym [10].

Indoximod (IND) jest inhibitorem 2,3-dioksygenazy indolowej-1 (IDO1) – białka zaangażowanego w metabolizm tryptofanu szlakiem kinureninowym. IND zwiększając lokalne stężenie TRP i obniżając powstawanie jego pochodnych w mikrośrodowisku komórek nowotworowych przywraca aktywność między innymi limfocytom T aktywując przeciwnowotworowe mechanizmy odpornościowe [73,74]. Nieliczne doniesienia sugerują, że IDO1 oraz metabolity TRP wywierają także bezpośredni wpływ na komórki nowotworowe zwiększając ich żywotność [59,63,64]. Jednak mechanizm działania IND niezależny od procesów immunologicznych jest słabo poznany.

Wyniki dotychchczasowych badań sugerują, że skuteczna terapia onkologiczna często jest wynikiem kompleksowego podejścia do procesu nowotworowego. Oprócz klasycznych leków mających bezpośredni punkt uchwytu w zmutowanych komórkach, istotną rolę odgrywają mechanizmy związane z aktywacją układu immunologicznego pacjenta. W dostępnej literaturze wykazano, że IND wywiera efekt synergistyczny w połączeniu z czynnikami genotoksycznymi i inhibitorami immunosupresyjnego szlaku PD-1/PD-L [29,74,79]. Między innymi takie cechy wykazuje MM-129.

W oparciu o powyższe doniesienia, celem pracy była ocena:

- przeciwnowotworowej aktywności łącznego podania MM-129 i IND wobec komórek raka jelita grubego w modelu *Danio rerio*

- żywotności komórek raka jelita grubego DLD-1 oraz HT-29 po ekspozycji na MM-129 oraz indoximod

- wpływu łącznego podania MM-129 i indoximodu na proces apoptozy

- wpływu łącznego podania MM-129 i indoximodu na ekspresję białek zaangażowanych w sygnalizację wewnątrzkomórkową oraz 2,3-dioksygenazy indolowej-1.

Rozdział 4. Realizacja celów naukowych, materiały i metody badawcze, podsumowanie wyników badań i dyskusja

Materiały i metody badawcze

W ramach niniejszej rozprawy przeprowadzono badania z użyciem komórek raka jelita grubego linii DLD-1 oraz HT-29. Komórki pozyskano z Amerykańskiej Kolekcji Hodowli Komórkowych (American Type Culture Collection ATCC, Manassas, VA). Komórki linii DLD-1 hodowane były w podłożu RPMI 1640 (Sigma Aldrich), komórki linii HT-29 w podłożu McCoy's 5a (Sigma, USA). Obie linie komórkowe umieszczono w inkubatorze (Heraeus), zapewniając następujące parametry wzrostu: 5% CO^2 (normoksja), wilgotność względna 95%, temperatura 37^0C.

Badane komórki inkubowano przez 24 godziny z MM-129 w stężeniu 10 µM, indoximodem w stężeniu 200 µM oraz z kombinacją tych związków MM-129 (10 µM) + IND (200 µM). Stężenia związków dobrano na podstawie danych literaturowych [10,80].Grupę kontrolną stanowiły komórki inkubowane w obecności rozpuszczalnika DMSO.

W pierwszym etapie oceniono wpływ związków na proces nowotworowy w modelu zebrafish (*Danio rerio,* danio pręgowany). Ksenografty utworzono według następującego protokołu: komórki linii DLD-1 oraz HT-29 wyznakowano za pomocą CM-Dil ((Thermo Fisher Scientific Inc., Waltham, MA), a następnie ostrzyknięto nimi larwy zebrafish (48hpf). Tak utworzone ksenografty DLD-1 oraz HT-29 inkubowano z testowanymi związkami przez 48 godzin. Następnie komórki nowotworowe zobrazowano przy użyciu systemu EVOS M5000 z filtrem Cy5 (wzbudzenie: 628 nm; emisja: 692 nm). Dodatkowo wyizolowano RNA i analizowano ekspresję ludzkiego oraz rybiego genu GAPDH z użyciem metody qRT-PCR. Test ten przeprowadzono za pomocą instrumentu LightCycler® 480 II (Roche, Bazylea, Szwajcaria) na 96-dołkowych płytkach przy użyciu PowerUp SYBR Green Master Mix (Applied Biosystems). Względna ekspresja mRNA została znormalizowana.

Następnie oceniono właściwości antyproliferacyjne połączenia związków wykorzystując w tym celu embriony zebrafish. Sześciodołkową płytkę wypełniono pożywką E3, a następnie w każdym dołku umieszczono 20 embrionów i poddano je 3-godzinnemu działaniu MM-129 (10 µM), indoximodu (200 µM) oraz kombinacji tych związków MM-129 (10 µM) + IND (200 µM). Eksperymenty prowadzono wobec grupy kontrolnej inkubowanej w obecności DMSO (rozpuszczalnik MM-129), którego stężenie nie przekroczyło 0.1%. W czasie trwania eksperymentu obserwowano wszystkie zarodki, a następnie dokumentowano zachodzące zmiany przy użyciu mikroskopu stereoskopowego V8 (Zeiss, Jena, Germany). Zdjęcia wykonywane były co 15min. Każdy test przeprowadzono w trzech niezależnych powtórzeniach.

W celu potwierdzenia wyników otrzymanych w teście proliferacji z użyciem embrionów zebrafish wykonano klasyczny test MTT oceniający żywotność komórek narażonych na działanie MM-129, IND oraz ich kombinacji MM-129 (10 µM) + IND (200 µM) w warunkach *in vitro.* Zasada testu opiera się na pomiarze aktywności oksydoredukcyjnej

mitochondriów. W żywych komórkach żółty rozpuszczalny w wodzie bromek 3-(4,5-dimetylo-2-tiazolilo)-2,5-difenylo-2H- tetrazoliowy (MTT) uległ redukcji do fioletowego nierozpuszczalnego w wodzie formazanu. Pomiaru absorbancji dokonano przy długości fali równej 570 nm. Żywotność komórek hodowanych w obecności MM-129 (10 µM) i IND (200 µM) obliczono jako procent komórek kontrolnych. Wartości próbek uzyskano z trzech niezależnych eksperymentów przeprowadzonych w dwóch powtórzeniach (n = 6).

W kolejnym etapie przeprowadzono badania, których celem była ocena indukcji i przebiegu apoptozy. Proces ten analizowano przy użyciu komercyjnego zestawu Detection Kit II (BD Pharmingen, San Jose, CA, USA) stosując metodę cytometrii przepływowej (cytometr przepływowy BD FACSCanto II, San Jose, CA, USA). Komórki obu linii, po 24-godzinnej inkubacji ze związkami, poddano barwieniu z wykorzystaniem aneksyny V-FITC i jodku propidyny (PI). Podwójne barwienie pozwoliło na identyfikację żywych, wczesno- i późnoapoptotycznych komórek oraz komórek nekrotycznych. Testy prowadzono wobec trzech grup kontroli pozytywnej: pierwsza zawierała komórki kontrolne i jodek propidyny, druga komórki kontrolne i aneksynę V-FITC, trzecia komórki kontrolne i PI oraz aneksynę V-FITC. Kontrolę negatywną stanowiły komórki hodowane w podłożu niezawierającym związków. Test przeprowadzono w trzech niezależnych powtórzeniach.

Z wykorzystaniem techniki cytometrii przepływowej zbadano również wpływ MM-129 (10 µM), IND (200 µM) oraz kombinacji tych związków MM-129 (10 µM) + IND (200 µM) na zmiany w potencjale transbłonowym mitochondriów (MMP). Posłużono się przy tym komercyjnym zestawem JC-1 MitoScreen kit (BD Biosciences) oraz oprogramowaniem FACSDiva (BD Biosciences Systems, San Jose, CA, USA). Po 24-godzinnej inkubacji komórki z badanymi związkami zawieszono w roztworze lipofilnego barwnika JC-1. W żywych komórkach związek ten uległ agregacji w macierzy mitochondrialnej, w komórkach apoptotycznych i nekrotycznych doszło do jego dyfuzji, co uwidoczniło się jako fluorescencyjne zielone zabarwienie komórek. Test przeprowadzono w trzech niezależnych powtórzeniach.

W celu szczegółowej oceny procesu apoptozy badano aktywację kaspaz: -8, -10 oraz -3/7 przy pomocy odpowiednich zestawów (kaspaza-8: FLICA Caspase-8 Assay Kit, kaspaza-10: FLICA Caspase-10 Assay Kit, kaspaza-3/7: FLICA Caspase -3/7 Assay Kit, Mont-Royal, QC, Kanada). Doświadczenie przeprowadzono przy użyciu cytometru przepływowego BD FACSCanto II, a wyniki analizowano za pomocą oprogramowania FACSDiva (wersja 6.1.3, BD Biosciences Systems, San Jose, Kalifornia, USA). Wartości uzyskano z trzech niezależnych eksperymentów przeprowadzonych w dwóch powtórzeniach (n = 6).

W ostatnim etapie badań przeprowadzono ocenę ekspresji białek (AKT oraz IDO1) z wykorzystaniem metody elektroforezy kapilarnej i immunodetekcji. Po oznaczeniu stężenia białka całkowitego metodą kwasu bicynchonowego (BCA) (Thermo Fisher Scientific, Waltham, MA, USA) próbki doprowadzono do jednakowego stężenia białka (0,4 mg/ml). Następnie dokonano rozdziału elektroforetycznego i immunodetekcji z użyciem następujących pierwszorzędowych przeciwciał monoklonalnych: mysie anty-IDO1 (Sigma-Aldrich, #SAB3701446, 1:100), mysie anty-AKT (Sigma-Aldrich, #05-591, 1:100) i mysia anty-β-aktyna (Sigma Aldrich, #A2228, 1:100). W tym celu posłużono się modułem

separacyjnym Jess 12–230 kDa (ProteinSimple, San Jose, Kalifornia, USA). Wyniki przedstawiono w postaci obrazów wygenerowanych przez kompatybilne oprogramowanie Compass (Compass for SW v5.0.1). Przeprowadzono normalizację białka wobec β-aktyny. Wartości uzyskano z trzech niezależnych eksperymentów przeprowadzonych w dwóch powtórzeniach *(n = 6).*

Do analizy uzyskanych wyników wykorzystano test normalności Shapiro – Wilka. Porównania grupowe przeprowadzono przy użyciu jednokierunkowej analizy wariancji (ANOVA), a istotne różnice między grupami oceniono za pomocą testu Tukey-Kramera. Obliczenia przeprowadzono za pomocą oprogramowania GraphPad 6 Prism (GraphPad Software, Inc., La Jolla, CA). Różnice uznano za istotne statystycznie, gdy $p<0.05$.

Podsumowanie wyników i dyskusja

Wyniki badań stanowiących podstawę niniejszej rozprawy opublikowano na łamach czasopisma **Cancers** – artykuł zatytułowany: *Assessment of an Anticancer Effect of the Simultaneous Administration of MM-129 and Indoximod in the Colorectal Cancer Model*. Wykazano, że jednoczesne podanie MM-129 i indoximodu hamowało rozwój nowotworu jelita grubego w ksenograftach danio pręgowanego. Połączenie tych związków nasiliło proces apoptozy w mechanizmie zależnym od kaspaz, wpłynęło na obniżenie potencjału mitochondrialnego komórek CRC i nasiliło eksternalizację fosfatydyloseryny. Dodatkowo, zastosowana kombinacja związków zaburzyła ekspresję kinazy białkowej B (AKT) oraz 2,3-dioksygenazy indolowej-1 (IDO1).

W pierwszym etapie badań analizowano wpływ łącznego podania MM-129 i IND na rozwój raka jelita grubego w modelu *Danio rerio*. Organizm ten chętnie wykorzystywany jest jako model onkologiczny ze względu na swoje unikatowe cechy takie jak wysoka homologia genów z genami ludzkimi, przezroczyste ciało i łatwość hodowli [81]. Transparentne ciało danio pręgowanego pozwala obserwować zjawiska zachodzące na poziomie pojedynczej komórki, co nie jest możliwe w organizmach pokrytych skórą i sierścią. Dorosłe osobniki zebrafish składają jednorazowo do 300 jaj, co sprawia, że łatwe staje się pozyskanie dużej liczby organizmów [82]. Prowadzenie eksperymentów na licznej próbie jest niezbędne, by zapewnić istotność statystyczną, a tym samym zwiększyć rzetelność otrzymanych wyników. Co szczególnie ważne, danio pręgowany ma rozwinięty układ odpornościowy, dzięki czemu stał się odpowiednim modelem do badań immunologicznych. W organizmie tym zidentyfikowano limfocyty T różnicujące się w dwie populacje: przeciwnowotworową Th1 i pronowotworową Th2 [83,84]. Odzwierciedla to procesy zachodzące u ludzi [85,86]. Ponadto układ immunologiczny zebrafish wyposażony jest w inne komórki odpornościowe takie jak makrofagi, komórki NK i neutrofile [87–89]. Umożliwia to prowadzenie zaawansowanych analiz i poznawanie zawiłych interakcji zachodzących między poszczególnymi elementami układu odpornościowego, co nie jest możliwe w prostych modelach komórkowych. Ponieważ IND jest inhibitorem enzymu ścieżki kinureninowej należy podkreślić, że enzymy metabolizujące TRP zostały zidentyfikowane w organizmie danio pręgowanego, a ich udział w patogenezie różnych zaburzeń był uprzednio analizowany [90–

93]. W przeprowadzonym eksperymencie wykazano, że łączne podanie MM-129 i IND istotnie obniżyło ekspresję ludzkiego genu GAPDH, co świadczyło o spadku liczby komórek nowotworowych. Zastosowana metoda qRT-PCR jest czulsza w porównaniu do obrazowania *in vivo* i z tego względu została wykorzystana w niniejszej analizie [94]. W przedstawionej rozprawie potwierdzono, że połączenie MM-129 i IND, czyli celowanie w procesy zachodzące bezpośrednio w komórkach nowotworowych z jednoczesnym hamowaniem ekspresji enzymu ścieżki kinureninowej, wywołało silniejszą odpowiedź przeciwnowotworową wobec komórek raka jelita grubego. Jest to zgodne z doniesieniami opisującymi wzrost skuteczności leczenia przy jednoczesnym zastosowaniu cytostatyków z inhibitorami KP [95,96].

Wcześniejsze badania pozwoliły stwierdzić, że MM-129 wywiera działanie cytostatyczne wobec komórek CRC [10]. Nieliczne dane sugerują także, że IDO1 bezpośrednio nasila podziały komórkowe [97]. Z tego względu w kolejnym etapie badań oceniono wpływ MM-129 i IND na proces podziału i żywotność komórek raka jelita grubego. W tym celu posłużono się dwiema metodami – testem z użyciem zarodków zebrafish oraz klasycznym testem MTT w hodowlach komórkowych. Mikroskopowa obserwacja podziałów komórkowych zarodków danio pręgowanego jest prostym i skutecznym narzędziem stosowanym do analizy antyproliferacyjnego potencjału związków. Może ona służyć jako metoda uzupełniająca względem standardowych testów żywotności w warunkach *in vitro* [98]. W eksperymencie wykazano, że oba związki zaburzały podziały komórkowe zarodków, przy czym zastosowanie MM-129 hamowało ten proces na wcześniejszym etapie w porównaniu do IND. Przy jednoczesnej inkubacji zarodków w obecności MM-129 i IND zahamowanie proliferacji obserwowano w tym samym punkcie czasowym, jak po ekspozycji na MM-129. W celu weryfikacji tych obserwacji przeprowadzono test żywotności komórek CRC po 24-godzinnej inkubacji z badanymi związkami. W tym etapie również stwierdzono, że oba związki stosowane oddzielnie wywierały działanie cytotoksyczne, ale efekt ten był bardziej widoczny w grupie narażonej na MM-129 w porównaniu do komórek inkubowanych z IND. Hill i wsp. opisali spadek żywotności komórek nowotworowych po zahamowaniu ekspresji IDO1, co jest zgodne z przedstawioną obserwacją wskazującą na antyproliferacyjny potencjał IND [99]. Narażenie komórek raka jelita grubego na MM-129 i IND nie spowodowało nasilenia działania cytotoksycznego w porównaniu do komórek eksponowanych wyłącznie na MM-129. Podobne wyniki opisali Maletzki i wsp., którzy wskazali, że wstępna inkubacja komórek CRC z IND nie zwiększyła ich chemowrażliwości, ani nie wpłynęła na wzrost cytotoksyczności związków powszechnie stosowanych w terapii raka jelita grubego - 5-fluorouracylu (5-FU), gemcytabiny i irynotekanu [100]. Wyniki eksperymentu z wykorzystaniem komórek raka szyjki macicy oraz raka sutka wskazały na odmienny efekt. Autorzy doniesienia opisali nasilenie cytotoksyczności paklitakselu przy jednoczesnym zastosowaniu proleku indoximodu (NLG-919) kompleksowanego cyklodekstryną [101]. Przytoczony wyżej Maletzki i wsp. odnotowali, że poziom ekspresji IDO1 wpłynął odwrotnie proporcjonalnie na wrażliwość komórek na indoximod. Różnice w poziomie ekspresji IDO1 w różnych typach komórek nowotworowych mogą tłumaczyć rozbieżności pomiędzy wynikami opisanymi w niniejszej rozprawie i wnioskami przedstawionymi przez inne zespoły badawcze [100].

Apoptoza jest programowaną śmiercią komórki, a szlaki ją indukujące stanowią ważny element terapii onkologicznej. Kaspazy -8 i -10 jako składowe szlaku zewnętrznego inicjują apoptozę poprzez receptory śmierci. Interakcja ligand/receptor śmierci prowadzi do kumulacji receptorów w błonie komórkowej, co skutkuje rekrutacją białek adaptorowych. Następnie proteiny te prowadzą do aktywacji prokaspazy-8 i prokaspazy-10. W przeciwieństwie do kaspaz -8 i -10, kaspaza 3/7 zaliczana jest do białek efektorowych, co oznacza, że prowadzi do całkowitej śmierci komórkowej. Kaspazy efektorowe są aktywowane przez kaspazy inicjatorowe. Wyniki przedstawione w niniejszej rozprawie pozwoliły stwierdzić, że jednoczesne zastosowanie MM-129 i IND indukowało apoptozę w komórkach raka jelita grubego w mechanizmie zależnym od aktywacji kaspaz: -8, -10 oraz -3/7. Dodatkowo pod wpływem 24–godzinnej inkubacji komórek CRC z kombinacją związków doszło do spadku potencjału błony mitochondrialnej oraz eksternalizacji fosfatydyloseryny. Wskazane zjawiska są uznanymi markerami zachodzącej śmierci komórkowej. Przedstawione w tej rozprawie wyniki są zbieżne z obserwacjami innych zespołów badawczych. Inkubacja komórek raka szyjki macicy z doksorubicyną i IND spowodowała znaczący wzrost aktywności kaspazy-3 i nasilenie apoptozy komórek HeLa [102]. Łączne zastosowanie gemcytabiny z kurkuminą, naturalnym inhibitorem IDO1, spowodowało intensyfikację apoptozy komórek nowotworowych, a Maleki Vareki i wsp. opisali wzrost wrażliwości komórek gruczolakoraka płuc na proapoptotyczne działanie gemcytabiny przy jednoczesnym zahamowaniu ekspresji IDO1 [100,103,104]. Również inne inhibitory IDO1, takie jak epacadostat oraz 1-L-MT, powiązano z aktywacją kaspaz w komórkach raka płaskonabłonkowego jamy ustnej i raka jelita grubego [64,105]. Doniesienia te wskazują na zaangażowanie enzymu KP w procesy życiowe komórek nowotworowych.

Dane literaturowe wskazują, że pochodne TRP nasilają aktywację szlaku sygnałowego zależnego od AKT, to znaczy kinazy silnie zaangażowanej w metabolizm, przeżycie, wzrost i podziały komórek nowotworowych [97,106]. Dało to podstawy by sądzić, że zastosowanie inhibitora IDO1 może zahamować rozwój komórek nowotworowych lub intensyfikować działanie cytostatyków. W poprzednich badaniach stwierdzono, że MM-129 ograniczał proliferację komórek CRC poprzez szlak PI3K/AKT [1]. Analiza wpływu łącznego podania MM-129 i IND na ekspresję AKT wykazała jej spadek po 24-godzinnej ekspozycji komórek na testowane związki. Santhanam i wsp. opisali nasilone podziały komórek CRC w mechanizmie zależnym od AKT i zahamowanie tego procesu po podaniu inhibitora IDO1 [107]. Wskazano również, że podanie związków cytotoksycznych wraz z inhibicją IDO1 wywarło efekt synergistyczny i znacząco obniżyło ekspresję kinazy AKT w komórkach CRC, co popiera wyniki opisane w przedstawionej rozprawie [97].

W ostatnim etapie zbadano bezpośredni wpływ kombinacji obu związków na ekspresję enzymu 2,3-dioksygenazy indolowej-1. Aktywność MM-129 wobec elementów szlaku kinureninowego nie była wcześniej opisana, brakuje również doniesień o działaniu pochodnych 1,2,4-triazyny na elementy tej ścieżki. Otrzymane wyniki pozwoliły jednoznacznie stwierdzić, że tylko IND hamował ekspresję IDO1 w komórkach raka jelita grubego, natomiast MM-129 nie wywierał takiego wpływu. Przeprowadzona analiza dowiodła także proapoptotycznych właściwości obu związków. Wykorzystanie dwóch różnych „punktów uchwytu” działania MM-129 i IND skutkowało nasileniem działania związków. Do uzyskania pełnej odpowiedzi

przeciwnowotworowej wynikającej z hamowania IDO1 niezbędna jest obecność funkcjonalnego układu immunologicznego, więc całkowity efekt terapeutyczny mógł być obserwowany jedynie w złożonym modelu eksperymentalnym. Uzyskane wyniki dały podstawy do planowania kolejnych badań, które będą mogły przyczynić się do opracowania innowacyjnej terapii wykorzystywanej w leczeniu raka jelita grubego.

Rozdział 5. Wnioski

1. Terapia skojarzona pochodną 1,2,4-triazyny (MM-129) oraz inhibitorem 2,3-dioksygenazy indolowej-1 (indoximodem) istotnie hamuje proliferację komórek raka jelita grubego w modelu zebrafish (*Danio rerio*).

2. Jednoczesna ekspozycja na MM-129 i indoximod zmniejsza żywotność komórek raka jelita grubego linii DLD-1 oraz HT-29 na drodze hamowania ekspresji kinazy białkowej B (AKT).

3. Inkubacja komórek raka jelita grubego w obecności MM-129 i indoximodu skutkuje nasileniem apoptozy w mechanizmie zależnym od kaspaz.

4. Aktywność przeciwnowotworowa leków cytostatycznych może być istotnie wyższa przy jednoczesnej inhibicji ścieżki kinureninowej.

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Rozdział 7. Streszczenie w języku polskim

Cząsteczką, która wykazuje cechy klasycznego cytostatyku, a jednocześnie obniża ekspresję czynnika PD-L1 jest pochodna 1,2,4-triazyny: MM-129. Wykazano, że związek ten nasila ekspresję białka p53, obniża ekspresję CDK2, aktywuje kaspazy i hamuje kluczowe szlaki sygnalizacji wewnątrzkomórkowej (mTOR, AKT, BTK) w komórkach raka jelita grubego (CRC). Skutkuje to istotnym działaniem antyproliferacyjnym i proapoptotycznym. Warto zaznaczyć, że pomimo tak szerokiej aktywności farmakologicznej MM-129 jest dobrze tolerowany przez organizm zwierząt doświadczalnych, ponadto posiada stosunkowo niski potencjał toksyczności wobec narządów miąższowych oraz szpiku kostnego.

Ścieżka kinureninowa (KP) jest podstawowym szlakiem metabolizmu tryptofanu (TRP). Stała się ona potencjalnym celem terapeutycznym nowoczesnego, lecz wciąż eksperymentalnego leczenia onkologicznego. Wysoka aktywność 2,3-dioksygenazy indolowej-1 (IDO1) – enzymu inicjującego przemiany TRP szlakiem kinureninowym powoduje różnicowanie limfocytów T do subpopulacji immunosupresyjnej. Nieliczne doniesienia sugerują, że IDO1 wywiera także bezpośredni wpływ na procesy zachodzące w komórkach nowotworowych, zwiększając ich żywotność, ruchliwość i inwazyjność. Wysoka ekspresja IDO1 koreluje z gorszymi prognozami przeżycia pacjentów, przez co inhibitory tego enzymu mogą być obiecującymi związkami wspomagającymi klasyczną terapię przeciwnowotworową.

Jednym z inhibitorów IDO1 jest indoximod (IND), którego działanie farmakologiczne ściśle związane jest ze wzrostem TRP i spadkiem stężenia jego metabolitów (kinurenin) w mikrośrodowisku komórek nowotworowych. Poprzez takie działanie IND przywraca aktywność między innymi limfocytom T nasilając przeciwnowotworowe mechanizmy odpornościowe. Słabo poznane są natomiast efekty IND wywierane niezależnie od odpowiedzi immunologicznej, na procesy zachodzące w komórkach nowotworowych. Wnioski z wcześniejszych badań wskazują, że optymalny efekt wobec komórek nowotworowych uzyskiwany jest przy jednoczesnym stosowaniu IND z czynnikami genotoksycznymi oraz inhibitorami szlaku PD-1/PD-L1. Cechy takie wykazuje MM-129.

Opierając się na powyższych doniesieniach przeprowadzono analizę, której celem była ocena wpływu jednoczesnego podania MM-129 i IND na rozwój komórek raka jelita grubego. Badania przeprowadzono w doświadczalnym modelu danio pręgowanego, a także w warunkach *in vitro* z wykorzystaniem dwóch linii komórkowych CRC: DLD-1 oraz HT-29.

Uzyskane wyniki wskazały, że terapia skojarzona MM-129 oraz IND znacząco hamowała rozwój nowotworu jelita grubego w organizmie danio pręgowanego, indukowała apoptozę w mechanizmie zależnym od kaspaz, obniżyła ekspresję białka AKT i zmniejszyła żywotność w hodowli komórek raka jelita grubego. Przedstawione dane poszerzyły wiedzę o mechanizmach działania obu związków oraz wskazały, że efekt cytostatyczny wobec komórek raka jelita grubego był istotnie nasilony przy jednoczesnym hamowaniu aktywności 2,3-dioksygenazy indolowej. Doniesienia te stanowią podstawę do dalszych badań nad opracowaniem innowacyjnej terapii o potencjalnym zastosowaniu klinicznym.

Streszczenie w języku angielskim

A molecule that exerts the features of a classic cytostatic and may reduce the expression of the PD-L1 protein is MM-129 – a derivative of 1,2,4 triazine. It has been shown that this compound increases the expression of the p53 protein, reduces the expression of CDK2, activates caspases, and inhibits key intracellular signaling pathways (mTOR, AKT, BTK) in colorectal cancer (CRC) cells. This results in significant antiproliferative and proapoptotic effects. It is worth noting that despite such a wide pharmacological activity, MM-129 is well tolerated by the experimental animals, and has a relatively low toxicity potential towards parenchymal organs and bone marrow.

The kynurenine pathway (KP) is the main pathway of tryptophan (TRP) metabolism. It has become a potential therapeutic target of innovative, but still experimental oncological treatment. High activity of indoleamine 2,3-dioxygenase-1 (IDO1) - an enzyme that initiates TRP transformation via this pathway, causes the differentiation of T lymphocytes into an immunosuppressive subpopulation. Few reports suggest that IDO1 has a direct impact on the processes ongoing in cancer cells, increasing their viability, motility, and invasiveness. High IDO1 expression correlates with worse patient survival prognosis, which makes inhibitors of this enzyme promising compounds supporting classical anticancer therapy.

One of the IDO1 inhibitors is indoximod (IND). Its pharmacological effect is related to the increase of TRP and the decrease of its metabolites (kynurenines) in the cancer cells microenvironment. Through this action, IND restores the activity of T lymphocytes, which intensifies the anticancer immune response. On the other hand, the effects of IND exerted directly on processes occurring in cancer cells are poorly understood. Conclusions from previous studies indicate that the optimal anticancer effect of IND is achieved after its combination with genotoxic agents and inhibitors of the PD-1/PD-L1 pathway. MM-129 possesses such features.

Based on those reports, an analysis was performed to assess the impact of simultaneous administration of MM-129 and IND on the development of colorectal cancer cells. The research was carried out in an experimental zebrafish model, as well as *in vitro* using two CRC cell lines: DLD-1 and HT-29.

The obtained results indicated that the combination of MM-129 and IND significantly inhibited the development of colorectal cancer in zebrafish, induced apoptosis in a caspase-dependent mechanism, reduced the expression of AKT protein, and decreased the viability of colorectal cancer cells. The presented data expanded the knowledge about the mechanisms of action of both compounds and indicated that the cytostatic effect toward colorectal cancer cells was significantly enhanced while inhibiting the activity of indoleamine -2,3-dioxygenase-1. These reports constitute the basis for further research on the development of an innovative therapy with potential clinical application.

Review

Not Only Immune Escape—The Confusing Role of the TRP Metabolic Pathway in Carcinogenesis

Iwona Kwiatkowska [1,*], Justyna Magdalena Hermanowicz [1,2], Alicja Przybyszewska-Podstawka [3] and Dariusz Pawlak [1]

[1] Department of Pharmacodynamics, Medical University of Bialystok, Mickiewicza 2C, 15-222 Bialystok, Poland; justyna.hermanowicz@umb.edu.pl (J.M.H.); dariusz.pawlak@umb.edu.pl (D.P.)
[2] Department of Clinical Pharmacy, Medical University of Bialystok, Mickiewicza 2C, 15-222 Bialystok, Poland
[3] Department of Biochemistry and Molecular Biology, Medical University of Lublin, Chodźki 1, 20-093 Lublin, Poland; alicjaprzybyszewska@umlub.pl
* Correspondence: iwona.kwiatkowska@umb.edu.pl; Tel./Fax: +48-857485601

Simple Summary: Recently, mechanisms that lead to immune escape by cancer cells have been under great investigation. Elements involved in the tryptophan metabolism pathway and its derivatives are considered factors that play a wide role in silencing the immune system. However, it seems that those agents contribute to tumorigenesis through a direct impact on cancer cells. This study aimed to gather available data about the kynurenine pathway and its modulating effects on disease development through the impact on immune and cancer cells. This allows for an understanding of the complexity of this metabolic pathway in the context of carcinogenesis and indicates ambiguities that may explain the current failure of therapy with the use of compounds inhibiting tryptophan metabolism. The collected data not only help us to understand the pathogenesis of cancer but also provide the basis for the development of new therapeutic strategies in oncology.

Abstract: Background: The recently discovered phenomenon that cancer cells can avoid immune response has gained scientists' interest. One of the pathways involved in this process is tryptophan (TRP) metabolism through the kynurenine pathway (KP). Individual components involved in TRP conversion seem to contribute to cancerogenesis both through a direct impact on cancer cells and the modulation of immune cell functionality. Due to this fact, this pathway may serve as a target for immunotherapy and attempts are being made to create novel compounds effective in cancer treatment. However, the results obtained from clinical trials are not satisfactory, which raises questions about the exact role of KP elements in tumorigenesis. An increasing number of experiments reveal that TRP metabolites may either be tumor promoters and suppressors and this is why further research in this field is highly needed. The aim of this study is to present KP as a modulator of cancer development through multiple mechanisms and to point to its ambiguity, which may be a reason for failures in treatment based on the inhibition of tryptophan metabolism

Keywords: kynurenine pathway; tryptophan; epithelial–mesenchymal transition; carcinogenesis; circulating tumor cells

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1. Introduction

The intensive development of science in the field of immuno-oncology gives hope for a thorough understanding of the changes taking place in the body during the development of neoplasms. One of the pathways that has been particularly strongly studied in this context is the metabolism of tryptophan (TRP). Efforts are being made to assign a pro- or anti-tumor role to individual elements of this metabolic pathway. However, the complexity and multiplicity of intertwined processes still leave more questions than answers and represent an open field for future research. This publication summarizes the available data on the direct and indirect effects of components of the TRP metabolic pathway on carcinogenesis.

2. Mechanisms Involved in Immune Evasion

For many years, the role of the immune system in cancer development has been under detailed scientific investigation. It is known that immune cells have an ability to recognize developing malignant cells. On the other hand, the enhanced activity of regulatory Tcells (Treg) leads to a decreased immune response and facilitates tumor growth. Moreover, cancer cells secrete factors that favor immunosuppressive microenvironment development, which further enables them to avoid the immune mechanism. This phenomenon is called "immunoediting" and is based on the hypothesis, that the immune system can be both—a tumor suppressor and promotor. It is divided into three phases—elimination, equilibrium, and escape. The first stage is based on observations that cancer cells express specific antigens that are recognized by dendritic cells (DCs) and presented to lymphocytes T, which in turn eliminate pathological cells through cytotoxic mechanisms [1]. In this step, tumor cells can be recognized also by macrophages and natural killers, which altogether leads to Tcell activation [2]. If the immune system eliminates all of the abnormal cells, the whole process of tumor development stops. However, some growing cells seem to be resistant to host immune mechanisms, which leads to the second phase—equilibrium. In this step, dynamic processes remain in balance, but cancer cells gain features that allow them to avoid immune recognition with the following destruction. Factors such as IL-12, IL-23 (interleukin 12, 23) and the elements of adaptive immune response keep tumor cells in a silent state [3]. However, if malignant cells obtain superiority, they progress to the third phase of immunoediting—escape—and become clinically apparent. To reach this state, cancer cells expand multiple mechanisms which facilitate their immunological evasion. One of them is based on a decreased expression of MHC-I (major histocompatibility complex, class I), with the following disturbances in antigen expression or presentation. This leads to inhibition in an antigenicity and moderates a recognition of tumor cells by immune factors. Another way to silence host defense response toward developing malignant cells is the production of molecules, which serve as immune inhibitors. Among them, programmed death-ligand-1 (PD-L1) is one of the most studied and well-known molecules, which already serves as a pharmacological target point in clinical practice. This ligand, after binding to its receptor PD-1 (programmed cell death protein 1) on immune cells, exerts multiple effects on them. Enhanced conversion of CD4+ Tcells into immunosuppressive Treg, decreased cytotoxicity of CD8+ Tcells, reprogramming macrophages into M2 subtypes, which inhibit immunity, are among well-studied effects acquired after PD-L1/PD-1 pathway activation [4,5]. The third and the most complex mechanism triggered by cancer cells for immune evasion is the excretion of prosurvival factors and molecules, which enable the production of an immunosuppressive microenvironment. It can be obtained by the secretion of cytokines with the following recruitment of Treg and MDSCs (myeloid-derived suppressor cells), and changes in amino-acids metabolism. Arginine, glutamine, leucine, and tryptophan are among those which are now under investigation and establishment of their function will help to better understand the biology of cancer [6–8]. Due to the broad role of each of the listed amino-acids, it is not possible to describe them in detail in one manuscript. For this reason, the given paper focuses selectively on one of them—TRP—and its metabolism via the kynurenine pathway (KP) as a factor enhancing tumor development.

3. Tryptophan Metabolism and Its Modulators

Tryptophan, an endogenous amino-acid essential for proper organism development in the course of further metabolic transformations, is converted into indole, with the participation of intestinal microflora, into serotonin (5-HT) under the influence of TRP hydroxylase 2 enzymes, and in the highest level is metabolized through kynurenine pathway [9]. The latter involved two isoforms of indoleamine oxidases (IDO1, IDO2) and tryptophan 2,3-oxidase (TDO2) which are rate-limiting enzymes, that degrade TRP into kynurenine (Kyn). The next steps lead to the transformation of Kyn into kynurenic acid (KYNA), 3-hydroxykynurenine (3-HKYN), antranilic acid (AA) and further production of xanturenic (XA), picolinic (PA) and quinolinic (QUIN) acids. In the final step, active NAD^+

(nicotinamide adenine dinucleotide) arises. Most TRP metabolites are active and exert a multiple and differentiated role in cancer development, which is described in detail below. Here, it should be emphasized that malignant cells can produce individual elements of KP, as well as factors that enhance the activity of this pathway. IDO1 expression is observed in almost all human tissues and its expression upraises with age, IDO2 at the highest level can be observed in the liver, epididymis and brain, and TDO2 except the liver and brain, can be found in the placenta [10]. All of them may be detected in different types of cancers with various severity. Those three enzymes, as rate-limiting factors of TRP metabolism, gained the greatest scientific interest and are now under intense development as a potential therapeutic goal in cancer immunotherapy. Understanding which factors take part in controlling KP elements can shed a light on new therapeutic strategies in oncology. The main molecules involved in IDO-1 activity regulation are proinflammatory agents, i.e., lipopolysaccharides, pathogen-associated molecular patterns, TGF-β (transforming growth factor-beta), and IFN-γ (interferon-gamma) at the forefront. Chronic inflammation is a hallmark of cancer, so the involvement of the aforementioned factors in both processes, i.e., inflammation and expression of TRP catabolizing enzymes, suggests that kynurenine pathway elements play a role in carcinogenesis. An additional argument indicating the involvement of KP in the development of tumorigenesis is an observed correlation between the presence of the known proto-oncogene MYC and overexpression of tryptophan transporters and its increased intracellular transport. Furthermore, the level of KP enzymes is significantly higher in the presence of MYC, when compared to a knock-out cell line [11]. Another oncogene, whose activity influences IDO1 regulation, is the c-KIT proto-oncogene. Balachandran et al. showed that the inhibition of KIT signaling significantly decreases IDO1 activity, confirming the role of this oncogene in KP regulation [12]. Conversely, the high activity of tumor suppressor protein Bin-1 was connected with low IDO1 expression and better prognosis for patients [13], which additionally links the TRP metabolite pathway with tumor development.

An expression of TDO2 is controlled by corticosteroids, and it was shown that cortisol increases gene transcription of this protein [14]. Other hormones, such as estrogen and testosterone, seem to have no impact on TDO expression [15,16]. Still, TRP itself induces TDO2 expression and thus its high dietary intake is a stimulator for this protein. Moreover, in mice models, a high-fat diet was indicated as a factor uprising liver TDO2 level [17]. This may point to a potential role of a balanced diet in cancer prevention. In regards to cancer development and KP regulation, the role of the active form of vitamin B6 (PLP) cannot be overlooked, as its low serum level is correlated with a higher risk of disease occurrence. PLP is a cofactor of KP enzymes, i.e., kynureninase and kynurenine aminotraspherases, which take part in the transformation of Kyn to AA and HK to HAA. Therefore, low B6 supply and its systemic deficiency lead to the accumulation of procancerous metabolites [18]. The gathered information shows how complex and multifactorial the tryptophan metabolism pathway is. Moreover, most of the formed metabolites enhance cancerogenesis, but some of them seem to play a protective role, which makes the whole pathway more challenging to use as a therapeutic target. The further part of this manuscript describes the known aspects of KP elements in cancerogenesis modulation (Figure 1).

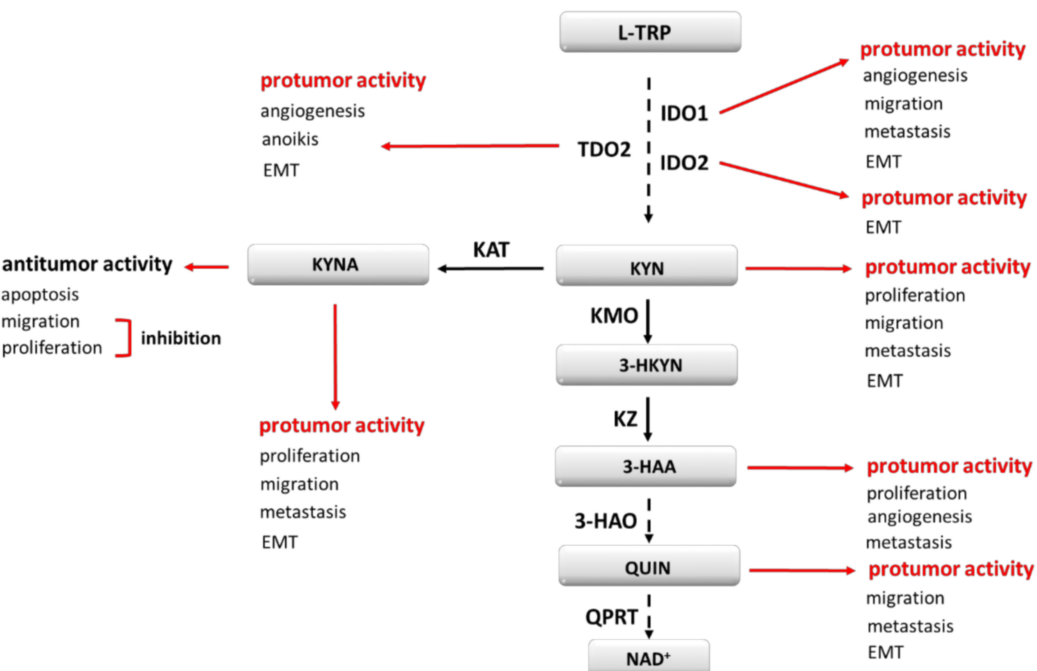

Figure 1. Kynurenine pathway elements as cancerogenesis modulators. EMT—epithelial-to-mesenchymal transition; 3-HAA—3-hydroxyanthranilic acid; 3-HAO—3-hydroxyanthranilate-3,4-dioxygenase; 3-HKYN—3-hydroxykynurenine; IDO1, IDO2—indoleamine oxidase 1, 2; KAT—kynurenine aminotransferase; KMO—kynurenine 3-monooxygenase; KYNA—kynurenine acid; KYN—kynurenine; KZ—kynureninase; L-TRP—tryptophan; NAD^+—nicotinamide adenine dinucleotide; QPRT—quinolinic acid phosphoribosyltransferase; QUIN—quinolinic acid; TDO2–tryptophan 2,3-oxidase.

4. IDO1 and Its Role in Cancer Development

IDO1, the best-studied enzyme of all KP, seems to play one of the most complex roles in cancerogenesis. TRP depletion in local milieu IDO1 affects immunological cells and silences immune response. The activation of GCN-2 kinase (general control nonderepressible 2 kinase) and the inhibition of mTOR (mechanistic target of rapamycin) pathway lead to an immunosuppressive phenotype and facilitated immunosurveillance escape by cancer cells. It has been discovered that IDO1 has a direct impact on malignant cells and enhances tumorigenesis by increasing angiogenesis and metastasis. Moreover, this enzyme conditions metabolite production which contributes to oncogenesis either through AhR (aryl hydrocarbon receptor) activation or in other mechanisms described below. Due to that fact, IDO1, as a pharmacological target, has a high potential in oncology and its inhibition gives an opportunity for increased therapeutic successes. The depletion of TRP affects dendritic cells and two populations of lymphocyte T—immunosuppressive regulatory Tcells (Treg) and cytotoxic lymphocyte T (Tc). TRP deficiency, through the activation of GNC-2, is a signal enhancing the expression of inhibitory receptors (ILT3, ILT4) in DCs [19]. Those cells, as antigen-presenting cells (APCs) to lymphocytes T, as well take part in the differentiation of naïve Tcells in chosen subpopulations. The overexpression of ILTs, especially ILT3 and ILT4 on DCs, induces the differentiation of CD8+ and CD4+ Tcells into Treg [20]. Additionally, in CD4+ Th cells, the presence of ILTs enhances their anergy, thus inhibits the antitumor role. A high expression of ILTs essential for Treg induction [21] altogether favors the immunosuppressive phenotype. In general, those receptors are suspected to be a key factor inducing tolerogenicity [22] thus developing immunological tolerance in host organisms. The role of immunoglobulin-like transcript receptors in cancer development has been reported previously [23]. In few cancer types, such as breast, colorectal, non-small cell lung cancer, and renal cell carcinoma, high expression of those proteins was generally connected with more advanced stages of the disease, more often occurring metastasis, and poor prognosis for patients. The mentioned research points on the acti-

vation of ERK1/2 (extracellular signal-regulated kinases) signaling pathway, enhanced epithelial-to-mesenchymal transition (EMT), and increase in VEGF (vascular endothelial growth factor) level, which altogether contributes to augmented motility, angiogenesis, and invasiveness of cancer cells [24–27]. The gathered information shows that TRP depletion affects immune cells, which has a direct impact on cancer cells, their increased motility, metastasis potential and results in patients' worse prognosis and overall survival. Another structure affected by GCN-2 activation exerted by IDO-1-induced TRP depletion is ζ-chain of T-cell receptor (TCR) in CD8+ Tcells. This structure is a key agent which conditions the occurrence of signaling from TCR [28], with the following activation of CD8+ and their full antitumor immune response [29]. Therefore, its downregulation in case of non-sufficient TRP level interrupts the cascade of events with the following impairment in lymphocyte proliferation and cytokine production [30]. To fully understand how the downregulation of the zeta chain in TCR affects tumor development, it is necessary to describe the role of CD8+ lymphocytes in this process. Those cells are at the highest level responsible for the direct killing of tumor cells through the secretion of cell membrane perforating molecules, i.e., cathepsin C, perforin, granzymes. The second mechanism, induced by CD8+ Tcells, which leads to cell apoptosis, is their expression of the Fas ligand, which, after binding to its receptor on the targeted cell, induces caspases and endonucleases leading to DNA impairment [31]. Previously, it was shown that a low number of CD8+ Tcells correlates with enhanced tumor growth and poor prognosis [32,33]. Detailed research points to a zeta chain as a key factor mediating antitumor response. In the case of oral cancer and Hodgkin's Disease, a low level of the zeta chain in TCR in peripheral Tcells is correlated with a more advanced stage of disease [34,35]. Moreover, enhancing TCR signaling by binding immunoglobulin superfamily member 4 (IGSF4) to the zeta chain significantly decreased tumor size and weight in murine models with implanted melanoma cells and reduces the occurrence of metastatic colonies. [36]. Still, the high activity of IDO1, and thus a decreased TRP level leads to abnormal activity of immunosuppressive Tregs, whose pro-tumorigenic activity is based on crosstalk with other immune cells, as well as on a direct impact on cancer cells. Those cells express CD73 and CD39 endonucleotidases, which take part in adenosine production [37]. The overexpression of the latter was connected to an increased number of occurring liver metastasis in colorectal cancer (CRC) in the murine model and correlates with the worst prognosis and a poor outcome in patients [38]. Adenosine, whose production from ATP and AMP is enhanced in the presence of Treg, affects tumor development by activating PI3K/Akt/mTOR pathway and upregulating metalloproteinases that stimulate invasiveness and migration capacity of malignant cells [39]. Additionally, the whole loop leading to adenosine excretion leads to angiogenesis, caused by an intensified production of VEGF [40]. Besides the direct act on processes promoting oncogenesis, an elevated number of Tregs, and thus IDO1 activity, leads to the development of the immunosuppressive tumor microenvironment. High activity of Tregs limits interleukin 2 (IL-2) production, with the following CD8+ lymphocyte inhibition [41]. Other cells from the immune system, whose activity is at least in part controlled by Tregs are cancer-associated fibroblasts (CAFs), macrophage type 2 (M2) cells, regulatory B cells (Bregs), and myeloid-derived suppressor cells [42]. It should be emphasized here that the latter can secrete IDO1 [43], which loops the course of events even more. All of them are considered to be tumor promoters, their activity being increased under the impact of Treg. The outcomes of the experiments confirm the crucial role of Treg in cancerogenesis. In a few cancer types, i.e., gastric, breast, renal a higher intratumoral Treg level correlates with a worse prognosis [44–46]. Additionally, it was reported that high Treg activity contributes to chemoresistance [47,48]. On the other hand, clinical reports indicate the inhibition of inflammatory response by Treg, which in a further perspective leads to a decreased level of occurring tumor [49]. Taking into account the double role of Treg in cancer development, and IDO1 effect on both subpopulations of lymphocytes—the downregulation of CD8+ cytotoxic Tcells and the upregulation of immunosuppressive Treg it seems reasonable to focus on the ratio between these lines as the most proper prognostic factor in the

context of IDO1 activity [50]. The outcomes from oncological patient samples confirm that a high CD8+/Treg ratio, and hence a low IDO1 activity, is associated with a more favorable prognosis [51–53]. The described effects are the results obtained by the activation of GCN-2 kinase. However, it was mentioned before that TRP depletion affects mTOR kinase, with its inhibition. This results in a decreased number of cytotoxic and helper T cells and an increased number of immunosuppressive Treg in the general population. Besides an indirect impact of IDO1 on cancer cells, it excretes its own direct effect. In bladder cancer cells, IDO1 inhibition leads to a limited colony formation, an increased E-cadherin expression with a concomitantly reduced N-cadherin and vimentin presence. This in total points to IDO1 being a promoter of epithelial-to-mesenchymal transition and a factor that facilitates a gain in motility capacity by cancer cells. Moreover, in the same experiment, the authors show a reduced ability for tubule formation by HVUECs and thus decreased angiogenesis after silencing IDO1 [54]. Those results are in the line with the results obtained by Pan et al., who reported decreased cell invasiveness and migratory ability in lung cancer cells after IDO1 silencing [55]. The same authors discovered that IDO1 presence conditions sufficient vessel density and the progression of vessel mimicry, which in short is unnecessary for the proper angiogenesis process. Another trial focused on lung cancer cells confirms that IDO1 activity is crucial for metastasis occurrence, and its inhibition improves patient outcomes [56]. The described data point to mutual permeating of immunological processes and cancer development, as well as indicate IDO1 as a significant factor that takes part in this mechanism.

5. TDO2, IDO2 and Their Role in Cancer Development

Two other rate-limiting enzymes are less studied than IDO1. Nevertheless, it is known that they are as well involved in maintaining oncogenesis. Their effect can partially be explained by similar to IDO1 activity toward TRP metabolism with its depletion in the local environment and the accumulation of immunosuppressive metabolites. From the pharmacological perspective, these overlapping events provide a reason for cancer cell resistance to IDO1 inhibition, as its role is taken over by the other two enzymes. However, the available data indicate that the expression level of IDO1, IDO2 and TDO2 differs between cancer types and that the occurrence of each enzyme alone can be an independent prognosis factor. This knowledge suggests that, at least in part, TDO2 or IDO2 exert a tumor-promoting role through different mechanisms than those described in IDO1 activity. In metastatic uveal melanoma, TDO2 but not IDO1 was found to be expressed in cancer tissue, in a constitutive manner [57]. In the same cell line, TNF-∝ (tumor necrosis factor-alfa) was pointed as a factor, which upregulates TDO2 expression, but has no impact on the IDO1 level. Additionally, in the case of triple-negative breast cancer, TDO2 seems to play a major role in disease progression, surpassing the importance of IDO1 [58]. Hsu et al. showed that TDO2 is expressed in lung cancer-associated fibroblasts which after knockdown disturb DCs differentiation and response from Th2 [59]. The role of the latter in cancer progression is based mainly on interleukin secretion. Th2 in a tumor microenvironment is a great source of IL-4, IL-5 and IL-13. Both IL-4 and IL-13, when in excess, have been connected with more aggressive cancers, enhanced metastasis, proliferation, and tumor growth. Detailed studies point to multiple mechanisms which are regulated by those interleukins. Well-known tumor-promoting signaling pathways, such as ERK1/2, Akt, mTOR, and STAT6 are among those induced by the mentioned factors. Moreover, it has been proved that IL-4 induces an expression of antiapoptotic proteins, such as Bax, BCL-xl, xFLIP, and contributes to sustained cancer growth through the induction of expression of glucose transporter—GLUT1 [60–65]. The aspect which cannot be omitted in the context of Th2 derived interleukins in cancer development is their role in macrophage polarization. Both IL-4 and IL-13 are involved in the differentiation of macrophages into pro-tumorigenic M2 [66], which are classified as tumor-associated macrophages (TAMs) [67]. A high presence of TAMs in the tumor microenvironment is associated with a poor prognosis in different cancer types as NSCLC, pancreatic carcinoma,

ovarian cancer or gastric cancer [68–71]. Considering their involvement in cancerogenesis, it is worth mentioning, that they play a role at each stage of the ongoing process [72]. By the secretion of inflammation-promoting factors, i.e., TNF, IFN-g, ROS (reactive oxygen species) contribute to the establishment of the mutagenic microenvironment and facilitate the development of damaged cells [73]. In human glioma, ovarian cancer and clear cell renal carcinoma M2 have been reported to activate STAT3 signaling with enhanced proliferation and sustained survival of tumor cells [74,75]. As a source of proangiogenic factors, such as VEGF-A, and VEGF-C M2 contribute to angio- and lymphangiogenesis, respectively [76]. Their presence in the tumor microenvironment is connected with changes in the expression of EMT markers, such as E-cadherin, β-catenin, vimentin, and snail [77], which points to their involvement in aggressive phenotype development. TAMs enable metastasis formation and increase tumor cell invasiveness, via upregulation of metalloproteinases expression, which was reported both in vivo and in patient samples [78–80]. Noteworthy is the fact, that M2 can secrete exosomes, which contain specific miRNA or molecules such as ApoE and integrins, that in the next steps activate migration-inducing signaling pathways [81–83]. Moreover, the presence of M2 in a tumor microenvironment is considered as one of the factors responsible for the occurrence of chemoresistance [84,85] and its targeting could improve the efficacy of treatment [86]. The role of TDO2 in tumor development is not restricted only to the modulation of immune cell activity. It has been shown, that this enzyme is involved in enhancing the survival of circulating tumor cells (CTCs), through the participation in the development of resistance to anoikis (a form of programmed cell death) [87]. Due to AhR involvement in this process, the whole pathway is described below.

The last known rate-limiting enzyme involved in TRP metabolism is IDO2. Its role in tumorigenesis is significantly less examined than IDO1, thus a lot of questions about its involvement in cancerogenesis remain. Nevertheless, an important contribution of both IDO1 and IDO2 in cancer development, not mentioned before, is their involvement in NAD^+ production. This dinucleotide as a final product of TRP metabolism is used as a source of energy for maintaining cells' functions and viability. In a tumor microenvironment, an increased level of NAD^+ has been shown to exert an immunosuppressive effect by the inhibition of T cell survival, proliferation and cytotoxic activity [88]. Additionally, its further metabolism takes part in enhancing immune evasion by promoting PD-L1 expression in tumor cells [89]. Its upraised level is connected with a poor prognosis and decreased overall survival in the number of solid tumors, i.e., non–small cell lung cancer, renal cell cancer, ovarian cancer, gastric cancers [90]. This ligand works through the PD-1 receptor expressed on different cell types, including activated Tcells. After interaction, the signaling from TCR attenuates [91], which is described above in the context of maintained tumor development. Additionally, recent reports suggest that PD-L1 has a direct impact on cancer cells, therefore it intensifies tumorigenesis not only via the modulation of the immune response. The correlation between an elevated level of PD-L1 and enhanced EMT was reported in a few cancer types, i.e., head and neck squamous cell carcinoma, lung adenocarcinoma, gastric cancer [92–94]. Wang et al. showed that in renal cancer cells PD-L1 overexpression induces the expression of SREBP-1c, a factor involved in cell lipogenesis. The authors connected this event with EMT induction and intensified cancer cell migration [95]. Other immune-independent effects caused by PD-L1 include the activation of mTORC1 and Ras/ERK and signaling cascades, with the following sustained growth of melanoma and ovarian cancer cells and enhanced EMT in glioblastoma multiforme, respectively [96,97]. Moreover, Mandarano et al. showed a correlation between upregulated IDO2 and PD-L1 levels, which indicates the existence of a link between IDO2 activity and enhanced cancerogenesis in a PD-L1-dependent manner [98]. Besides affecting PD-L1 expression, NAD^+ contributes to stem cell proliferation and pluripotency [99]. However, it still needs to be confirmed if this nucleotide plays a role in maintaining the viability of cancer stem cells. There still exists a high need to define other mechanisms of IDO2 via which it contributes to cancerogenesis. However, reports showing an upraised level of this enzyme in human

cancer tissues suggest that it is an important factor, which cancer cells use to maintain their survival in a host organism.

6. AhR Agonists—Carcinogenesis Modulators

The abovementioned effects are not only the ones that allow us to categorize the described enzymes as tumor modulators. Their wide role is also based on the production of metabolites that are involved in oncogenesis. Kynurenine, 3-hydroxykynurenine and kynurenic acid are characterized by a common feature—aryl hydrocarbon receptor activation. AhR is a transcriptional factor, which after interacting with a ligand translocates from the cytoplasm to the nucleus and modulates there the expression of targeted genes. Its final effects are ligand-specific, but due to the topic of this manuscript, here, only the effects caused by TRP metabolites are described (Figure 2).

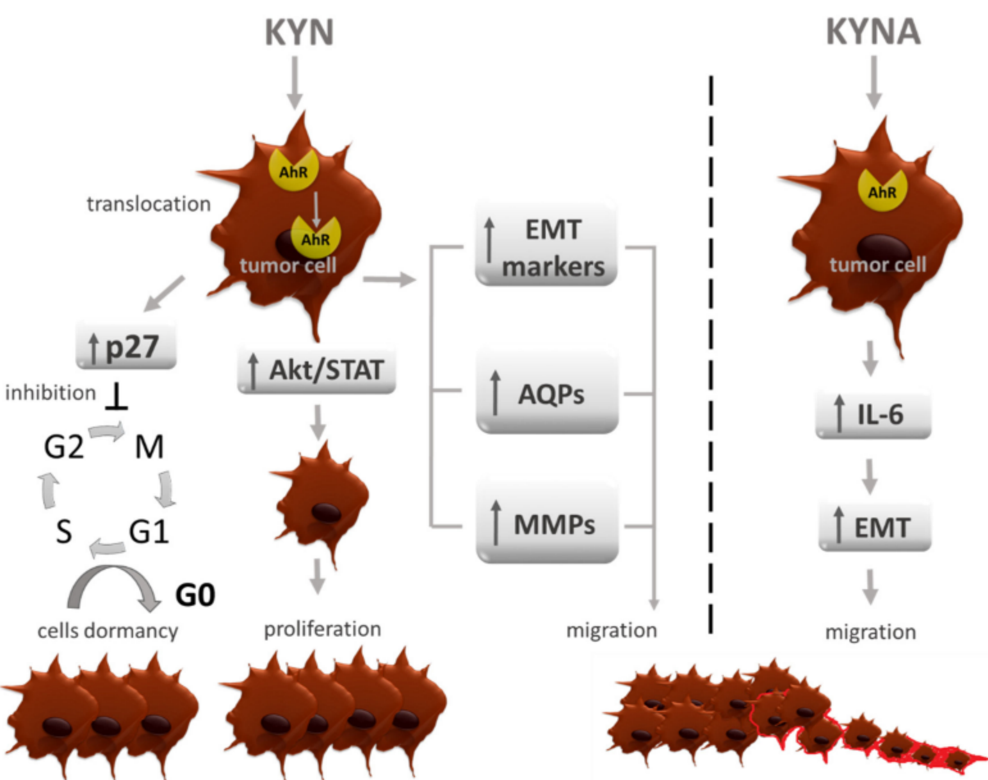

Figure 2. AhR—dependent pro-tumorigenic activity of TRP metabolites. AhR—aryl hydrocarbon receptor; Akt—protein kinase B; AQP4—aquaporin 4; EMT—epithelial-to-mesenchymal transition; IL-6—interleukin 6; KYN—kynurenine; KYNA—kynurenic acid; p27-tumor suppressor; MMPs—metalloproteinases; STAT3—signal transducer and activator of transcription 3.

It was suspected that AhR is a pro-tumor agent, although evidence suggests that, depending on cancer type it may have an opposite, tumor-suppressive role. Here it needs to be emphasized that, like enzymes, AhR activation can have a direct effect on cancer cells or contributes to immunosuppressive microenvironment development. Kynurenine was shown to induce Akt and STAT3 signaling pathways in renal cancer cells, and increase their proliferation, migration and drug resistance [100]. Enhanced migration after Kyn/AhR interaction was observed in HCC (hepatocellular carcinoma) and the authors linked it with changes in the level of EMT markers, as well as the overexpression of MMP9 [101]. Another reported mechanism, which contributes to migration, is the expression of aquaporin 4 (AQP4) observed in glioma cells after Kyn/AhR interaction [102]. Due to the regulation of cell volume, the contribution to filopodia formation and changes in shape, AQP4 enhances cell motility and metastasis [103,104]. Additionally, the high activity of this

water channel was correlated with increased VEGF expression and related angiogenesis, as well as upraised metalloproteinase-2 activity [105,106]. Moreover, circulating cells that are detached from the extracellular matrix are highly vulnerable to a specific cell death type called anoikis. D'Amato et al. showed that TDO2/Kyn/AhR axis contributes to gaining a resistance to anoikis by cancer cells and by these means promotes migrating cells survival and facilitates metastasis formation (Figure 3) [87].

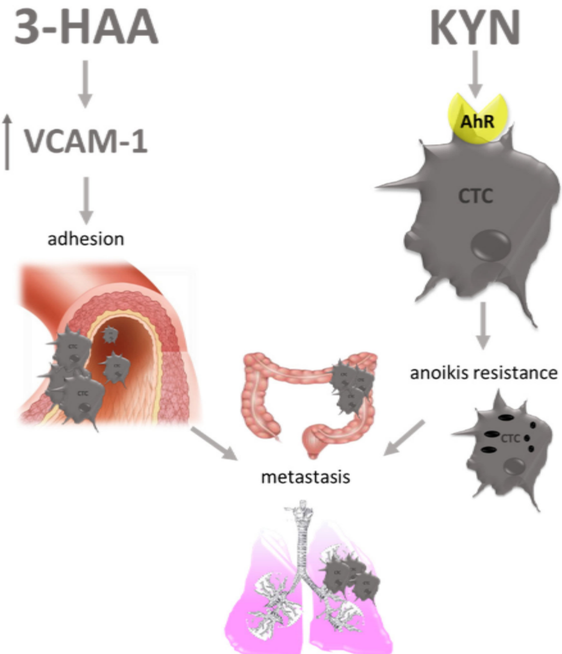

Figure 3. Effects of TRP metabolites on circulating tumor cells (CTCs). 3-HAA—3-hydroxyanthranilic acid; AhR—aryl hydrocarbon receptor; CTC—circulating tumor cell; KYN—kynurenine; VCAM-1—vascular cell adhesion molecule-1.

Kyn contributes to the development of resistance to classical apoptosis as well and that was reported in breast cancer cells [107]. Moreover, this metabolite in an AhR-depended mechanism causes cycle cell arrest in G0/G1 phase via the overexpression of cell-cycle inhibitor p27 in tumor repopulating cells (TRCs) [108]. This leads to cell dormancy—a state in which cells poorly proliferate and are highly resistant to pharmacological intervention (Figure 1). Shi et al. showed that blocking IDO1/Kyn/AhR pathway restores a cell cycle and induced apoptosis of TRCs [109], thus may be a proper strategy in disease relapse prevention. Additionally, TRCs release Kyn which then upregulates PD-1 expression on CD8+ Tcells by AhR activation (Figure 4) [110]. Li et al. showed that Kyn activates AhR expressed on DCs, and this interaction results in a switch from an immunogenic to tolerogenic subtype [111]. Tolerogenic DCs contribute to inducing Tcell differentiation into immunosuppressive Treg. Mezrch et al. confirmed that the Kyn/AhR axis is involved in Treg production by interaction with DCs [112].

Another metabolite—kynurenic acid—is also involved in increased cancer development, by immunomodulation. DiNatale at al. reported that KYNA treated MCF-7 cells overexpress IL-6, and this effect largely depends on AhR activation [113]. It was previously shown, that IL-6 expressing MCF-7 are characterized by the EMT phenotype and an increased metastatic potential [114–116], so in linking these two reports it is proper to consider KYNA/AhR interaction as a factor enhancing tumor invasiveness. The gathered data link AhR activation with ongoing cancerogenesis, concomitant inflammatory response and an immunosuppressive microenvironment. Nonetheless, in neuroblastoma Kyn via AhR activation lead to KISS1 gene overexpression and in this mechanism it was shown to decrease tumor metastasis and improve patient overall survival [117]. This is in the

line with a few other reports, which shows that AhR acts like a tumor suppressor in liver cancer, colorectal cancer, melanoma, and prostate cancer [118–121]. Although, this effect was obtained in a KP-independent mechanism, further research is still needed to reveal if any of TRP metabolites have a tumor suppressive role through AhR activation.

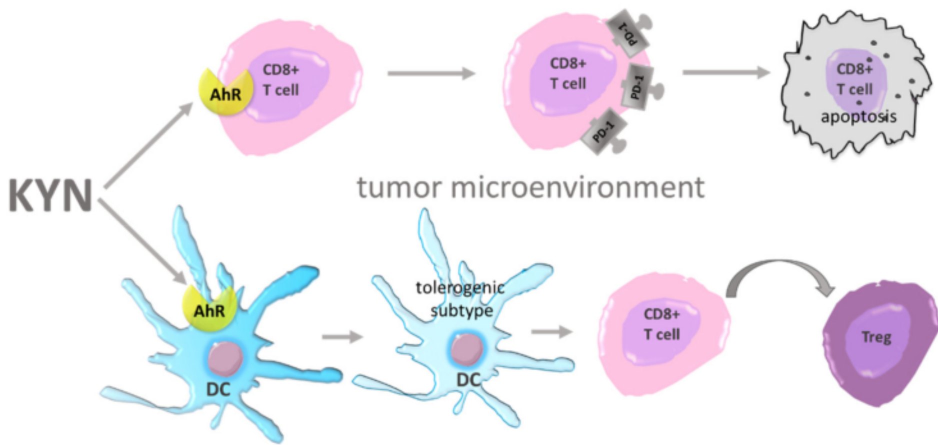

Figure 4. Immune evasion—impact of KYN on the tumor microenvironment. AhR—aryl hydrocarbon receptor; CD8+ T cell—lymphocyte T CD8+; DC—dendritic cell; KYN—kynurenine; PD-1—programmed death receptor 1; Treg—regulatory Tcells.

7. Other Carcinogenesis Modulating Effects of TRP Metabolites

Besides the fact that the effects exerted in mechanisms depend on AhR activation, individual metabolites are also involved in cancerogenesis in different ways (Figure 4). Experimental evidence points to a correlation between an upraised level of circulating TRP derivatives and a higher risk of cancer overall or a more advanced grade of the disease [122,123]. However, their exact role has not been well studied yet the contribution of TRP metabolites to cancerogenesis is burdened with high ambiguities and controversies. They are connected with an undiscovered character of some metabolites, which seems to be pro and anti-cancerous at the same time. Due to this fact, whole KP remains a great subject for further studies and the establishment of its role will help to understand the pathogenesis of cancer. TRP derivatives are considered immunomodulators, but some of them seem to directly affect cancer cells. This paragraph gathers known data about TRP metabolites, tries to explain their complex role in cancerogenesis, and illustrates gaps and needs which should be included in further examinations. 3-hydroxykynurenine and 3-hydroxyanthranilic acid are considered DNA damaging carcinogens. This effect is exerted by the production of H_2O_2 affected by DNA single-strand breaks [124]. This leads to genome instability, which is a well-known feature of cancer cells. Other metabolites, i.e., kynurenine, quinolinic acid and cinnarbinic acid—a direct metabolite of 3HAA—are as well considered as ROS generators [125], which further impair different structures leading to enhanced tumor development. Song et al. showed that Kyn-derived ROS induces caspase release in NK (natural killers) cells and leads to their apoptosis [126]. NK cells are elements of an innate immune response, which are responsible for maintaining an anti-tumor response. Through direct cytotoxic activity achieved by the secretion of perforines and grazymes and/or by the induction of Fas-depended apoptosis, they eliminate malignant cells from the host organism [127]. Their depletion in the tumor microenvironment is one of the mechanisms which cancer cells use to avoid immunosurveillance. In a murine model of NSCLC, NK depletion was correlated with spontaneous lung metastasis achieved without affecting primary tumor growth [128]. A similar conclusion comes from tests conducted on colorectal mouse xenografts treated with tofacitinib-NK-reducing compound. In those animals, increased lung metastasis after the treatment was observed [129]. Additionally, Aydin et al. showed that the pro-metastatic effect exerted by a reduced number of NK is achieved in a ROS-depended mechanism [130], which lends a weight of argument that ROS-

generating TRP metabolites enhance metastasis. Another effect leading to oxidative stress induction is mitochondrial dysfunction. In this context, QUIN was pointed as a harmful compound [131]. Mitochondrial failure has been connected with high aggressiveness, ongoing EMT and increased metastatic potential and chemoresistance of cancer cells [132]. Due to this, QUIN may be suspected to modulate these processes, but similarly to the abovementioned derivatives, its role is yet to be discovered. An elevated level of ROS is connected with all stages of tumor development, but detailed information about the contribution of TRP metabolites in specific processes has not been established as yet and this leads to a necessity to develop research in this direction.

Another ROS-independent tumor-promoting effect was reported by Bishnupuri and colleagues [133]. They show, that in colon cancer cells Kyn and QUIN activate PI3K/AKT/GSK3β which results in β-catenin translocation from the cytoplasm to the nucleus. In lung cancer cells kynurenine through Akt phosphorylation activates two other pathways—cAMP response element-binding protein (CREB) and with-no-lysine (K) protein kinases (WNK)—which were connected with the enhanced spheroid formation and migration, respectively [134]. Phosphorylation of a CREB signaling was previously connected with an enhanced expression of metalloproteinases 2 and 9, filopodia formation and an increased level of EMT markers [59,135]. On the other hand, the WNK kinases family affects downstream factors, including TGF-β or NF-kB (nuclear factor kappa B), known as tumor promotors [136]. Kynurenine acid) has been reported as an G protein-coupled receptor 35 (GPR35) agonist (Figure 5) [137,138].

This receptor was found to be overexpressed in breast cancer, lymph nodes of colon cancer patients, which is connected with higher tumor aggressiveness and poor prognosis [139,140]. Another evidence of GPR35 involvement in migration comes from the trial lead on intestinal epithelial cells intentionally damaged by chemotherapy. Wang et al. showed decreased cell migration after GPR35 inhibition [141]. GPR35 is also expressed on macrophages, and activated under KYNA treatment [142]. Pagano et al. reported that the activation of GPR35 in those cells is connected with enhanced neoangiogenesis, tumor tissue remodeling and enhanced tumor growth in murine colon cancer xenograft [143]. This outcome suggests that KYNA by affecting macrophages may have an impact on colon cancer cells. Its contribution to other tumor types still needs to be confirmed. The function of macrophages is also impaired by 3-HAA (Figure 5). This TRP metabolite inhibits PI3k/AKT and mTOR signaling pathways, which leads to inhibition of NF-kB activation and attenuates nitric oxide (NO) production. Moreover, it also enhances the differentiation of macrophages into the M2 phenotype [144], whose role in cancerogenesis is described above. Oh et al. also showed decreased NO production by 3-HAA stimulated macrophages. Detailed research on possible mechanisms revealed that 3-HAA enhances HO-1 expression, with the following inducible nitric oxide synthase (iNOS) inhibition and a decrease in NO production. Although the role of M2 in carcinogenesis is clear, the level of NO and macrophage iNOS activity is not so clear-cut and the role of 3-HAA should be further explored. Besides macrophages, 3-HAA as a strong immunomodulator affects other immune cells (Figure 6). In dendritic cells, it attenuates the production of IL-6, IL-12 and TNF-∝ and inhibits their maturation and activation by interrupting p-JNK and p-p38 signals. The authors also pointed to a decreased ability of DCs for stimulation of Tcell activation, proliferation and differentiation under the influence of 3-HAA (Figure 5) [145]. Moreover, 3-HAA directly affects Tcells. Piscianz et al. proved that 3-HAA induces cell death in activated Tcell subpopulation [146], which is a well-known mechanism used by cancer cells in immune escape [147]. Lee et al. showed selective Th1 but not Th2 apoptosis induced by 3-HAA and QUIN. This discovered mechanism depends on cytochrome c release and caspase activation as independent from Fas/Fas ligand interaction [148]. Moreover, 3-HAA alone eliminates already activated Tcells, by decreasing intracellular glutathione levels [149]. Altogether, 3-HAA leads to an increase of Th2 subpopulation, whose procancerous activity is described above. As mentioned at the beginning 3-HKYN and 3-HAA also induce strong apoptosis of CD4+ Tcells with a simultaneous increase of im-

munosuppressive CD4+ FoxP3 Tcell subpopulation [150]. These observations additionally enhance the pro-tumorigenic role of TRP metabolites.

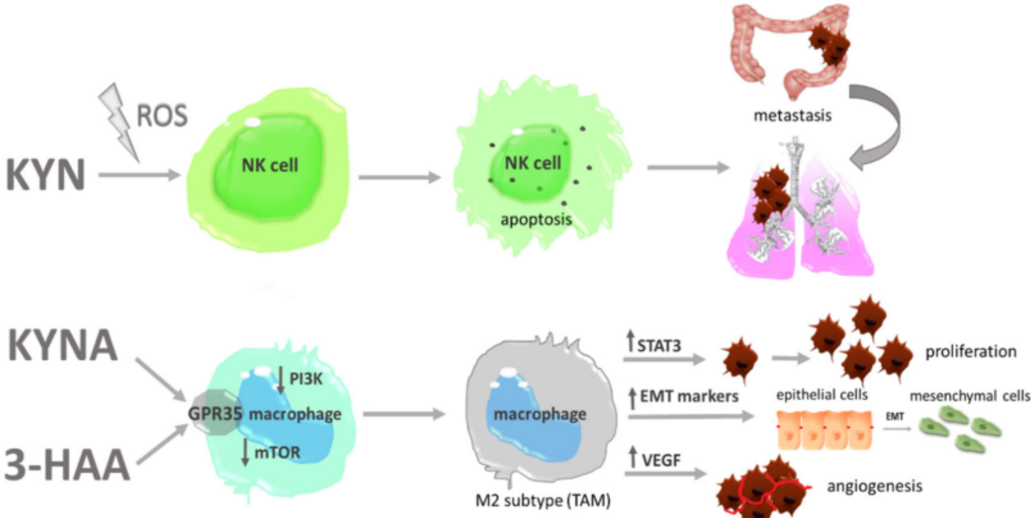

Figure 5. AhR—independent pro-tumorigenic activity of TRP metabolites. 3-HAA—3-hydroxyanthranilic acid; EMT—epithelial-to-mesenchymal transition; GPR35—G protein-coupled receptor 35; KYN—kynurenine; KYNA—kynurenic acid; mTOR—mechanistic target of rapamycin; NK cell—natural killer; PI3K—phosphatidylinositol 3 kinase; ROS—reactive oxygen species; STAT3—signal transducer and activator of transcription 3; TAMs—tumor associated macrophages; VEGF—vascular endothelial growth factor.

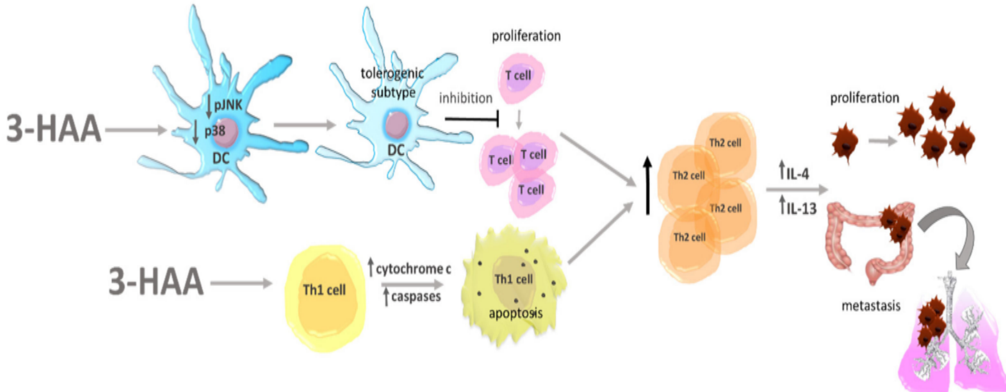

Figure 6. Impact of 3-HAA on the tumor microenvironment. 3-HAA—3-hydroxyanthranilic acid; DCs—dendritic cells; IL-4, 13—interleukins 4, 13; p38—phosphorylates specific transcription factor; pJKN—c-Jun N-terminal kinase; T cell—lymphocyte TTh—helpers lymphocytes T.

Nonetheless, as mentioned before, KP contribution to cancerogenesis is not clear. Walczak et al. gathered information about KYNA level in different cancers, and showed that depending on the type of disease, the level of this metabolite is decreased or upraised, but the mechanism of this phenomenon is not discovered [151]. However, this suggests that in some conditions, KYNA may serve as a tumor suppressor (Figure 7).

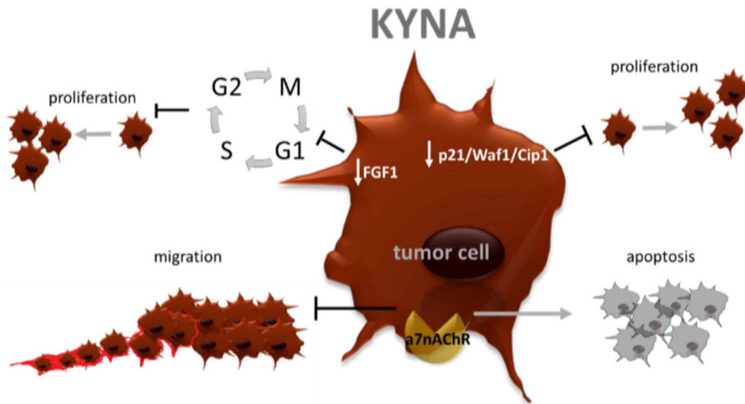

Figure 7. Antitumor effects of KYNA. a7nAChR—alpha-7 nicotinic receptor; FGF-1- fibroblast growth factor-1; KYNA—kynurenic acid; p21 Waf1/Cip1—cyclin-dependent kinase inhibitor 1.

In HT-29 colon cancer cells, this derivative was shown to inhibit p21 Waf1/Cip1 with following DNA synthesis inhibition and decreased proliferation [152]. The same mechanism was reported for renal cancer cells [153]. Moreover, the authors point to decreased migration of malignant cells after KYNA stimulation, which stays in a line with another experiment conducted on glioma cells. In these brain tumor cells described metabolite was pointed as a factor antagonizing glutamate activity, which resulted in DNA synthesis inhibition and reduced migration [154]. KYNA is also considered as an alpha-7 nicotinic acetylcholine receptor (a7nAChR) inhibitor [155]. This interaction mainly matters in neuronal disorder, nevertheless, recent reports emphasized the role of nicotinic acetylcholine receptors in oncogenesis. The blockage of a7nAChR reversed acetylcholine-induced cell migration and invasiveness in NSCLC cells and gastric cancer cells via blocking signaling by MEK/ERK pathway [156,157]. Moreover, in gastric cancer, cholangiocarcinoma, this receptor inhibition was connected with reduced expression of EMT markers [158]. Scientific reports also indicated increased apoptosis of breast cancer and cholangiocarcinoma cells after a7nAChR silencing [159]. KYNA is also considered a tumor suppressor due to the inhibition of fibroblast growth factor-1 (FGF-1) release [160], which is involved in MMPs activation, angiogenesis, tumor progression, promotion of cancer cells stemness and is associated with worst patients' prognosis [161–166]. The blockage of the FGF-1 signaling pathway causes a cell cycle arrest in phase G0/G1, thus decreased cancer cell proliferation. It also reduces tumor growth and occurring metastasis [167,168]. Another metabolite described before as a tumor promotor 3-HAA also seems to be a double-edged compound. In the human umbilical vein endothelial cells (HUVECs), which serve as a model for angiogenesis evaluation 3-HAA inhibits monocyte chemoattractant protein-1 (MCP-1) secretion and vascular cell adhesion molecule-1 (VCAM-1) expression [169]. These molecules belong to factors enhancing angiogenesis, cancer cell survival, invasiveness and metastasis in different tumor types [170–173]. VCAM-1 also facilitates circulating tumor cell adhesion to the vascular endothelium, and thus burrowing circulating cells in new organs [174]. Targeting these proteins and decreasing their level may help to limit metastasis, cancer progression and improve patient outcomes, thus 3-HAA by this mechanism may work as a tumor suppressor. Another TRP metabolite, with potential antitumor activity, is picolinic acid In the murine model Ehrlich ascites carcinoma treatment with PA significantly reduced tumor growth and improved longevity [175]. A similar result was obtained by Ruffman et al. who observed a therapeutic effect of PA in mice bearing MBL-2 lymphoma cells [176]. Apart from these old experiments, new ones are not available, and mechanisms through which PA exerts its antitumor effect are not well known. Gathered together this information illustrate that KP remains a puzzle for scientists focusing on cancer pathogenesis. However, the establishment of its exact role may help to develop an effective therapy for patients in the future.

8. Compounds Targeting Kynurenine Pathway in Clinical Trials

Nowadays, even without full knowledge about TRP metabolites in tumorigenesis, efforts are taken to develop compounds, which by KP modulation will serve as anticancer drugs. Due to the multistep process, the targeting of different parts of this pathway is being considered. Unfortunately, promising preclinical results more than once disappointed at the clinical stage. It may be connected with overlapping events and a huge number of factors contributing to TRP metabolism, and their ambiguous character. Due to that further studies need to be taken to reveal which pathways should be silenced, and which enhanced to ensure an effective therapy based on KP modulators. Perhaps a combination therapy with compounds targeting other metabolic pathways will be needed, and indeed recent clinical trials in huge majority examine a combination therapy. This paragraph summarizes, in short, KP modulators, which are under development as an anticancer therapy. One of the approaches focuses on IDO1, IDO2 and TDO2 inhibition, due to their pro-cancerogenous activity. Inhibitors are divided by their affinity to each enzyme, and thus from available drugs selective IDO1 or TDO2 inhibitors or a non-selective IDO1/TDO2 inhibitor can be distinguished. Epacadostat—a selective IDO1 inhibitor—is examined at the highest level [177,178]. Since monotherapy with epacadostat was not more favorable when compared to tamoxifen in ovarian cancer [179], the connection with other compounds has been tested. Phase III of the trial checking the efficacy of epacadostat with pembrolizumab versus pembrolizumab alone in melanoma showed no predominance in the drug combination [180]. A similar conclusion was reported by Kelly et al. who checked a combination of epacadostat and pembrolizumab in patients with advanced sarcoma [181]. A clinical trial evaluating the combination with another monoclonal antibody, atezolizumab, toward NSCLC also did not show satisfying results [182]. Those surprisingly disappointing results impeded research on IDO1 selective inhibitors, nevertheless, some novel compounds are tested in new combinations and indications. The effectiveness of one of them, BMS-986205 combined with biological compounds in endometrial cancer or endometrial carcinosarcoma, liver cancer, bladder cancer, melanoma, NSCLC, oral cavity squamous cell carcinoma, glioblastoma, kidney cancer and gastric cancer will be tested in clinical trials, which are now in the recruiting phase [183–190]. The trial comparing BMS-986205 with nivolumab to nivolumab alone efficacy is terminated, but the results are not available yet [191]. KHK2455 and LY3381916 are both in early development. The application of the first one in bladder cancer in combination with anti-PD-L1 therapy will be checked in a trial that is now in the phase of recruiting patients [192]. The second compound, also with anti-PD-L1 coadministration in different types of solid tumors was tasted in a terminated trial, the results of which are not available yet [193]. Due to the newly recognized role of TDO2 in cancer progression, molecules inhibiting its activity are also under development. There are not a lot of data available in this field, however, one selective TDO2 inhibitor—680C9—is in a preclinical test and it sensitizes glioblastoma cells on genotoxic treatment when combination therapy is applied [194]. Other selective TDO2 inhibitors are in early development and further investigation is highly needed [195,196]. The lack of the expected efficacy of selective inhibitors forced to raise a question about the cause of this effect. One hypothesis is based on the possibility of taking over the role of the inhibited enzyme by another, still an active one. Due to that fact, the compounds with inhibitor activity towards two enzymes are under enhanced development and include indoximod (1-MT), its prodrug NLG-802 and navoximod [197–199]. The clinical significance of 1-MT in different solid tumors and in acute myeloid leukemia was evaluated alone or in combination therapy [200–202]. Attempts have been made to co-administrate this compound with classical chemotherapy, such as nab-paclitaxel or gemcitabin for pancreatic cancer, temozolomid for brain cancer taxanes in breast cancer or docetaxel in solid tumors [203–206]. However, despite good tolerance, there is a lack of predominance of combination therapy [205]. Still, indoximod was reported to increase the effectiveness of DNA damaging chemotherapy, without changes in its toxicity [202]. Additionally, it was also showed that 1-MT improves the efficacy of radiotherapy treatment [207]. NLG-802 was evaluated only in Phase I for

solid tumors, however, the results of this trial are not available. Navoximod was tested in Phase I of the clinical trial combined with ateolizumab. The trial was conducted on patients with solid tumors generally. As before, a good toxicity profile was illustrated, however no benefits from the combination therapy were shown [208]. In turn, the outcomes from Phase Ib indicated that the monotherapy of recurrent advanced solid tumors resulted in stable disease response [209]. Other IDO/TDO2 inhibitors, such as HTI-1090, DN1406131 are in early development, and no final results are available yet [210,211]. Compounds such as RG70099, EPL-1410, CB548, CMG017 are in preclinical tests and show potential antitumor activity [212–214]. MK-7162 another compound targeting TRP catabolizing enzymes has no discovered mechanism, nevertheless, it is included in clinical trials for adult patients with solid tumors in a combination with pembrolizumab [215]. As of now, the results from this phase are not known. Another approach targeting KP enzymes is based on the occurrence of specific cytotoxic Tcells directed toward IDO1, IDO2, TDO2. Detailed studies lead to a discovery, that this Tcell subpopulation has a direct cytotoxic activity toward tumor cells and immunosuppressive DCs, Th2 [216–218]. For this reason, enzyme-derived epitopes are used as a vaccine to enhance response from effector Tcells. This therapeutic option is already in a clinical trial in NSCLC and melanoma in combination with temozolomide, ipilimumab, epacadostat [216,219–221]. The available results point to good safety profile, however a bigger group of patients is needed to confirm its efficacy. The next structure responsible for pro-tumoral effect of TRP metabolites is AhR and its inhibition may serve as a target in cancer treatment. In a preclinical test on mice xenografts with oral, breast and skin cancer HP163 reduces both tumor growth and a number of immunosuppressive cells [222]. Another small molecule AhR inhibitor—BAY218, used in the murine model—reduced tumor growth and enhanced immune response by increasing the infiltration of CD8+ T and NK cells with simultaneous reduction with regard to suppressive GR1+ myeloid cells and M2 macrophages [223]. A significant activity toward M2 macrophages under AhR inhibitor treatment was also observed by Garcia et al. [224]. They show in in vitro study that IDE-AhRi-1 administration leads to the full arrest of M2 suppressive effect and the enhancement of Tcell activity. The first clinical trial with the use of AhR inhibitor is in the phase of recruiting patients, hence any clinical significance of this strategy will be known in the future [225].

As observed, the whole KP is a very potent therapeutic target, however high hopes connected with IDO1 or TDO2 inhibition up to date turn out to be promising in preclinical tests and not highly effective in clinical practice. It is possible that the described complexity of KP and the dual role of metabolites in cancerogenesis is a reason for this failure. It shows how important it is to focus on further and detailed research on each TRP metabolite to find a golden mean. Positive results of the preclinical test (although disappointing in further steps) suggest that targeting KP is a potent strategy for the establishment of alternative treatment and improvement of needs. Moreover, in clinical practice, KP has one more application. Besides serving as a target for therapy, measurement of the level of the circulating metabolites or Kyn/TRP ratio is used as a biomarker of occurring cancer disease. Determination of these quantities in the blood or urine derived from patient samples has been previously used to define the advancement of the disease and helped to predict patient prognosis in the case of few cancer types, i.e., renal cell carcinoma, bladder cancer, breast cancer, colon cancer, prostate cancer or gastroesophageal cancer [226–231]. This additionally emphasizes the usefulness of the practical application of the kynurenine pathway in oncological diagnosis and treatment.

9. Other Aspects of TRP Metabolism

As mentioned at the beginning of this manuscript, KP is not the only pathway through which TRP is metabolized. The importance of the serotonin pathway in the context of enhancing oncogenesis has also increased significantly in recent years. The available data indicate an increased growth of cancer cells under the influence of serotonin in prostate cancer, breast cancer, small-cell lung cancer, colorectal cancer, cholangiocarcinoma,

hepatocellular carcinoma, and glioma [232]. Moreover, the activity of this monoamine has been associated with an intensified epithelial–mesenchymal transformation process, increased cell migration abilities, and thus an increase in metastatic potential [233]. In vascular endothelial cells, 5-HT activates angiogenic-signaling kinases, which confirms its involvement in the vascularization of growing cancerous tumors [234,235]. In addition, the discussed monoamine is also classified as an immunomodulator, however, its pro and anti-tumor role are yet to be established. By affecting macrophages, it contributes to the formation of the immunosuppressive M2 population, on the other hand, 5-HT increases the cytotoxicity of natural killers, contributing to the increased eradication of cancer cells [236]. The studies also showed increased proliferation of B lymphocytes under the influence of 5-HT, however, their pro or antitumor effect exerted in the tumor microenvironment is unclear [237,238]. A similar effect was observed on Tcell proliferation after 5-HT stimulation [236]. The involvement of the serotonin pathway in cancerogenesis is another extensive topic, and cannot be described in the given manuscript in detail. Nevertheless, it is important to keep in mind that the role of TRP catabolism in cancer development is not restricted to a kynurenine pathway.

10. Conclusions

The above information shows how extensive and complex the role of tryptophan metabolism in modulating oncogenesis is. The world of science is facing an enormous challenge that must be resolved in order to understand the role of this metabolic pathway in cancer development. Despite the growing number of sources of information about individuals' role and their involvement in cancerogenesis, a lot of them are still a puzzle and give an opportunity for further significant research. Overlapping immune mechanisms and a classic well-known process of tumorigenesis additionally emphasize a need for exploring the role of immunomodulators as tumor promotors or suppressors. Gaining this knowledge may change the face of oncological treatment in the future and improve patients' survival and quality of life.

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Article

Assessment of an Anticancer Effect of the Simultaneous Administration of MM-129 and Indoximod in the Colorectal Cancer Model

Iwona Kwiatkowska [1,*], Justyna Magdalena Hermanowicz [1,2], Robert Czarnomysy [3], Arkadiusz Surażyński [4], Krystyna Kowalczuk [5], Joanna Kałafut [6], Alicja Przybyszewska-Podstawka [6], Krzysztof Bielawski [3], Adolfo Rivero-Müller [6], Mariusz Mojzych [7] and Dariusz Pawlak [1]

[1] Department of Pharmacodynamics, Medical University of Bialystok, Mickiewicza 2C, 15-222 Bialystok, Poland; justyna.hermanowicz@umb.edu.pl (J.M.H.); dariusz.pawlak@umb.edu.pl (D.P.)
[2] Department of Clinical Pharmacy, Medical University of Bialystok, Mickiewicza 2C, 15-222 Bialystok, Poland
[3] Department of Synthesis and Technology of Drugs, Medical University of Bialystok, Mickiewicza 2C, 15-222 Bialystok, Poland; robert.czarnomysy@umb.edu.pl (R.C.); krzysztof.bielawski@umb.edu.pl (K.B.)
[4] Department of Medicinal Chemistry, Medical University of Bialystok, Mickiewicza 2C, 15-222 Bialystok, Poland; arkadiusz.surazynski@umb.edu.pl
[5] Department of Integrated Medical Care, Medical University of Bialystok, ul. M Skłodowskiej-Curie 7A, 15-096 Bialystok, Poland; krystyna.kowalczuk@umb.edu.pl
[6] Department of Biochemistry and Molecular Biology, Medical University of Lublin, Chodźki 1, 20-093 Lublin, Poland; joanna.kalafut@umlub.pl (J.K.); alicja.przybyszewska-podstawka@umlub.pl (A.P.-P.); adolfo.rivero-muller@umlub.pl (A.R.-M.)
[7] Faculty of Health Science, Collegium Medicum, The Mazovian Academy in Plock, Plac Dabrowskiego 2, 09-402 Plock, Poland; mmojzych@yahoo.com
* Correspondence: iwona.kwiatkowska@sd.umb.edu.pl

Simple Summary: The discovery of the involvement of the kynurenine pathway in carcinogenesis has prompted significant changes in pharmacotherapy strategies. One such approach involves the simultaneous targeting of an ongoing process in cancer cells and inhibiting the abovementioned pathway. Based on this approach, we investigated the anticancer effect of combining a 1,2,4-triazine derivative, MM-129, together with an inhibitor of the kynurenine pathway, indoximod, in a colon cancer model. The obtained results support the efficacy of this strategy, providing a basis for future in-depth analyses.

Abstract: (1) Background: The purpose of the given study was to examine the antitumor activity of the simultaneous administration of MM-129, a 1,2,4-triazine derivative, and indoximod (IND), the kynurenine pathway inhibitor, toward colon cancer. (2) Methods: The efficiency of the co-administration of the studied compounds was assessed in xenografted zebrafish embryos. Then, the effects of the combined administration of compounds on cellular processes such as cell viability, apoptosis, and intracellular signaling pathways were evaluated. In vitro studies were performed using two colorectal cancer cell lines, namely, DLD-1 and HT-29. (3) Results: The results indicated that the simultaneous application of MM-129 and indoximod induced a stronger inhibition of tumor growth in zebrafish xenografts. The combination of these compounds intensified the process of apoptosis by lowering the mitochondrial potential, enhancing the externalization of phosphatidylserine (PS) and activation of caspases. Additionally, the expression of protein kinase B (AKT) and indoleamine 2,3-dioxygenase-(1IDO1) was disrupted under the applied compound combination. (4) Conclusions: Simultaneous targeting of ongoing cell signaling that promotes tumor progression, along with inhibition of the kynurenine pathway enzyme IDO1, results in the enhancement of the antitumor effect of the tested compounds against the colon cancer cells.

Keywords: MM-129; indoximod; colon cancer; kynurenine pathway; immunooncology; zebrafish

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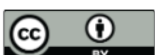

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1. Introduction

The kynurenine pathway (KP) is one of the tryptophan (TRP) metabolism pathways. Elements of the path include rate-limiting enzymes, such as IDO1, IDO2, and TDO2 (indoleamine 2,3-dioxygenase-1, indoleamine 2,3-dioxygenase-2, and tryptophan 2,3-dioxygenase), and arising metabolites, i.e., kynurenine and kynurenic acid. Mounting evidence indicates a major role of KP in oncogenesis via a specific process called immunoediting. This phenomenon assumes that tumor cells disrupt the microenvironment to promote immune evasion. Elements of KP are highly involved in this unfavorable process. For example, overexpression of IDO1, IDO2, and TDO2 results in drug resistance, enhanced metastasis formation, intense cancer cell proliferation, impeded apoptosis, and a poor prognosis for patients [1–5]. To explain the role of these enzymes in the development of immunosuppressive microenvironment, it should be mentioned that their overexpression and high enzymatic activity on cancer cells lead to a local depletion of TRP. This effect was previously linked to the impairment of T cells, dendritic cells, mastocytes, and macrophage function with concomitant intense tumor growth [6,7]. There is also some evidence of a precancerous effect of KP elements directly exerted on tumor cells. Indeed, IDO1 expression correlates with the enhanced aggressiveness of cancer cells via the dysregulation of the markers linked with epithelial-to-mesenchymal transition such as E-cadherin, N-cadherin, and vimentin [8,9]. Additionally, some researchers noticed an enhancement of angiogenesis in the presence of IDO1 [10]. All this has fueled the search for molecules that inhibit the activity of the KP pathway as an innovative approach to cancer treatment.

One such molecule is indoximod (IND). It exerts pleiotropic effects on immune regulation by reversing an inhibition of mTOR in T cells and restricting the activity of GCN2 kinase in effector T cells. That restores their proliferation and anticancer capability [11]. Via the modulation of aryl hydrocarbon receptor (AhR) activity on T cells, it promotes their differentiation into the Th subpopulation and diminishes their polarization into immunosuppressive Treg cells. It also leads to the upregulation of the transcription of RORC along with the downregulation of the transcription of FOXP3 factors [12]. In dendritic cells, IND, via AhR interaction, leads to a downregulation of IDO expression, which improves dendritic cells' ability for antigen presentation and antitumor response. Moreover, IND intensifies the efficacy of DNA-damaging chemotherapy [13] and exerts a stronger antitumor effect in combination with anti-PD-1/PD-L1 antibodies (programmed cell death protein-1/programmed death ligand-1) [14]. So far, research on the IND mechanism of action and effectiveness has focused mainly on its immunological effects. Reports on its impact on processes ongoing directly in cancer cells are limited.

In our previous study, we developed a small 1,2,4-triazine derivative (MM-129) and proved its anticancer activity in vitro in zebrafish and mouse xenografts [15]. We showed, that MM-129 exerts its favorable effect on colon cancer cells by inhibiting their crucial signaling pathways, such as PI3K/AKT/mTOR and Bruton's tyrosine kinase (BTK), together with decreasing PD-L1 expression [16]. Moreover, MM-129 enhances caspases activity with concomitant increased apoptosis of colon cancer cells. Additionally, we proved a favorable safety profile of the molecule [17].

Since it is known that IND enhances the effectiveness of compounds affecting the abovementioned pathways, and MM-129 combines the features of classic chemotherapeutics by blocking the immune checkpoint, we hypothesized that the co-administration of these compounds may exert a stronger antitumor effect. As a continuation of our previous research, we designed and conducted a study that aimed to verify this idea and to assess an antitumor activity of combined administration of MM-129 and IND in an experimental colon cancer model. In order to evaluate this, we established zebrafish xenografts using two lines of colorectal cancer (DLD-1 and HT-29). We also conducted mechanistic studies using zebrafish embryos and colon cancer cells (DLD-1 and HT-29) to evaluate the intracellular signaling pathways and other molecular events that might be involved in the antitumor effect exerted by the compound combination.

2. Materials and Methods

2.1. Zebrafish Drug Screening Assay

EU Directive 2010/63/EU of 22 September 2010 regulates the criteria for the inclusion of animals in scientific research. According to this document, zebrafish (*Danio rerio*) embryo and eleutheroembryo cultures, meaning their earliest life stages, are regarded as equivalent to in vitro cell culture. For this reason, they are not subject to the regulatory frameworks dealing with animal experiments. Nevertheless, experiments with free-feeding larvae older than 120 hpf (hours post-fertilization) of development are classified as animal experiments. This is why they require adequate permissions. The experiments planned in our study were conducted only on zebrafish larvae younger than 120 hpf; therefore, ethics approval was not required. The conditions provided to the zebrafish were as follows: 28.5 °C in E3 buffer in 30 L aquaria. That made a rate of one fish per liter of water. Light/darkness cycles were, respectively, 14/10 h. The zebrafish were fed in accordance with the guidelines established by the Research Animals Department of the Royal Society for the Prevention of Cruelty to Animals (RSPCA).

2.2. Zebrafish Xenograft Injection

Colon cancer DLD-1 and HT-29 cell lines were labeled with Vybrant Dil (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol. Stained cells were re-suspended in Dulbecco's modified Eagle's medium (DMEM) at the final concentration of 1×10^7 cells/mL. Zebrafish larvae were manually dechorionated 24 hpf and injected with cells 48 hpf. Cancer cells were loaded into a glass needle pulled by a P-1000 Next Generation Micropipette Puller (Sutter Instrument Company, Novato, CA, USA). Approximately 300 labeled cells were injected into the interior yolk space of each larva using an electronically regulated air-pressure microinjector (Narishige IM-300 Microinjector, Tokyo, Japan). After injection, the zebrafish larvae with transplanted cells (DLD-1 $n = 80$ and HT-29 $n = 80$) were randomly assigned to control and drug treatment groups. DLD-1 and HT-29 xenografts (72 hpf) were incubated with MM-129 (10 µM), IND (200 µM), or compound combination (MM-129 + IND) for 48 h. MM-129 was dissolved in dimethyl sulfoxide (DMSO), below 0.1%, and IND was dissolved in water. The control group was incubated in E3 buffer enriched with DMSO in the concentration 0.1%. The zebrafish xenografts were cultured in an E3 buffer at 34 °C until the end of the experiment (120 hpf).

2.3. In Vivo Imaging

The zebrafish larvae were anesthetized by 0.04 mg/mL ethyl 3-aminobenzoate methanesulfonate tricaine before imaging. Images of xenografts were acquired in 48 hpf and in 120 hpf, using an EVOS M5000 Imaging System with the filter Cy5 (excitation: 628 nm; emission: 692 nm).

2.4. RNA Extraction and Quantitative Analysis

Total ribonucleic acid (RNA) from 20 fish with xenografts in each studied group was isolated using an ExtractMe Total RNA kit (Blirt, Gdansk, Poland) according to the manufacturer's protocol. The isolated RNA was used for cDNA synthesis through a High-Capacity cDNA Reverse Transcription Kit with the addition of an RNase Inhibitor (Applied Biosystems, Waltham, MA, USA). Zebrafish *gpdh* (forward 5′-GTGGAGTCTACTGGTGTCTTC-3′, reverse 5′-GTGCAGGAGGCATTGCTTACA-3′) was used as a housekeeping gene. The human *GAPDH* gene (forward 5′-CTCTGCTCCTCCTGTTCGAC-3′, reverse 5′-GCCCAATAC GACCAAATCC-3′) was used to quantify the number of human cells in the xenografted zebrafish larvae. All primers used for qPCR were tested for specificity and sensitivity. Quantitative real-time polymerase chain reaction (qPCR) expression analysis was then performed with the LightCycler® 480 II instrument (Roche, Basel, Switzerland) in triplicate on 96-well plates using PowerUp SYBR Green Master Mix (Applied Biosystems). Relative mRNA expression was calculated using the delta CT subtraction and normalized [18,19].

2.5. Zebrafish Egg Proliferation Assay

Zebrafish embryos were obtained from mating adults, maintained and raised as described previously [15]. Zygote period cleaving eggs were transferred to six-well plastic cell culture plates filled with embryo medium E3. The eggs (20 per well) were exposed to MM-129 (10 µM), IND (200 µM), or compound combination (MM-129 + IND) for 3 h. The final volume of the medium in each well was 2 mL. DMSO was used as a MM-129 solvent, and water was used as an IND solvent. The final concentration of DMSO in the wells did not exceed the concentration above 0.1%, which means that it was within the range ensuring no harmful effects of the solvent. Embryos from the control group were incubated in an E3 medium in the presence of 0.1% DMSO. During the experiment, all embryos were observed and the moment when characteristic changes occurred in all embryos of a given group was selected as the point of triggering the effect of the test compound. Each test was independently repeated three times. Cell division and zebrafish eggs development were conducted with the use of SteREO Discovery. A V8 stereo microscope (Zeiss, Jena, Germany) was used. Photos of ongoing processes were taken once every 15 min within the first three hours of incubation.

2.6. Cell Culture

Two human colorectal adenocarcinoma cell lines DLD-1 (CCL-221) and HT-29 (HTB-38) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). They were chosen based on their features. The use of the DLD-1 line allows for the checking of the sensitivity of cells histologically the most similar to a primary tumor to the tested compounds. On the other hand, the inclusion of cells from the HT-29 line allows us to assess whether multidrug resistance, absorption of nutrients, and chemically induced differentiation of enterocytes emerge. DLD-1 cells (passage number range of 6–8) were cultured in RPMI 1640 medium (Sigma, St. Louis, MO, USA) and HT-29 cells in McCoy's 5a medium (ATCC). Both media were complemented with 10% of fetal bovine serum (FBS) and 1% antibiotics: penicillin/streptomycin. The cells were cultured in 100 mm plates (Sarstedt, Newton, NC, USA) and stored in the incubator. The conditions maintained in the incubator ensured optimal cell growth and were 5% CO_2, 37 °C, and humidity in a range of 90–95%. After obtaining 70–80% confluency (a subconfluent cell culture), the cells were detached with 0.05% trypsin and 0.02% EDTA phosphate-buffered saline without calcium and magnesium (Corning, Corning, NY, USA). The cells were treated with compound solutions that were prepared as follows: MM-129 was dissolved in DMSO, below 0.1%, and IND was dissolved in water.

2.7. Cell Viability Assay

Cytotoxicity of the tested compound combination was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. DLD-1 and HT-29 cells were seeded in six-well plates "Nunc" at a density of 5×10^5 cells/well and incubated for 24 h in optimal growth conditions. Next, MM-129 (10 µM), IND (200 µM), or a compound combination (MM-129 + IND), were added in duplicate, and the plates were incubated for another 24 h. After the incubation, the plates were washed with PBS three times. Then, 1 mL of PBS and 50 µL of MTT solution were added, and the incubation was continued for 15 min. MTT solution was prepared as follows: 5 mg of MTT was dissolved in 1 mL of PBS. MTT tetrazolium is converted in viable cells into purple crystals of formazan, which does not appear in dead cells. Next, the supernatant was removed, and formazan crystals were dissolved in DMSO. In the following step, Sorensen's buffer was added. After that the absorbance was measured at a wavelength of 570 nm. The absorbance result obtained in the control was taken as 100%, and the viability of the cells incubated with the tested compounds was shown as a percentage of the control cells. The values from the samples were obtained from three independent experiments performed in duplicate (n = 6).

2.8. Flow Cytometry Assessment of Annexin V Binding

Induction of apoptosis was examined by flow cytometry, with the use of an Apoptosis Detection Kit II. The test principle is based on the observation that during programmed cell death (PCD), the phosphatidylserine (PS) is transferred to the cell surface from the inner side of the cells. DLD-1 and HT-29 cells (70–80% of confluence) were incubated (24 h) with MM-129 (10 μM), IND (200 μM), or a compound combination (MM-129 + IND). After 24 h incubation with the given compounds, the cells were dyed with annexin V-FITC and propidium iodide (PI). Double staining allows for the identification of viable, early, late apoptotic, and necrotic cells. Cells in which apoptosis was induced by the addition of 2 μL of 3% formaldehyde were used as a positive control. The cells were placed in a refrigerator for 15 min to induce apoptosis. Three controls were made: the first contained control cells and propidium iodide; the second, control cells and annexin V-FITC; and the third, control cells and propidium iodide and annexin V-FITC. To establish a negative control, cells cultured in a medium without the tested compounds were used. The test was independently repeated three times. The experiment was performed using the BD FACSCanto II flow cytometer, and the results were parsed with FACSDiva software (version 6.1.3, BD Biosciences Systems, San Jose, CA, USA).

2.9. Analysis of Mitochondrial Membrane Potential

Flow cytometry was conducted to test occurring changes in the mitochondrial membrane potential (MMP). For this purpose, the JC-1 MitoScreen kit (BD Biosciences, San Jose, CA, USA) was used. The test principle is that in normal cells, lipophilic dye JC-1 (1,10,3,30-tetraethyl- 5,50,6,60-tetrachloroimidacarbocyanine iodide), which aggregates in the mitochondrial matrix. In turn, in apoptotic and necrotic cells, this dye diffuses out of mitochondria, which is manifested by the green fluorescent cell staining. DLD-1 and HT-29 colon cancer cell lines covering about 80% of the plate were incubated with MM-129 (10 μM), IND (200 μM), or compound combination (MM-129 + IND) for 24 h in an incubator, with the same conditions as described above, i.e., 37 °C and 5% CO_2. After the incubation time, the medium was removed. The cells were washed two times with the required buffer. Subsequently, the cells were suspended in a 10 μg/mL JC-1 dye and incubated in the dark for 15 min. Before the analysis, the cells were washed with PBS and then analyzed using a BD FacsCanto II flow cytometer. The results were assessed using BD FACSDiva software (version 6.1.3, BD Biosciences Systems, San Jose, CA, USA). The test was independently repeated three times.

2.10. Caspase Activity Assays

Caspase-8, -10, and -3/7 activity were assessed with the adequate kits (caspase-8: FLICA Caspase-8 Assay Kit, caspase-10: FLICA Caspase-10 Assay Kit, caspase-3/7: FLICA Caspase-3/7 Assay Kit, Mont-Royal, QC, Canada). DLD-1 and HT-29 (70–80% of confluence) were incubated with MM-129 (10 μM), IND (200 μM) or compound combination (MM-129 + IND) for 24 h. After the incubation time, the cells were washed twice with cold PBS. Next, the cells were resuspended in the required buffer (93 μL was gently mixed with 5 μL required FLICA and 2 μL Hoechst 33342) and incubated at 37 °C for 60 min. Afterward, the cells were washed twice with apoptosis wash buffer and centrifuged at 300× *g*. Cells from samples prepared in this manner were resuspended in 100 μL buffer and labeled with 10 μg/mL propidium iodide. The experiment was performed using the BD FACSCanto II flow cytometer. The results were analyzed with FACSDiva software (version 6.1.3, BD Biosciences Systems, San Jose, CA, USA). The values were obtained from three independent experiments performed in duplicate (n = 6).

2.11. Capillary Protein Separation and Immunodetection

DLD-1 and HT-29 cells were incubated for 24 h with MM-129 (10 μM), IND (200 μM), or compound combination (MM-129 + IND). After the incubation, cells were collected and pellets were lysed in 100 μL RIPA buffer enriched with protease and phosphatase

inhibitors (Sigma-Aldrich). Cellular membranes were disrupted by sonification. The level of total protein concentration was determined using the bicinchoninic acid (BCA) method according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). Samples were brought to equal amounts of protein (0.4 mg/mL) and were loaded into a cartridge. Protein samples were separated by capillary electrophoresis using the 12–230 kDa Jess Separation Module (ProteinSimple, San Jose, CA, USA) following the manufacturer's instructions. Target proteins were detected with the following monoclonal antibodies: mouse anti-IDO1 (Sigma-Aldrich, Cat# SAB3701446, 1:100), mouse anti-AKT (Sigma-Aldrich, Cat# 05-591, 1:100), and mouse anti-β-actin (Sigma Aldrich, Cat #A2228, 1:100). For detection, the anti-mouse module for the Jess (DM-002, ProteinSimple) kit was used, which includes luminol-S, peroxide, antibody diluent 2, streptavidin-HRP, and anti-mouse secondary antibody. Results are presented as blot images generated by the Compass software (Compass for SW software v5.0.1), based on chemiluminescence signal for each targeted protein. To confirm loaded protein level and to verify normalized protein amount, detection of housekeeping protein β-actin was performed. For statistical analysis the chemiluminescence of secondary antibody signal peaks were chosen. The values were obtained from three independent experiments done in duplicate (n = 6).

2.12. Statistical Analysis

Shapiro–Wilk's test of normality was used for data distribution analysis. The normally distributed data were expressed as mean ± standard deviation (SD). Multiple group comparisons were performed by one-way analysis of variance (ANOVA), and significant differences between the groups were assessed using the Tukey–Kramer test and column statistics. Calculations were performed using GraphPad 6 Prism software (La Jolla, CA, USA). The differences were considered statistically significant when * $p < 0.05$.

3. Results

3.1. Compound Combination Had a Favorable Impact on Tumor Growth in Zebrafish Xenografts

The antitumor activity of the compound combination (MM-129 + IND) was assessed in the zebrafish model of xenotransplantation. DLD-1 and HT-29 cells, stained with Vybrant Dil, were implemented into the yolk sac of 48 hpf zebrafish embryo (n = 80 each group). Established xenografts (72 hpf) were assigned to four groups: control, exposed to MM-129 (10 μM), exposed to IND (200 μM), or exposed to MM-129 + IND for 48 h. After the allotted time, the larvae were collected and RNA was extracted to quantify the presence of human cells vs. fish tissue by measuring the human *GAPDH* gene vs. zebrafish gadph gene via qRT-PCR. In both DLD-1 and HT-29 xenografts, 48 h exposition to MM-129 or IND alone resulted in a statistically significant reduction of the human *GAPDH* presence. In both groups of DLD-1 cells exposed to a single compound, we obtained a reduction in the presence of the human *GAPDH* gene at a comparable level. On the other hand, in HT-29 xenografts, IND alone resulted in a more pronounced decrease in *GAPDH* gene presence than individuals exposed to MM-129 alone. What is valuable is that the decrease in the investigated gene presence was strengthened after the co-administration of compounds; however, this was more noticeable in DLD-1 xenografts. This led us to conclude that the inhibition of tumor development was most evident in the MM-129 + IND group, and that even though both tested compounds bear antitumor potential, this property was enhanced by their co-administration (Figure 1).

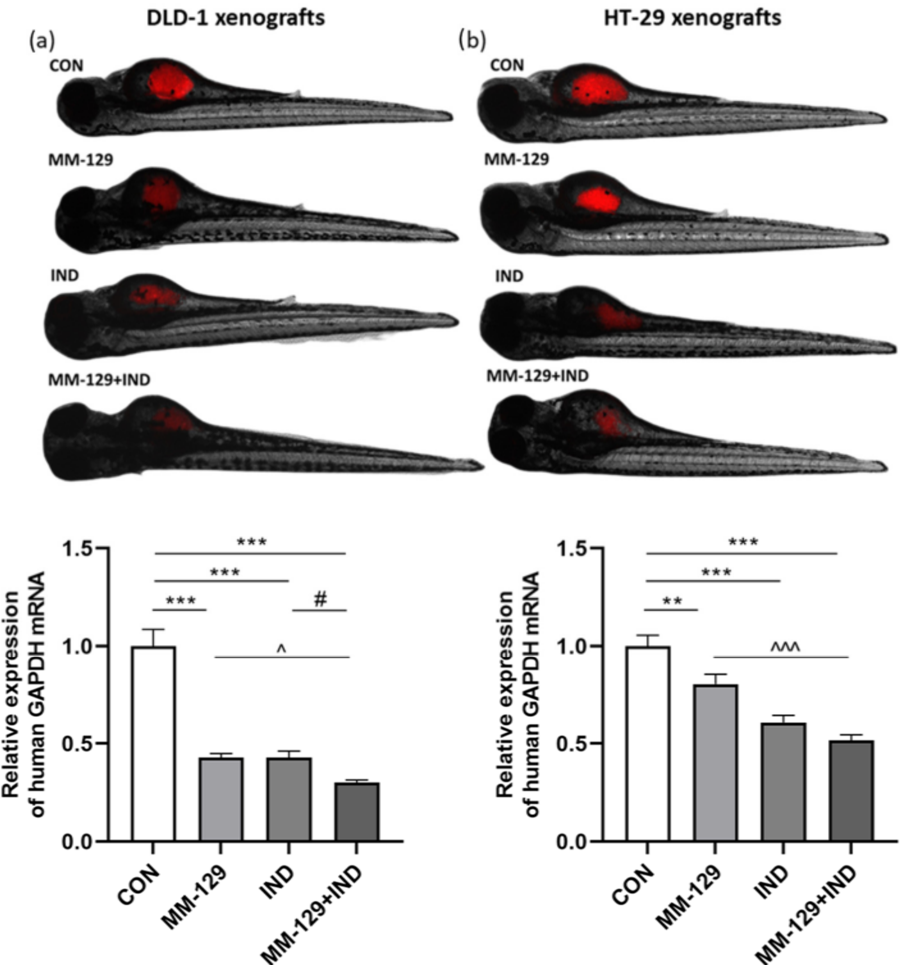

Figure 1. Impact of MM-129 (10 µM), IND (200 µM) or a combination of these agents (MM-129 + IND) on tumor development in DLD-1 (**a**), and HT-29 (**b**) zebrafish xenografts, and the expression of human *GAPDH* mRNA was determined at 120 hpf. Data were presented as mean ± standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA). n = 20 each group; ** $p < 0.01$, *** $p < 0.001$ vs. CON; ^ $p < 0.05$, ^^^ $p < 0.001$ vs. MM-129; # $p < 0.05$ vs. IND.

3.2. Antiproliferative Activity of Compound Combination in a Zebrafish Model

Zebrafish as a model organism in oncological research is also used to assess antiproliferative properties, whereas cell division is analyzed during embryogenesis. Briefly, in the first 45 min, zebrafish eggs form the stage of two cells, later in every 15 min the number of cells duplicate, creating the stages of 4, 8, and 64 cells within 1, 1.25, and 2 hpf, respectively. The fact that the cell division is easy to observe and document as microscopic photos makes it a widely used platform for evaluating the antiproliferative activity of compounds of interest.

In our experiment, we noticed a disruption in cell division in all experimental groups, while no disturbances were observed in the control group (Figure 2). However, the mentioned disturbances did not manifest at the same time spot. Only in the groups exposed to MM-129 and MM-129 + IND did we observe cell division arrest after 1 h. Cell division was also impaired by IND administrated alone; nevertheless, this effect occurred in the next stage of proliferation. The first changes in the development impairment were visible after 1.25 hpf, meaning on the stage of eight cells. After 2 h, we observed definitive proliferation inhibition in all experimental groups, while the development of untreated embryos was preserved without prejudice. This demonstrated the antiproliferative potential of both tested compounds; however, it suggested that MM-129 was more potent to inhibit cell division than IND alone.

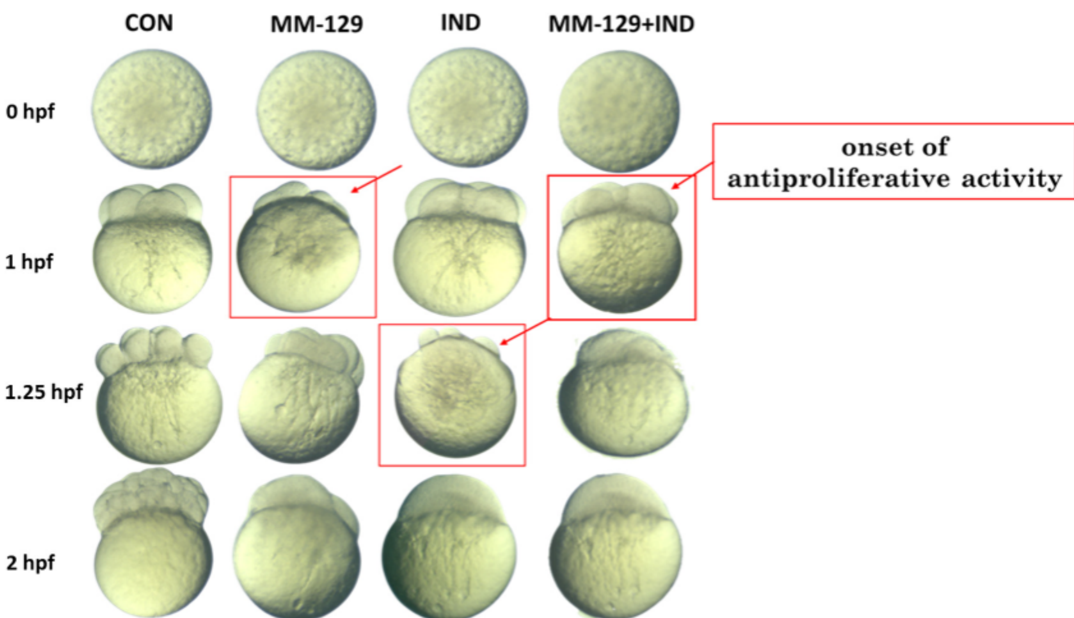

Figure 2. Effect of MM-129 (10 µM), IND (200 µM), and compound combination (MM-129 + IND) on cell division in the zebrafish embryo. Zebrafish eggs after 0, 1, 1.25, and 2 h of exposure to tested compounds; $n = 20$, hpf: hours post-fertilization.

3.3. Antiproliferative Activity of Compound Combination in Colon Cancer Cells

To verify observations made in zebrafish embryos, we conducted a classical MTT assay on DLD-1 and HT-29 cells. This allowed us to examine the effect of 24 h incubation with the tested compounds on the viability of cancer cells. In the course of the study, we found out that both compounds bore antiproliferative potential; however, when compared to the control, this effect was stronger in the group exposed to MM-129 than in the group exposed to IND alone. These results reflected observations made in zebrafish embryos. In the case of co-administration of MM-129 with IND, the obtained results were convergent for both tested cell lines and indicated a strong inhibition of cell viability. However, in the in vitro condition, the addition of IND did not increase the antiproliferative potential of MM-129 (Figure 3). The reason may be both the strong antiproliferative effect of MM-129 and the weak effect of IND in vitro, which requires the presence of immune cells to be fully effective.

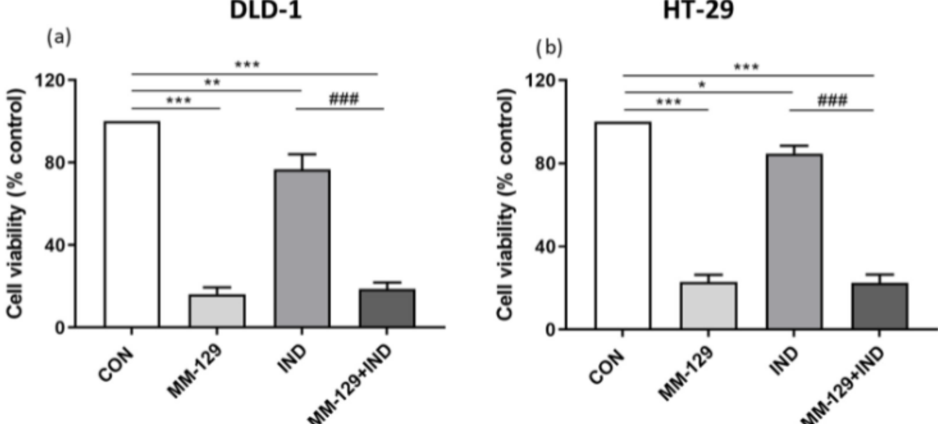

Figure 3. Viability of DLD-1 (**a**) and HT-29 (**b**) colon cancer cells treated for 24 h with MM-129 (10 µM), IND (200 µM), and compound combination (MM-129 + IND). Results are presented as mean values ± standard deviation (SD) from three independent experiments performed in duplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. CON; ### $p < 0.001$ vs. IND.

3.4. Compound Combination Enhanced Apoptosis via Decreasing Mitochondrial Membrane Potential and Phosphatidylserine Externalization

These results made us take further steps to test whether the compound combination exerts a significant impact on the molecular processes driving cancer development. For this purpose, we conducted several experiments to investigate changes in cytotoxicity. One of the well-known hallmarks of programmed cell death is a loss of mitochondrial membrane potential (MMP). As a consequence, the release of apoptogenic factors and loss of oxidative phosphorylation might be detected.

Using flow cytometry, we investigated the proapoptotic effect exerted in vitro after 24 h exposition on the proposed compound combination. In DLD-1, MM-129 and IND administrated alone caused a decrease in MMP in 87.1 ± 3% and 85.6 ± 7% of cells, respectively. The addition of IND to MM-129 resulted in a decrease in MMP in 97.8 ± 2% (^ $p < 0.05$ vs. MM-129; # $p < 0.05$ vs. IND). In the HT-29 line, MM-129 caused a stronger reduction of MMP than IND (85.6 ± 4% vs. 61.6 ± 6% respectively; && $p < 0.01$). In the MM-129 + IND group, we observed a higher percentage of cells with decreased MMP compared to the IND group (91.9 ± 6% vs. 61.6 ± 6% respectively, ### $p < 0.001$) (Figure 4a,b).

To describe in detail the changes in the apoptosis process under the influence of the compound combination, we investigated its impact on the externalization of phosphatidylserine. Briefly, the translocation of phosphatidylserine from the internal surface to the external surface of the plasma membrane is one of the recognized mechanisms that allows for the recognition and elimination of apoptotic cells [20]. Phosphatidylserine externalization might be assessed with the use of flow cytometry and the double staining method, using annexin V and propidium iodide (PI). It is known that early apoptotic cells bind only with annexin V, late-apoptotic cells bind with both of the dyes, necrotic cells bind only with PI, and the living population does not bind with any of the dyes. Due to the selective binding of dyes by cells in different states, the test allowed us to characterize the occurring cell death in detail and enabled us to conduct a more in-depth assessment of the impact of the tested approach on apoptosis.

In both cell lines exposed to a single compound, apoptosis (early and late) was detected in a higher percentage of cells incubated for 24 h with MM-129 than in those incubated with IND (respectively, 73.1 ± 4% vs. 63.3 ± 3% in DLD-1; & $p < 0.05$; 65.2 ± 3% vs. 51.2 ± 2% in HT-29; & $p < 0.05$). In DLD-1, we observed an increase in apoptotic cells after the combined administration compared to MM-129 (^ $p < 0.05$) and IND (### $p < 0.001$) alone (Figure 4c–f).

3.5. The Addition of IND Intensified Caspase Activation Exerted by MM-129

Caspases are key regulators of programmed cell death, and there are two ways to induce this process: intrinsic and external. Initiator caspase-8 and -10 are part of the extrinsic pathway, which is activated via death receptors. This is after ligand binding death receptors accumulate in the cell membrane, which leads to the recruitment of adapter proteins. Subsequently, these proteins interact with procaspase-8 and procaspase-10 via the death effector domain, which in turn results in an activation of caspase-8 and caspase-10. In our study, we observed that in vitro, in both DLD-1 and HT-29, MM-129 activated caspase-8 and -10 stronger than IND applied alone. Then, 24 h incubation with both tested compounds resulted in similar level of caspase-8 activation in both cell lines and amounted to about 95% for DLD-1 (95.03% ± 0.4) and HT-29 (95.97% ± 1.4) cells. A similar trend occurred in the case of caspase-10 activation under the combined molecules. After 24 h incubation with MM-129 + IND, the rate of DLD-1 cells with an active caspase-10 was 92.13% ± 4.3, while in HT-29 cells, the value remained at 95.07% ± 0.7 (Figure 5b,c,e,f).

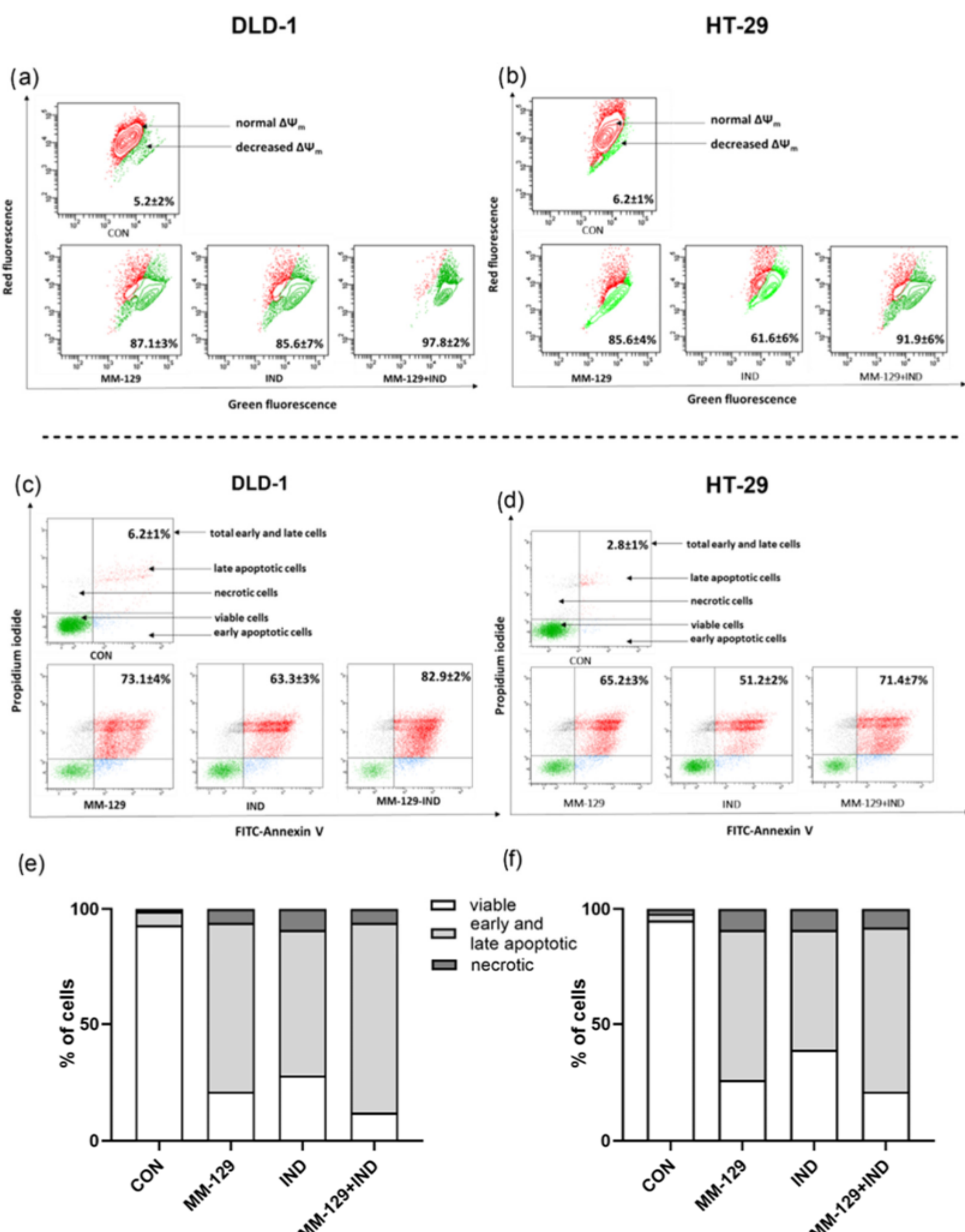

Figure 4. Representative dot-plots illustrating the loss of mitochondrial membrane potential, ΔΨm. (**a**,**b**) Flow cytometry dot-plots for the annexin V-FITC-propidium iodide assay (**c**,**d**) and quantitative chart illustrating the distribution of live, early, and late apoptotic and necrotic cells (**e**,**f**) of DLD-1 (**a**,**c**,**e**) and HT-29 (**b**,**d**,**f**) after 24 h exposition to MM-129 (10 µM), IND (200 µM), and MM-129 + IND. Values were obtained from three independent experiments.

To fully describe the caspases' activation in the latter stage of our research, we verified whether the tested compounds activated caspase-3/7. In contrast to the described above caspases-8 and -10, this is an effector caspase, which means that it affects the breakdown of cellular proteins and leads to complete cell death. It is also directly activated by the initiator caspases through the cleavage. With the use of flow cytometry, we checked if our compound combination was potent enough to activate a full caspase cascade. The obtained results revealed that MM-129 was generally more potent to activate caspase 3/7 in both tested colon cancer cell lines than IND alone. However, as in the previously observed changes in initiator caspase activation, co-administration of both molecules resulted in

stronger caspase 3/7 activation in both DLD-1 and HT-29 cell lines. After 24 h exposure to both compounds, we detected an active form of caspase 3/7 in 95.26% ± 1.2 of DLD-1 and 95.46% ± 3.1 HT-29, which was significantly higher when compared to the control group (Figure 5a,d).

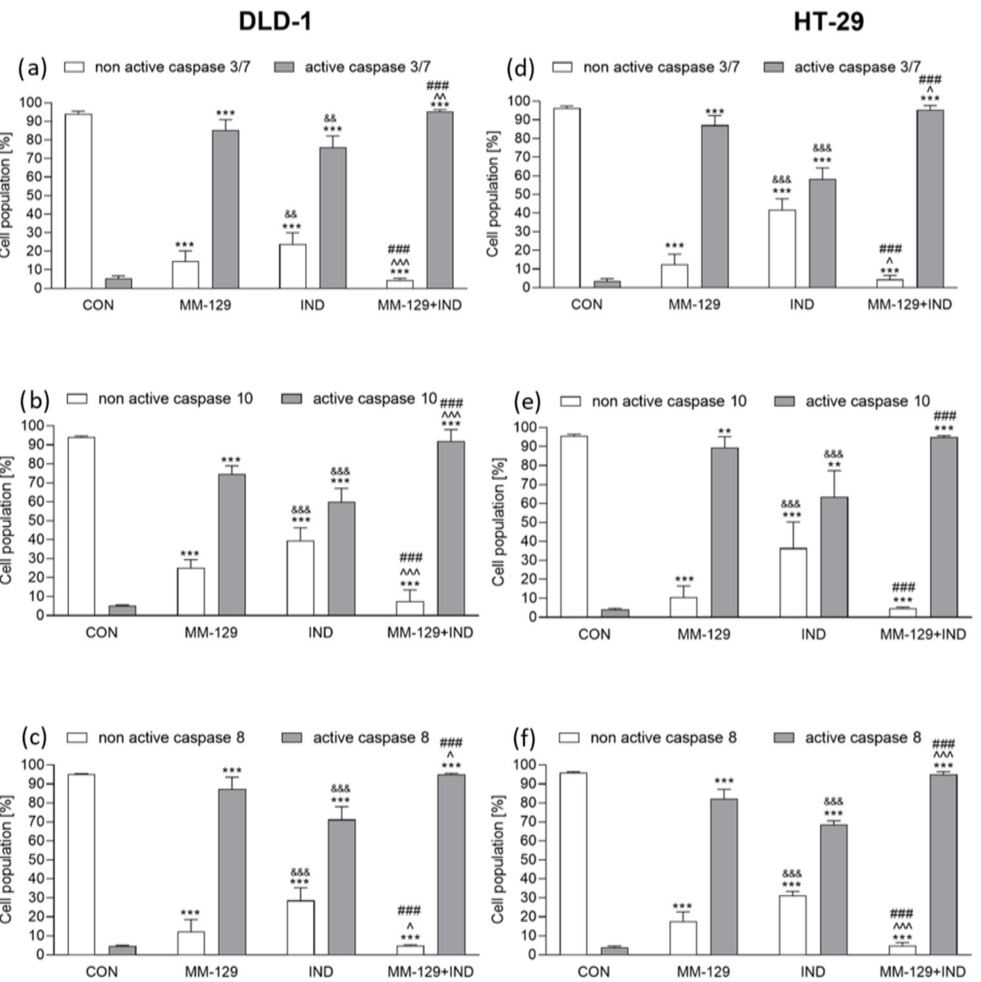

Figure 5. Flow cytometric analysis of caspase-3/7, caspase-10, and caspase-8 activation in DLD-1 (**a**–**c**) and HT-29 (**d**–**f**) colon cancer cells exposed to MM-129 (10 µM), IND (200 µM), and MM-129 + IND for 24 h. Values from three independent experiments performed in duplicate were presented as mean ± standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA). ** $p < 0.01$, *** $p < 0.001$ vs. CON; ^ $p < 0.05$, ^^ $p < 0.01$, ^^^ $p < 0.001$vs. MM-129; ### $p < 0.001$ vs. IND; && $p < 0.01$, &&& $p < 0.001$ IND vs. MM-129.

3.6. AKT Signaling Pathway Was Impaired under the Impact of Compound Combination

The abovementioned results indicated an antiproliferative and proapoptotic potential of the proposed compound combination. That prompted us to determine which of the signaling pathways was involved in causing such effects. It is well known that the PI3K/AKT pathway is highly involved in several cellular processes, necessary for cell survival. Among them, metabolism, proliferation, and apoptosis should be listed. Many of the anticancer drugs target this pathway. Due to this fact, we decided to test whether the proposed compound combination affects the expression of AKT protein kinase. What is interesting is that the obtained results demonstrated a strong inhibition of the protein's expression in both cell lines under the simultaneous administration of MM-129 + IND. While we observed a favorable effect of MM-129 in both tested cell lines, IND used alone was insufficient to inhibit the AKT expression in DLD-1 cells, but it caused its downregulation in HT-29. Despite this, co-administration of the compounds resulted in AKT inhibition at a

level comparable to the effect caused by the 1,2,4-triazine derivative. This suggests that the inhibition of AKT expression exerted by MM-129 was not impaired under the influence of IND (Figure 6).

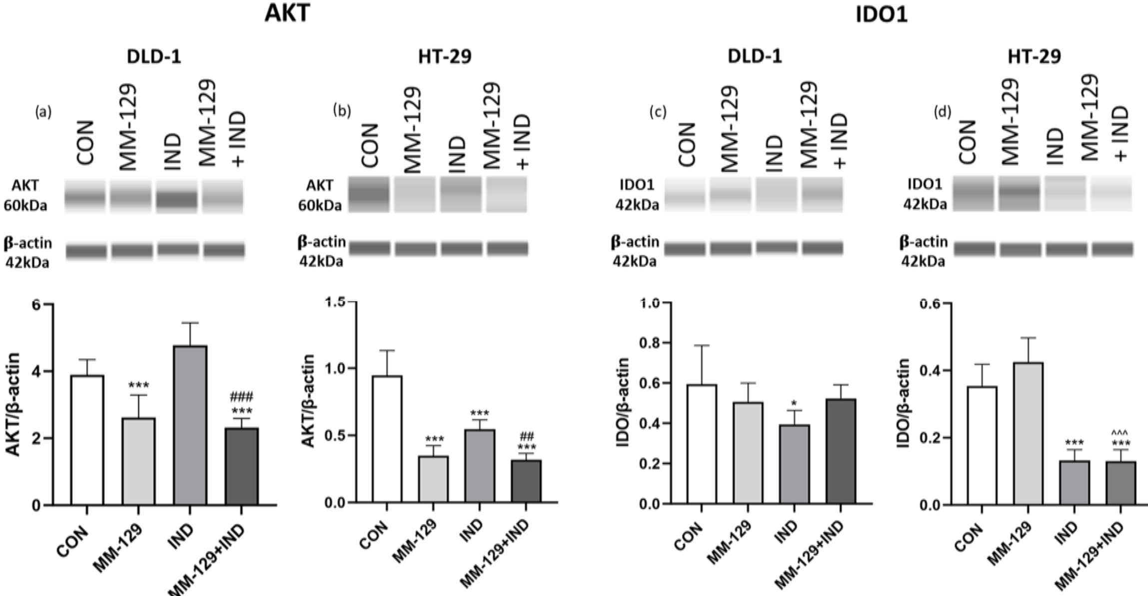

Figure 6. The downregulation of protein kinase B (AKT) (**a**,**b**) and indoleamine 2,3-dioxygenase-1 (IDO1) (**c**,**d**) exerted by MM-129 (10 μM), IND (200 μM), or MM-129 + IND toward DLD-1 and HT-29 colorectal cancer cells after 24 h exposition. The values were obtained from three independent experiments performed in duplicate. * $p < 0.05$, *** $p < 0.001$ vs. CON; ^^^ $p < 0.01$ vs. MM-129; ## $p < 0.01$, ### $p < 0.001$ vs. IND. Images and quantification were obtained by capillary protein separation and immunodetection. The uncropped blots are shown in the supplemental materials.

3.7. Indoximod but Not MM-129 Downregulated IDO1 Expression

Indoximod was developed as an inhibitor of the kynurenine pathway. However, available studies focus mostly on the effects exerted by IND on the immune cells' functions. The number of studies evaluating its direct effect on cancer cells is still limited. Additionally, the effect of MM-129 on the TRP metabolic pathway has not yet been studied. Taking these gaps into consideration, we included in our study an experiment that let us investigate whether the proposed compound combination is potent enough to inhibit the expression of the limiting enzyme of the TRP metabolite pathway. For this purpose, we checked whether the expression of IDO1 protein in DLD-1 and HT-29 colorectal cancer lines decreased under the impact of the tested compound combination. The presented results indicate that only IND was responsible for IDO1 inhibition. Interestingly, HT-29 cells turned out to be more sensitive to the proposed compound combination than DLD-1. In the former, exposure to both the KP inhibitor and its combination with MM-129 resulted in strong inhibition of the IDO1 enzyme. In DLD-1 cells, only the use of IND alone resulted in a weak decrease in IDO expression, and the combination of compounds did not exert a superior effect (Figure 6).

4. Discussion

In our previous studies, we showed that MM-129 exerts an antitumor effect toward colon cancer zebrafish and mouse xenografts [15]. Due to the increasing interest of scientists in the role of immunological processes in cancer development, we extended our current research and checked whether the combination of 1,2,4-triazine derivative MM-129 with a kynurenine pathway inhibitor—indoximod (IND)—would result in a stronger anticancer response. Previous reports on the effects of IND focused on its immunological effects. However, it is not clear whether IND has a direct effect on the processes ongoing in colorectal cancer cells. In this study, we have presented, for the first time, that this compound

combination exerted a stronger antitumor effect in the zebrafish model of colorectal cancer, which was particularly noticeable in DLD-1 xenografts. Moreover, we have shown that in vitro combination of compounds enhances apoptosis through decreasing mitochondrial potential, enhanced phosphatidylserine externalization, and caspase activation. Finally, we detected that only IND is responsible for the IDO1 inhibition.

Zebrafish (*Danio rerio*) is used in oncological research thanks to its unique features. Among them is its transparent body, which allows for observations of phenomena at the single-cell level, ease of obtaining a large number of offspring, the possibility to obtain reliable results in a short time, and high gene homology to humans (approximately 70% of genes, including non-coding region), with these underlined as the most valuable [21–23]. However, this is not a model free from limitations. The temperature for conducting experiments using zebrafish is lower than the human body temperature, which may affect the observed processes. Also, drug administration through direct absorption from the zebrafish medium may impact the precision in compound dosing [24]. The model also poses technical difficulties: due to its small size, manipulation of the equipment and implantation of cells is highly challenging. In the presented study, simultaneous incubation of zebrafish colon cancer xenografts with MM-129 and IND resulted in a significant reduction of tumor cells manifested as a strong reduction of a human *GAPDH* gene expression. We assessed it by the qRT-PCR method due to its higher sensitivity [19], and further supported it by imaging. So far, it has been established that in vivo IND empowers T-cell activity toward eliminating cancer cells. It is manifested as inhibition of immunosuppressive Treg proliferation, enhanced differentiation of suppressor Tregs into anti-cancer Th17, and activation of effector T cells [13,25]. IND as a KP inhibitor reverses the effects of TRP depletion in the tumor microenvironment, which results in the reactivation of MAP4K3 and subsequent activation of mTORC1 activity, together with the inactivation of GCN2 kinase in T helper cells [26]. Those events are linked with favorable antitumor response [27–30]. Zebrafish as a model organism is relevant for immunological studies due to the presence of T cells, which are potent to differentiate into two subpopulations: antitumor Th1-like and protumor Th2-like [31,32]. This reflects processes similar to those ongoing in humans. Also, its immune system contains other immune cells, such as macrophages, natural killer (NK) cells, and neutrophils, which expand the scope of the use of zebrafish in immunooncological research [33–36]. Equally important, in the context of investigating KP's role in cancer development, is the fact that TRP metabolizing enzymes were previously detected in zebrafish, and their involvement in various disorders was already studied using this model [37–40]. In the given study, we confirmed that the combination of tested molecules, meaning simultaneous targeting of cancer cells and KP enzyme, exerts a stronger antitumor response toward colon cancer xenografts than any of the used compounds alone. This is in line with reports, which reveal an enhanced tumor regression under the impact of KP inhibitors combined with chemotherapeutic agents [41–43]. Favorable results give a base to plan further experiments, focusing on exact immunologically driven processes responsible for an antitumor effect. This aspect will be the subject of our future study.

MM-129, meaning a 1,2,4-triazine derivative, is similar to roscovitine in terms of structure [15]. To the best of our knowledge, there are no reports that would describe 1,2,4-triazine derivatives or roscovitine as factors that influence IDO1 expression or any other KP elements. To verify if MM-129 influences the impact of IND toward IDO1, we assessed an expression of this protein in DLD-1 and HT-29 cells after 24 h with both tested molecules. What is highly important is that our results have shown that only IND downregulates IDO1 expression in both tested colon cancer cell lines. Due to the lack of the impact of MM-129 on the KP enzyme, we concluded that this molecule inhibited cancer growth only by disrupting processes ongoing directly in the malignant cells, whereas IND was additionally responsible for inhibiting the expression of the kynurenine pathway enzyme.

While investigating in detail the IDO1 role in colon cancer development, we found a report that suggests that the enzyme is directly involved in colorectal cancer (CRC) proliferation, and that its inhibition improves colon cancer management [44]. Based on this,

together with the abovementioned observation of IDO1 downregulation, we conducted experiments that verified whether the tested molecule combination exerts a favorable antiproliferative effect. For this reason, we conducted a test with the use of zebrafish embryos. It is a simple, reliable, and highly efficient tool for cancer drug screening and it was pointed as an assay complementary to commonly used MTT [45]. In our experiment, we showed that both compounds disturbed cell proliferation of zebrafish embryos; however, MM-129 exerted an antiproliferative effect on the earlier stage of cell division than IND used alone. The exposition on the compound combination resulted in an inhibition of cell proliferation at the same point of cell division as detected after the exposition on MM-129 only. This suggests that the antiproliferative effect observed in both groups exposed to tested molecules alone was not diminished by their co-administration. To verify these observations, we conducted a classical MTT assay and checked the cytotoxic effect exerted after 24 h of DLD-1 and HT-29 incubation with the tested compounds. At this stage, similarly to results obtained in zebrafish embryos, we showed an antiproliferative effect exerted by both compounds. Results obtained in the MTT test coincided with the outcome of the previous experiment and indicate an antiproliferative effect of both compounds when used alone, with MM-129 exerting a stronger reduction of cell viability. Again, the addition of IND to 1,2,4-triazine derivative did not reverse its effects. Reduced proliferation of cancer cells after IDO1 inhibition was previously reported by Hill et al., which supports our findings of the antiproliferative potential of IND [46]. We showed a lack of an overriding cytotoxic effect of the compound combination, which stays in line with the conclusion reported by Maletziki et al. In their research, they highlighted that even preconditioning of CRC cells with IND neither increases their chemosensitivity nor increases the cytotoxicity of 5-fluorouracil (5-FU), gemcitabine, and irinotecan [47]. Interestingly, in breast cancer cells, NLG-919, an indoximod prodrug, complexed with cyclodextrin, enhanced the cytotoxic effect of paclitaxel toward HeLa, and the authors observed it as a dose-depended effect [48]. Looking for discrepancies between their results and our observations, one can point to different levels of IDO1 expression between breast and colorectal cells. The abovementioned Maletziki et al. study reported that the level of IDO1 expression inversely affects cell sensitivity to indoximod, and higher expression of IDO1 in colorectal cancer cells than in breast cancer cells was previously observed [47,49]. This suggests also that the sensitivity of malignant cells to treatment with IDO1 inhibitors varies between tumor types and points to the complex role of the kynurenine pathway in cancerogenesis.

The obtained results led us to investigate molecular processes that could be affected by the simultaneous administration of MM-129 and IND. By the Western blot test, we assessed protein expression and noticed that in DLD-1 and HT-29 colon cancer cells, expression of AKT kinase decreased after 24 h exposure to the combination of molecules. It is well known that AKT is highly involved in cell proliferation, and it serves as one of the potential targets in colon cancer therapy [50]. In our previous studies, we showed that MM-129 was potent to decrease the expression of AKT in CRC cells. However, currently, there are no data on whether indoximod exerts any direct effect on this protein expression in colon cancer cells. The results obtained in the given study differed between cell lines and indicated that IND alone was potent to inhibit AKT expression in HT-29 but did not exert this effect in DLD-1. It is worth noticing that Bishnupri et al. reported a link between KP, IDO1 inhibitors, and dysregulation of the AKT signaling pathway in colorectal cancer cells. They noticed that KP metabolites increased the activation of AKT in CRC cancers, and they suggested that IDO1 inhibition synergized with the cytotoxic chemotherapeutics targeting the AKT pathway [5]. In addition, Santhanam et al. reported an increased CRC proliferation in the AKT-dependent manner under the impact of kynurenine—the first metabolite of TRP in the kynurenine pathway. This effect was reversed after the exposition of an IDO1 inhibitor, INCB24360 [51].

Finally, we checked whether the proposed compound combination exerted a proapoptotic effect. For that purpose, we observed changes in mitochondrial membrane potential;

externalization of phosphatidylserine; and activation of caspases, i.e., known markers of cell death. The obtained results allowed us to conclude that the simultaneous use of both compounds impacted the induction of apoptosis in colon cancer cells. The available data also show that human tumor cells are more sensitive to gemcitabine with simultaneous IDO1 downregulation [52]. Interestingly, a combination of gemcitabine with curcumin evoked more intense apoptosis in cancer cells [47]. The latter was previously identified as a kynurenine pathway inhibitor in cancer cells [53]. In this given study, we also showed that the proapoptotic effect of molecule combination was additionally exerted via an intense caspase activation. Available reports demonstrate that epacadostat, as well as 1-L-MT, another IDO1 inhibitor, led to the activation of a caspase in cancer cells [54,55]. A similar result to this one was obtained in HeLa cells, in which simultaneous use of IND with doxorubicin resulted in a significant increase in caspase-3 activity with concomitant apoptosis of these cells [56].

In the presented study, we validated that simultaneous targeting cancer cell along with downregulation of IDO1 expression results in strong antitumor effects in zebrafish xenografts. Also, both tested compounds showed proapoptotic effects to varying extents, whereas only IND inhibited IDO1 expression. This extension of action might be a reason for the favorable results observed in Danio rerio. Since the anticancer effect of IDO1 inhibition requires the presence of immunological mechanisms, a clear effect of potentiating the anticancer effect of the combination of compounds is difficult to demonstrate in vitro. However, in a complex organism, the effect of the KP enzyme inhibitor is revealed, intensifying the anticancer effect exerted by MM-129. Moreover, the presented observations indicate the proapoptotic effect of IND on colorectal cancer cells, which expands the current knowledge about its mechanism of action.

In the end, we would like to highlight the difference in response between DLD-1 and HT-29 cells to the proapoptotic effects of the tested compounds. DLD-1 cells turned out to respond better to the proapoptotic effects of both the combination of compounds and MM-129 or IND administrated alone. This was particularly visible in the reduction of mitochondrial potential. In this experiment, both compounds administered separately decreased mitochondrial potential to a similar extent. We also observed a stronger reduction of human *GAPDH* mRNA in DLD-1 zebrafish xenografts under the combined administration of compounds than in HT-29 xenografts. As mentioned previously, DLD-1 cells are histologically the most similar to a primary tumor, whereas the HT-29 line lets us assess whether multidrug resistance, absorption of nutrients, and chemically induced differentiation of enterocytes occur. Stronger sensitivity to apoptosis of DLD-1 when compared to HT-29 cells was reported in previous reports, which stays in line with the presented results [57–60]. Various mechanisms, such as a higher expression of glucose regulated protein 78 in DLD-1 or a stronger expression of mitochondrial cyclooxygenase-2 (COX-2) in HT-29, were reported as a reason of this discrepancy [58,60]. What is highly interesting is that Cesario et al. conducted a study that revealed an interplay between IDO1 and COX-2 in cancer [61]. In our study, we noted a stronger downregulation of IDO1 expression in HT-29 cells, one that might shape our future work on existing crosstalk. On the other hand, in the zebrafish DLD-1 xenografts exposed to MM-129 or IND alone, we observed an inhibition of tumor development at a similar level, but in HT-29 xenografts, IND alone inhibited tumor development more strongly than MM-129 alone compared to the control group. We also observed different response of DLD-1 and HT-29 cells on IDO1 inhibition by IND. Confronting these observations, the question arises as to whether there is a relationship between the sensitivity of cells to apoptosis and the role of elements of the KP pathway in the development of colorectal cancer. These intriguing results require in-depth research and will become the subject of our further work focusing on the role of KP-dependent immune processes involved in the development of colorectal cancer.

When considering the future usefulness of the presented results, the question arises about the possibility of their practical application. By discussing this issue from a broader perspective, we would like to refer to reports regarding the development of new compounds

potentially useful in CRC therapy. One of the biggest challenges in oncology is targeted therapy. For this purpose, new forms of drugs are created and successfully tested, including liposomes, nanoparticles, and cubosomes [62–65]. Their use allows for the achievement of preferential accumulation in nonspecific cancer cells, as well as a reduction of toxicity in other vital organs. MM-129, as a new molecule, has not yet been tested for possible formulations into modern forms of the drug. On the other hand, IND and its prodrug have already been subjected to such tests [66]. What is particularly important is that nanoparticles and liposomes that were created contained IND together with compounds classified as classical chemotherapy [67,68]. Their use was associated with greater effectiveness in experimental models [12]. This gives rise to the hypothesis that the tested compound combination will have a chance of exerting anticancer effects in a moder formulation. Following the approach of targeted therapy and maximizing the effectiveness and safety of therapy, we plan to conduct research that will bring us closer to developing this form of combination of compounds. We hope that this will increase its clinical potential.

5. Conclusions

To sum up, we showed for the first time that the combination of the 1,2,4-triazine derivative MM-129 with the inhibitor of the kynurenine pathway, indoximod, results in a stronger antitumor response toward colon cancer cells. This indicates that simultaneous targeting of processes ongoing in the tumor cell, together with the inhibition of the kynurenine pathway enzyme, is a right and promising approach in designing future therapeutic options in the fight against colorectal cancer. Undoubtedly, the presented studies need further investigation, especially with the deep focus on immunologically driven changes responsible for an antitumor response.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers16010122/s1, Full pictures of the Western blots.

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Białystok, 27.02.2024

Iwona Kwiatkowska
Uniwersytet Medyczny w Białymstoku
Zakład Farmakodynamiki
Ul. Mickiewicza 2C
15-089 Białystok

Oświadczenie autora

Oświadczam, iż mój udział w przygotowaniu publikacji:

1. Kwiatkowska, I., Hermanowicz, J. M., Przybyszewska-Podstawka, A., & Pawlak, D. (2021). Not Only Immune Escape-The Confusing Role of the TRP Metabolic Pathway in Carcinogenesis. Cancers, 13(11), 2667. https://doi.org/10.3390/cancers13112667

wchodzącej w skład mojej rozprawy doktorskiej polegał na * opracowaniu koncepcji, zebraniu i przeglądzie literatury, analizie i interpretacji danych oraz przygotowaniu oryginalnej wersji manuskryptu, co określam jako 80% udziału w przygotowaniu wyżej wymienionej publikacji.

2. Kwiatkowska, I., Hermanowicz, J. M., Czarnomysy, R., Surażyński, A., Kowalczuk, K., Kałafut, J., Przybyszewska-Podstawka, A., Bielawski, K., Rivero-Müller, A., Mojzych, M., & Pawlak, D. (2023). Assessment of an Anticancer Effect of the Simultaneous Administration of MM-129 and Indoximod in the Colorectal Cancer Model. Cancers, 16(1), 122. https://doi.org/10.3390/cancers16010122

wchodzącej w skład mojej rozprawy doktorskiej polegał na * opracowaniu koncepcji i planu badań oraz metod badawczych, prowadzeniu części eksperymentalnej badań, analizie statystycznej wyników i ich interpretacji oraz przygotowaniu oryginalnej wersji manuskryptu, co określam jako 60% udziału w przygotowaniu wyżej wymienionej publikacji.

Iwona Kwiatkowska
....
Podpis autora rozprawy doktorskiej (czytelny)

Dariusz Pawlak
....
Podpis promotora (czytelny)

**W przypadku każdej z włączonych do cyklu prac zaleca się złożenie oświadczenia przez autora wskazujące na jego merytoryczny oraz procentowy wkład w powstanie pracy [np. twórca hipotezy badawczej, pomysłodawca badań, wykonanie specyficznych badań (np. przeprowadzenie konkretnych doświadczeń, opracowanie i zebranie danych, wykonanie zestawień statystycznych itp.), wykonanie analizy wyników, przygotowanie manuskryptu artykułu, i inne]. Określenie wkładu autora powinno być na tyle precyzyjne, aby umożliwić dokładną ocenę jego udziału i roli w powstaniu każdej z prac.*

Białystok, 27.02.2024r.

Dariusz Pawlak
Uniwersytet Medyczny w Białymstoku
Zakład Farmakodynamiki
Ul. Mickiewicza 2C
15-089 Białystok

Oświadczenie współautora

Oświadczam, iż mój udział w przygotowaniu publikacji:

1. Kwiatkowska, I., Hermanowicz, J. M., Przybyszewska-Podstawka, A., & Pawlak, D. (2021). Not Only Immune Escape-The Confusing Role of the TRP Metabolic Pathway in Carcinogenesis. Cancers, 13(11), 2667.

wchodzącej w skład rozprawy doktorskiej Pani mgr Iwony Kwiatkowskiej polegał na nadzorze merytorycznym i edycji manuskryptu.*

2. Kwiatkowska, I., Hermanowicz, J. M., Czarnomysy, R., Surażyński, A., Kowalczuk, K., Kałafut, J., Przybyszewska-Podstawka, A., Bielawski, K., Rivero-Müller, A., Mojzych, M., & Pawlak, D. (2023). Assessment of an Anticancer Effect of the Simultaneous Administration of MM-129 and Indoximod in the Colorectal Cancer Model. Cancers, 16(1), 122.

wchodzącej w skład rozprawy doktorskiej Pani mgr Iwony Kwiatkowskiej polegał na nadzorze merytorycznym, a także na pozyskaniu funduszy i zarządzaniu projektem.*

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Dariusz Pawlak
....
Podpis (czytelny)

W przypadku prac dwu- lub wieloautorskich zaleca się złożenie oświadczenia **przez współautora wskazujące na jego merytoryczny (a NIE procentowy) wkład w powstanie pracy [np. twórca hipotezy badawczej, pomysłodawca badań, wykonanie specyficznych badań (np. przeprowadzenie konkretnych doświadczeń, opracowanie i zebranie danych, wykonanie zestawień statystycznych itp.), wykonanie analizy wyników, przygotowanie manuskryptu artykułu, i inne]. Określenie wkładu danego współautora powinno być na tyle precyzyjne, aby umożliwić dokładną ocenę jego udziału i roli w powstaniu każdej pracy.*

Białystok, 27.02.2024r.

Justyna Magdalena Hermanowicz
Uniwersytet Medyczny w Białymstoku
Zakład Farmakodynamiki
Ul. Mickiewicza 2C
15-089 Białystok

Oświadczenie współautora

Oświadczam, iż mój udział w przygotowaniu publikacji:

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wchodzącej w skład rozprawy doktorskiej Pani mgr Iwony Kwiatkowskiej polegał na współuczestnictwie w opracowaniu koncepcji i przygotowaniu oryginalnej wersji manuskryptu, a także a także na pozyskaniu funduszy i zarządzaniu projektem.*

2. Kwiatkowska, I., Hermanowicz, J. M., Czarnomysy, R., Surażyński, A., Kowalczuk, K., Kałafut, J., Przybyszewska-Podstawka, A., Bielawski, K., Rivero-Müller, A., Mojzych, M., & Pawlak, D. (2023). Assessment of an Anticancer Effect of the Simultaneous Administration of MM-129 and Indoximod in the Colorectal Cancer Model. Cancers, 16(1), 122.

wchodzącej w skład rozprawy doktorskiej Pani mgr Iwony Kwiatkowskiej polegał na współuczestnictwie w prowadzeniu części eksperymentalnej badań, a także na nadzorze merytorycznym i edycji manuskryptu oraz na pozyskaniu funduszy i zarządzaniu projektem.*

Jednocześnie wyrażam zgodę na przedłożenie wyżej wymienionej pracy/prac przez Panią/ Pana mgr Iwony Kwiatkowskiej jako część rozprawy doktorskiej w formie spójnego tematycznie cyklu prac opublikowanych w czasopismach naukowych.

Justyna Hermanowicz
....
Podpis (czytelny)

W przypadku prac dwu- lub wieloautorskich zaleca się złożenie oświadczenia* ***przez współautora* *wskazujące na jego merytoryczny (a NIE procentowy) wkład w powstanie pracy [np. twórca hipotezy badawczej, pomysłodawca badań, wykonanie specyficznych badań (np. przeprowadzenie konkretnych doświadczeń, opracowanie i zebranie danych, wykonanie zestawień statystycznych itp.), wykonanie analizy wyników, przygotowanie manuskryptu artykułu, i inne]. Określenie wkładu danego współautora powinno być na tyle precyzyjne, aby umożliwić dokładną ocenę jego udziału i roli w powstaniu każdej pracy.*

Białystok, 27.02.2024r.

Alicja Przybyszewska- Podstawka
Uniwersytet Medyczny w Lublinie
Zakład Biochemii i Biologii Molekularnej
ul. Chodźki 1
20-093 Lublin

Oświadczenie współautora

Oświadczam, iż mój udział w przygotowaniu publikacji:

1. Kwiatkowska, I., Hermanowicz, J. M., Przybyszewska-Podstawka, A., & Pawlak, D. (2021). Not Only Immune Escape-The Confusing Role of the TRP Metabolic Pathway in Carcinogenesis. Cancers, 13(11), 2667.

wchodzącej w skład rozprawy doktorskiej Pani mgr Iwony Kwiatkowskiej polegał na współudziale w przygotowaniu oryginalnej wersji manuskryptu (oprawa graficzna).*

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Alicja Przybyszewska-Podstawka
....
Podpis (czytelny)

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Białystok, 27.02.2024r.

Robert Czarnomysy
Uniwersytet Medyczny w Białymstoku
Zakład Syntezy i Technologii Środków Leczniczych
ul. Jana Kilińskiego 1
15-089 Białystok

Oświadczenie współautora

Oświadczam, iż mój udział w przygotowaniu publikacji:

2. Kwiatkowska, I., Hermanowicz, J. M., Czarnomysy, R., Surażyński, A., Kowalczuk, K., Kalafut, J., Przybyszewska-Podstawka, A., Bielawski, K., Rivero-Müller, A., Mojzych, M., & Pawlak, D. (2023). Assessment of an Anticancer Effect of the Simultaneous Administration of MM-129 and Indoximod in the Colorectal Cancer Model. Cancers, 16(1), 122.

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ADIUNKT
Zakład Syntezy i Technologii
Środków Leczniczych
dr hab. Robert Czarnomysy
....
Podpis (czytelny)

W przypadku prac dwu- lub wieloautorskich zaleca się złożenie oświadczenia* ***przez współautora* *wskazujące na jego merytoryczny (a NIE procentowy) wkład w powstanie pracy [np. twórca hipotezy badawczej, pomysłodawca badań, wykonanie specyficznych badań (np. przeprowadzenie konkretnych doświadczeń, opracowanie i zebranie danych, wykonanie zestawień statystycznych itp.), wykonanie analizy wyników, przygotowanie manuskryptu artykułu, i inne]. Określenie wkładu danego współautora powinno być na tyle precyzyjne, aby umożliwić dokładną ocenę jego udziału i roli w powstaniu każdej pracy.*

Białystok, 27.02.2024r.

Arkadiusz Surażyński
Uniwersytet Medyczny w Białymstoku
Zakład Chemii Leków
ul. Mickiewicza 2D
15-222 Białystok

Oświadczenie współautora

Oświadczam, iż mój udział w przygotowaniu publikacji:

2. Kwiatkowska, I., Hermanowicz, J. M., Czarnomysy, R., Surażyński, A., Kowalczuk, K., Kałafut, J., Przybyszewska-Podstawka, A., Bielawski, K., Rivero-Müller, A., Mojzych, M., & Pawlak, D. (2023). Assessment of an Anticancer Effect of the Simultaneous Administration of MM-129 and Indoximod in the Colorectal Cancer Model. Cancers, 16(1), 122.

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Arkadiusz Surażyński
....
Podpis (czytelny)

W przypadku prac dwu- lub wieloautorskich zaleca się złożenie oświadczenia* ***przez współautora* *wskazujące na jego merytoryczny (a NIE procentowy) wkład w powstanie pracy [np. twórca hipotezy badawczej, pomysłodawca badań, wykonanie specyficznych badań (np. przeprowadzenie konkretnych doświadczeń, opracowanie i zebranie danych, wykonanie zestawień statystycznych itp.), wykonanie analizy wyników, przygotowanie manuskryptu artykułu, i inne]. Określenie wkładu danego współautora powinno być na tyle precyzyjne, aby umożliwić dokładną ocenę jego udziału i roli w powstaniu każdej pracy.*

Białystok, 27.02.2024r.

Krystyna Kowalczuk
Uniwersytet Medyczny w Białymstoku
Zakład Zintegrowanej Opieki Medycznej
ul. Marii Skłodowskiej-Curie 7A
15-096 Białystok

Oświadczenie współautora

Oświadczam, iż mój udział w przygotowaniu publikacji:

2. Kwiatkowska, I., Hermanowicz, J. M., Czarnomysy, R., Surażyński, A., Kowalczuk, K., Kałafut, J., Przybyszewska-Podstawka, A., Bielawski, K., Rivero-Müller, A., Mojzych, M., & Pawlak, D. (2023). Assessment of an Anticancer Effect of the Simultaneous Administration of MM-129 and Indoximod in the Colorectal Cancer Model. Cancers, 16(1), 122.

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Krystyna Kowalczuk
....
Podpis (czytelny)

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Białystok, 27.02.2024r.

Joanna Kałafut
Uniwersytet Medyczny w Lublinie
Zakład Biochemii i Biologii Molekularnej
ul. Chodźki 1
20-093 Lublin

Oświadczenie współautora

Oświadczam, iż mój udział w przygotowaniu publikacji:

2. Kwiatkowska, I., Hermanowicz, J. M., Czarnomysy, R., Surażyński, A., Kowalczuk, K., Kałafut, J., Przybyszewska-Podstawka, A., Bielawski, K., Rivero-Müller, A., Mojzych, M., & Pawlak, D. (2023). Assessment of an Anticancer Effect of the Simultaneous Administration of MM-129 and Indoximod in the Colorectal Cancer Model. Cancers, 16(1), 122.

wchodzącej w skład rozprawy doktorskiej Pani mgr Iwony Kwiatkowskiej polegał na współudziale w prowadzeniu części eksperymentalnej badań. *

Jednocześnie wyrażam zgodę na przedłożenie wyżej wymienionej pracy/prac przez Panią/ Pana mgr Iwony Kwiatkowskiej jako część rozprawy doktorskiej w formie spójnego tematycznie cyklu prac opublikowanych w czasopismach naukowych.

Joanna Kałafut
....
Podpis (czytelny)

W przypadku prac dwu- lub wieloautorskich zaleca się złożenie oświadczenia* ***przez współautora* *wskazujące na jego merytoryczny (a NIE procentowy) wkład w powstanie pracy [np. twórca hipotezy badawczej, pomysłodawca badań, wykonanie specyficznych badań (np. przeprowadzenie konkretnych doświadczeń, opracowanie i zebranie danych, wykonanie zestawień statystycznych itp.), wykonanie analizy wyników, przygotowanie manuskryptu artykułu, i inne]. Określenie wkładu danego współautora powinno być na tyle precyzyjne, aby umożliwić dokładną ocenę jego udziału i roli w powstaniu każdej pracy.*

Białystok, 27.02.2024r.

Krzysztof Bielawski
Uniwersytet Medyczny w Białymstoku
Zakład Syntezy i Technologii Środków Leczniczych
ul. Jana Kilińskiego 1
15-089 Białystok

Oświadczenie współautora

Oświadczam, iż mój udział w przygotowaniu publikacji:

2. Kwiatkowska, I., Hermanowicz, J. M., Czarnomysy, R., Surażyński, A., Kowalczuk, K., Kałafut, J., Przybyszewska-Podstawka, A., Bielawski, K., Rivero-Müller, A., Mojzych, M., & Pawlak, D. (2023). Assessment of an Anticancer Effect of the Simultaneous Administration of MM-129 and Indoximod in the Colorectal Cancer Model. Cancers, 16(1), 122.

wchodzącej w skład rozprawy doktorskiej Pani mgr Iwony Kwiatkowskiej polegał na współudziale w części eksperymentalnej badań. *

Jednocześnie wyrażam zgodę na przedłożenie wyżej wymienionej pracy/prac przez Panią/ Pana mgr Iwony Kwiatkowskiej jako część rozprawy doktorskiej w formie spójnego tematycznie cyklu prac opublikowanych w czasopismach naukowych.

KIEROWNIK
Zakładu Syntezy i Technologii
Środków Leczniczych
prof. dr hab. Krzysztof Bielawski

Podpis (czytelny)

W przypadku prac dwu- lub wieloautorskich zaleca się złożenie oświadczenia* ***przez współautora* *wskazujące na jego merytoryczny (a NIE procentowy) wkład w powstanie pracy [np. twórca hipotezy badawczej, pomysłodawca badań, wykonanie specyficznych badań (np. przeprowadzenie konkretnych doświadczeń, opracowanie i zebranie danych, wykonanie zestawień statystycznych itp.), wykonanie analizy wyników, przygotowanie manuskryptu artykułu, i inne]. Określenie wkładu danego współautora powinno być na tyle precyzyjne, aby umożliwić dokładną ocenę jego udziału i roli w powstaniu każdej pracy.*

Białystok, 27.02.2024r.

Adolfo Rivero-Müller
Uniwersytet Medyczny w Lublinie
Zakład Biochemii i Biologii Molekularnej
ul. Chodźki 1
20-093 Lublin

Oświadczenie współautora

Oświadczam, iż mój udział w przygotowaniu publikacji:

2. Kwiatkowska, I., Hermanowicz, J. M., Czarnomysy, R., Surażyński, A., Kowalczuk, K., Kałafut, J., Przybyszewska-Podstawka, A., Bielawski, K., Rivero-Müller, A., Mojzych, M., & Pawlak, D. (2023). Assessment of an Anticancer Effect of the Simultaneous Administration of MM-129 and Indoximod in the Colorectal Cancer Model. Cancers, 16(1), 122.

wchodzącej w skład rozprawy doktorskiej Pani mgr Iwony Kwiatkowskiej polegał na nadzorze merytorycznym. *

Jednocześnie wyrażam zgodę na przedłożenie wyżej wymienionej pracy/prac przez Panią/ Pana mgr Iwony Kwiatkowskiej jako część rozprawy doktorskiej w formie spójnego tematycznie cyklu prac opublikowanych w czasopismach naukowych.

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Podpis (czytelny)

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prof. dr hab. Mariusz Mojzych

Instytut Nauk Chemicznych

08-110 Siedlce, ul. 3 Maja 54, 25 643 11 13, kom. 666-037-451, e-mail: mariusz.mojzych@uws.edu.pl

Siedlce, 28.02.2024 r.

Oświadczenie współautora

Oświadczam, iż mój udział w przygotowaniu publikacji:

2. Kwiatkowska, I., Hermanowicz, J. M., Czarnomysy, R., Surażyński, A., Kowalczuk, K., Kałafut, J., Przybyszewska-Podstawka, A., Bielawski, K., Rivero-Müller, A., Mojzych, M., & Pawlak, D. **(2023)** *Assessment of an Anticancer Effect of the Simultaneous Administration of MM-129 and Indoximod in the Colorectal Cancer Model. Cancers, 16(1),* 122.

wchodzącej w skład rozprawy doktorskiej Pani mgr Iwony Kwiatkowskiej polegał na współudziale w części eksperymentalnej badań. *

Jednocześnie wyrażam zgodę na przedłożenie wyżej wymienionej pracy przez Panią mgr Iwony Kwiatkowskiej jako część rozprawy doktorskiej w formie spójnego tematycznie cyklu prac opublikowanych w czasopismach naukowych.

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Podpis (czytelny)

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.

KOMISJA BIOETYCZNA
PRZY UNIWERSYTECIE MEDYCZNYM W BIAŁYMSTOKU
ul. Jana Kilińskiego 1
15-089 Białystok
tel. 85 748 54 07
komisjabioetyczna@umb.edu.pl

Białystok, dn. 5.03.2024 r.

APK.002.191.2024

Sz. P.
mgr Iwona Kwiatkowska
Zakład Farmakodynamiki
Uniwersytet Medyczny w Białymstoku

Komisja Bioetyczna przy Uniwersytecie Medycznym w Białystoku po zapoznaniu się z pismem z dnia 27.02.2024 r., oraz załączoną specyfikacją badań z wykorzystaniem komercyjnych linii komórkowych, stanowiących podstawę Pani rozprawy doktorskiej, informuję, że nie ma w tym przypadku konieczności uzyskiwania zgody Komisji Bioetycznej.

Przedstawione karty charakterystyki linii komórkowych gwarantują dochowanie należytej staranności w przestrzeganiu przepisów pozyskania materiału biologicznego, jak też przestrzegania norm etycznych.

Z poważaniem
prof. dr hab. Otylia Kowal-Bielecka

Przewodnicząca Komisji Bioetycznej
przy Uniwersytecie Medycznym
w Białymstoku

Rozdział 13. Dorobek naukowy

Łączna wartość Impact Factor: 34.093
Łączna ilość punktów MNiSW: 820.000

Lista publikacji stanowiących rozprawę doktorską:

1. Kwiatkowska, I., Hermanowicz, J. M., Przybyszewska-Podstawka, A., Pawlak, D. (2021). Not Only Immune Escape-The Confusing Role of the TRP Metabolic Pathway in Carcinogenesis. Cancers, 13(11), 2667. https://doi.org/10.3390/cancers13112667

2. Kwiatkowska, I., Hermanowicz, J. M., Czarnomysy, R., Surażyński, A., Kowalczuk, K., Kałafut, J., Przybyszewska-Podstawka, A., Bielawski, K., Rivero-Müller, A., Mojzych, M., Pawlak, D. (2023). Assessment of an Anticancer Effect of the Simultaneous Administration of MM-129 and Indoximod in the Colorectal Cancer Model. Cancers, 16(1), 122. https://doi.org/10.3390/cancers16010122

Wykaz innych publikacji:

1. Rożkiewicz, D., Hermanowicz, J. M., Kwiatkowska, I., Krupa, A., Pawlak, D. (2023). Bruton's Tyrosine Kinase Inhibitors (BTKIs): Review of Preclinical Studies and Evaluation of Clinical Trials. Molecules (Basel, Switzerland), 28(5), 2400. https://doi.org/10.3390/molecules28052400

2. Kwiatkowska, I., Hermanowicz, J. M., Iwińska, Ż., Kowalczuk, K., Iwanowska, J., Pawlak, D. (2022). Zebrafish-An Optimal Model in Experimental Oncology. Molecules (Basel, Switzerland), 27(13), 4223. https://doi.org/10.3390/molecules27134223

3. Hermanowicz, J. M., Pawlak, K., Sieklucka, B., Czarnomysy, R., Kwiatkowska, I., Kazberuk, A., Surażyński, A., Mojzych, M., Pawlak, D. (2021). MM-129 as a Novel Inhibitor Targeting PI3K/AKT/mTOR and PD-L1 in Colorectal Cancer. Cancers, 13(13), 3203. https://doi.org/10.3390/cancers13133203

4. Kwiatkowska, I., Hermanowicz, J. M., Myśliwiec, M., Pawlak, D. (2020). Oxidative Storm Induced by Tryptophan Metabolites: Missing Link between Atherosclerosis and Chronic Kidney Disease. Oxidative medicine and cellular longevity, 2020, 6656033. https://doi.org/10.1155/2020/6656033

5. Hermanowicz, J. M., Kwiatkowska, I., Pawlak, D. (2020). Important players in carcinogenesis as potential targets in cancer therapy: an update. Oncotarget, 11(32), 3078–3101. https://doi.org/10.18632/oncotarget.27689

Wykaz doniesień zjazdowych:

1. Kwiatkowska, I., Hermanowicz, J., Pawlak, D. Proapoptotic activity of MM-129 and indoximod in experimental colon cancer model. 12th International Drug Chemistry Conference, Antalya, Turkiye, March 07-10, 2024. (Prezentacja ustna)

2. Hermanowicz, J., Kwiatkowska, I., Pawlak, D. MM-129 and indoximod combination as a new approach against colorectal cancer. 12th International Drug Chemistry Conference, Antalya, Turkiye, March 07-10, 2024. (Poster)

3. Kwiatkowska, I., Hermanowicz, J., Czarnomysy, R., Surażyński, A., Pawlak, D. MM-129: a derivative with a dual mechanism of action as an innovative molecule with antitumor activity against colon cancer cells. 10th Jubilee International Conference on Radiation in Various Fields of Research, Herceg Novi, Montenegro, June 13-17, 2022. (Prezentacja ustna)

4. Hermanowicz, J., Kwiatkowska, I., Pawlak, D. MM-129 as a new potential candidate against colon cancer - assessment of toxicity. 10th Jubilee International Conference on Radiation in Various Fields of Research, Herceg Novi, Montenegro, June 13-17, 2022. (Poster)

5. Kwiatkowska I., Rakowska, N. MM-129 and indoximod as an innovative anticancer therapy in an experimental zebrafish model. 16th BIMC, Bialystok International Medical Congress for Young Scientists, Bialystok, Poland, 6th-7th of May 2022. (Prezentacja ustna, wystąpienie nagrodzone 3. miejscem.)

6. Kwiatkowska, I., Hermanowicz, J., Czarnomysy, R., Bielawska, A., Mojzych, M., Bielawski, K., Pawlak, D. Proapoptotic activity of MM-129 in experimental colon cancer models. ACCORD 2022. Interdisciplinary Conference on Drug Sciences "Synergy of interdisciplinary innovations" Warsaw May 26-28 2022. (Prezentacja ustna)

7. Hermanowicz, J., Kwiatkowska, I., Surażyński, A., Pawlak, D., MM-129 a novel potential anticancer agent against colorectal cancer. ACCORD 2022. Interdisciplinary Conference on Drug Sciences "Synergy of interdisciplinary innovations" Warsaw May 26-28 2022. (Poster)

Wykaz innych aktywności:

1. Stypendystka programu Blue Book realizowanego w Dyrektoriacie Generalnym ds. Zdrowia i Bezpieczeństwa Żywności DG SANTE Komisji Europejskiej.
Luxembourg, 1.03 – 31.07. 2023r.

2. Stypendystka w projekcie badawczym „Opracowanie małocząsteczkowej pochodnej 1,2,4-triazyny (MM-129) – ocena aktywności przeciwnowotworowej i bezpieczeństwa

stosowania w doświadczalnym modelu raka jelita grubego". Narodowe Centrum Nauki, OPUS 16. Uniwersytet Medyczny w Białymstoku, Polska, 9.11.2020 – 27.06.2022r.

3. Uczestniczka studiów realizowanych w ramach Programu Erasmus+. Faculty of Pharmacy, University of Ljubljana.
Lubljana, Slovenia, 11.02. – 30.06.2019r.

4. Stażystka Programu Erasmus +. Staż realizowany w szpitalnej aptece onkologicznej Oncopharmacie AP-HM, Hôpital de la Timone.
Marseille, France, 23.07.2018 – 22.09.2018r.

5. Uczestniczka programu Student Exchange Program organizowanego przez International Pharmaceutical Students' Association (IPSA). Staż realizowany w Hematology Center after prof. R. Yeolyan, CJSC of RA Ministry of Health.
Yerevan, Armenia, 17.07 – 28.07. 2017r.

6. Członek sekcji „Młoda Farmacja" Polskiego Towarzystwa Farmaceutycznego 2014 – 2019.