UNIWERSYTET MEDYCZNY
IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU

Katedra Morfologii i Embriologii Człowieka

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PRACA DOKTORSKA

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Działanie cytotoksyczne i proapoptotyczne resweratrolu na wybrane nowotwory przewodu pokarmowego w badaniach *in vitro*

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Spis treści

1. Wykaz publikacji stanowiących pracę doktorską

Poniżej przedstawiono spis publikacji będących podstawą pracy doktorskiej pt.: „Działanie cytotoksyczne i proapoptotyczne resweratrolu na wybrane nowotwory przewodu pokarmowego w badaniach *in vitro*". Sumaryczny IF cyklu zgodny z rokiem publikacji jest równy 11,725, a punktacja MNiSW wynosi 295.

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III. **Katarzyna Ratajczak**, Natalia Glatzel-Plucińska, Katarzyna Ratajczak-Wielgomas, Katarzyna Nowińska, Sylwia Borska. Effect of Resveratrol Treatment on Human Pancreatic Cancer Cells through Alterations of Bcl-2 Family Members.
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2. Wprowadzenie

Choroby nowotworowe uznawane są za jedną z głównych przyczyn zgonów na całym świecie. Nowotwory przewodu pokarmowego z reguły wykrywane są zbyt późno, co spowodowane jest brakiem charakterystycznych objawów podczas rozwoju choroby. Fakt ten z kolei przekłada się na ograniczenie możliwości leczenia [1]. Szczególne zagrożenie stanowi rak trzustki, uważany za jeden z najbardziej agresywnych nowotworów [2]. Objawy kliniczne raka trzustki pojawiają się na zaawansowanym etapie rozwoju choroby, co bardzo często uniemożliwia wprowadzenie efektywnej terapii. Dodatkowo, w momencie diagnozy obecne są liczne przerzuty, zarówno miejscowe jak i odległe [3], [4]. Postępowanie w leczeniu raka trzustki jest uzależnione od stopnia zaawansowania choroby. Leczeniem przynoszącym najlepsze efekty są zabiegi chirurgiczne oraz radioterapia, pod warunkiem, że choroba została zdiagnozowana na wczesnym etapie. Standardem leczenia u pacjentów z przerzutami jest chemioterapia [5]–[7].

Podziały komórek prawidłowych podlegają ścisłym regulacjom, które zostają zaburzone w przypadku kancerogenezy [8]. Podczas rozwoju nowotworu, komórki charakteryzują się przede wszystkim nieograniczonym potencjałem proliferacji, w wyniku zaburzenia kontroli cyklu komórkowego oraz hamowania apoptozy [9]. Dlatego duże zainteresowanie budzą substancje wpływające na co najmniej jeden z tych procesów.

W chemioterapii wielu nowotworów stosowane są leki cytostatyczne, które charakteryzują się wysoką efektywnością w stosunku do komórek o wysokim potencjale do podziałów, działające na nie i będące w różnych fazach cyklu komórkowego. W przypadku raka trzustki chemioterapia wykazuje dosyć niską skuteczność, powodując jednocześnie wiele działań niepożądanych, co stanowi niezwykle istotny czynnik limitujący zastosowanie tej formy leczenia. Przyczyną słabej odpowiedzi na terapię w raku trzustki jest oporność na cytostatyki, zarówno pierwotna, jak również nabyta w trakcie leczenia [10], [11]. Dodatkowo, chemioterapeutyki jako silnie toksyczne związki prowadzą do uszkodzenia nie tylko komórek nowotworowych, ale także komórek prawidłowych w wielu narządach, co prowadzi do licznych powikłań.

Niestety, pomimo postępu w wykrywaniu oraz leczeniu nowotworów, rokowania dla pacjentów ze zdiagnozowanym rakiem trzustki nadal pozostają bardzo złe, a pięcioletnie przeżycie pacjentów od momentu rozpoznania choroby osiąga zaledwie 9% [12], [13].
Obecnie stosowane metody leczenia często okazują się niewystarczające, co skłania do poszukiwania alternatywnych metod terapeutycznych. Dodatkowo, występowanie licznych

skutków ubocznych podczas stosowania chemioterapii skłania do poszukiwania związków zdolnych do niszczenia komórek nowotworowych, przy jednocześnie niskiej toksyczności względem komórek prawidłowych. Takich cech poszukuje się badając substancje pochodzenia naturalnego, wykazujące zróżnicowane właściwości lecznicze, niekiedy umożliwiające wykorzystanie ich w leczeniu różnych chorób [8]. Najczęściej są to związki polifenolowe, których powszechne występowanie w pokarmach roślinnych sprawia, że są one obecne w codziennej diecie. Jako metabolity wtórne roślin znajdują zastosowanie m.in. jako środki ochrony przed czynnikami chorobotwórczymi (wirusami, bakteriami, grzybami) oraz promieniowaniem UV [14], [15]. Podobnych właściwości ochronnych polifenoli upatruje się w kontekście prewencji różnych chorób, szczególnie nowotworowych. W tym celu przeprowadza się szereg badań *in vitro*, oceniających wpływ danego związku na wzrost komórek, regulację cyklu komórkowego, czy też zdolność do indukcji procesu apoptozy.

Jednym z przedstawicieli tej grupy związków jest resweratrol o budowie stilbenu (3,5,4'-trihydroksystilben). Polifenol ten jest obecny w wielu roślinach jadalnych (maliny, borówki, czarna porzeczka, kakaowiec), a jednym z głównych jego źródeł jest skórka ciemnych winogron [16], [17]. W naturze resweratrol występuje w postaci dwóch izomerów: *cis* i *tran*s, przy czym w badaniach naukowych wykorzystywany jest izomer *trans*, charakteryzujący się wyższą aktywnością biologiczną oraz stabilnością [18]. Wskutek działania czynników takich jak: wysokie pH czy promieniowanie UV, forma *trans*- ulega przekształceniu do formy *cis*-resweratrolu [19]. Już na początku lat 90. XX wieku prozdrowotne właściwości tego związku zostały zauważone i opisane w tak zwanym „paradoksie francuskim". Dotyczył on zależności pomiędzy spożyciem czerwonego wina, a niską zapadalnością na choroby wieńcowe wśród mieszkańców basenu Morza Śródziemnego, zwłaszcza w obecności wysokotłuszczowej diety, a korzystne efekty przypisano resweratrolowi – składnikowi czerwonego wina [20], [21]. Kolejne lata przyniosły coraz większą liczbę badań, świadczących o plejotropowym charakterze tego związku. Sugeruje się, że obok działania kardioprotekcyjnego, resweratrol wykazuje m.in. działanie przeciwutleniające oraz przeciwzapalne. W wielu badaniach szczególną uwagę zwrócono na różne aspekty właściwości przeciwnowotworowych resweratrolu, które po raz pierwszy zostały opisane w 1997 roku przez zespół Jang'a. W badaniach tych wykazano, że resweratrol wykazuje zdolność do hamowania rozwoju nowotworu na wszystkich etapach procesu kancerogenezy: inicjacji, promocji oraz progresji [22]. Jego działanie przeciwnowotworowe polega przede wszystkim na zdolności hamowania wzrostu i proliferacji komórek nowotworowych oraz pobudzeniu ich do programowanej śmierci, głównie apoptozy [23].

Bardzo duże nadzieje pokłada się w możliwości zastosowania resweratrolu w leczeniu raka trzustki, a badania naukowe pokazują, że oczekiwania te nie są bezpodstawne, choć mechanizm jego działania nie został jeszcze do końca poznany. Doniesienia naukowe wskazują na ważną rolę resweratrolu we wpływie na regulację szlaków biochemicznych, aktywność enzymów, a tym samym hamowanie proliferacji i indukcji procesu apoptozy w komórkach nowotworowych trzustki [24]–[26].

Podsumowując, przeciwnowotworowa aktywność resweratrolu związana jest przede wszystkim z jego oddziaływaniem na geny oraz białka związane z procesem proliferacji i apoptozy. Obiecujące wyniki wielu badań w modelu *in vitro* zachęcają naukowców do poszukiwania m.in. sposobu na zwiększenie biodostępności resweratrolu, by móc wykorzystać wyniki tych badań w praktyce klinicznej.

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3. Założenia i cel pracy

Choroby nowotworowe stanowią bardzo poważny problem współczesnej medycyny. Rak trzustki, ze względu na brak charakterystycznych objawów w początkowej fazie rozwoju choroby, budzi wiele niepokoju. Dostępne badania naukowe pokazują, że w zapobieganiu oraz leczeniu wielu typów nowotworów istotne znaczenie mają związki pochodzenia naturalnego, głównie ze względu na niski odsetek skutków ubocznych w porównaniu do tradycyjnych metod leczenia, takich jak np. chemioterapia. Grupą najczęściej badanych związków naturalnych są związki z grupy polifenoli, w tym resweratrol. Związek ten ze względu na bardzo zróżnicowany szereg aktywności biologicznych znalazł zastosowanie w leczeniu wspomagającym wielu chorób, w tym chorób nowotworowych.

Ze względu na dużą toksyczność standardowej chemioterapii, wszelkie wysiłki związane z identyfikacją mechanizmów działania polifenoli w celu wykorzystania ich w leczeniu nowotworów są bardzo ważne, zarówno z farmakologicznego jak i medycznego punktu widzenia.

Celem niniejszej pracy było wykazanie w badaniach *in vitro* cytotoksycznego i proapoptotycznego działania resweratrolu na wybrane nowotwory przewodu pokarmowego.

Celem pracy *Expression of genes and proteins of multidrug resistance in gastric cancer cells treated with resveratrol* była ocena wpływu reswratrolu na zmiany ekspresji genów związanych z opornością wielolekową w komórkach raka żołądka wrażliwych i opornych na działanie cytostatyków. Celem pracy poglądowej *Cytotoxic and proapoptotic effects of resveratrol in In vitro studies on selected types of gastrointestinal cancers* było przedstawienie leczniczych właściwości resweratrolu oraz dokonanie przeglądu dostępnych wyników badań w modelu *in vitro* nad antyproliferacyjnym i proapoptotycznym działaniem reswertarolu w nowotworach przewodu pokarmowego. Celem publikacji *Effect of resveratrol treatment on human pancreatic cancer cells through alterations of Bcl-2 family members* było wykazanie w badaniach *in vitro* cytotoksycznego i proapoptotycznego działania resweratrolu na komórki nowotworowe i prawidłowe trzustki, przede wszystkim w odniesieniu do wpływu na białka z rodziny Bcl-2.

4. Streszczenie

Pomimo postępu w dziedzinie profilaktyki i wczesnego wykrywania zmian nowotworowych, nowotwory nadal pozostają jedną z głównych przyczyn śmierci na całym świecie. Szczególnie niebezpieczne są nowotwory trzustki, ze względu na brak charakterystycznych objawów, pozwalających na postawienie diagnozy na wczesnym etapie rozwoju choroby. Tym samym późne rozpoznanie przekłada się na brak możliwości wdrożenia skutecznego leczenia.

Stosowanie cytostatyków w leczeniu większości nowotworów przynosi pożądane efekty w stosunku do komórek nowotworowych. Jednak w przypadku nowotworów trzustki ta forma leczenia charakteryzuje się niską skutecznością, prowadząc jednocześnie do licznych skutków ubocznych. Stąd też poszukuje się związków o działaniu toksycznym dla komórek nowotworowych przy jednocześnie niskiej toksyczności względem komórek prawidłowych. Przykładem takich substancji są związki polifenolowe, obecne w wielu produktach spożywczych, wchodzących w skład naszej codziennej diety. Jednym z przedstawicieli tej grupy związków jest resweratrol.

Resveratrol (3,5,4'-trihydroksystilben) jest fitoaleksyną występującą w wielu roślinach, takich jak maliny, borówki, czarna porzeczka. Głównym jego źródłem są ciemne winogrona. Wyróżnia się dwie formy stereoizomeryczne *cis* i *trans,* przy czym formą bardziej aktywną jest forma *trans*. Resweratrol wykazuje szereg zróżnicowanych aktywności biologicznych, w tym aktywność przeciwnowotworową. Działanie przeciwnowotworowe resweratrolu polega między innymi na hamowaniu proliferacji komórek w poszczególnych fazach cyklu komórkowego oraz indukcji procesu apoptozy.

Badania w pracy (Katarzyna Mieszała, Małgorzata Rudewicz, Agnieszka Gomułkiewicz, Katarzyna Ratajczak-Wielgomas, Jędrzej Grzegrzółka, Piotr Dzięgiel, Sylwia Borska. Expression of genes and proteins of multidrug resistance in gastric cancer cells treated with resveratrol. *Oncol.Lett. 2018,* Vol.15, s. 5825-5832) wykonano na trzech liniach nowotworowych żołądka, wrażliwych (EPG85-257P) i opornych na działanie cytostatyków: daunorubicyny (EPG85-257RDB) i mitoksantronu (EPG85-257RNOV). Za pomocą reakcji real-time PCR w badanych liniach komórkowych dokonano oceny zmian ekspresji genów związanych z opornością wielolekową (MDR, *multidrug resistance*) w wyniku zastosowania różnych stężeń resweratrolu. Następnie za pomocą metody Western Blot określono poziom białek kodowanych przez te geny. Wykorzystując metodę immunofluorescencji potwierdzono

obecność poszczególnych białek w liniach komórkowych. Wyniki przeprowadzonych badań poddano analizie statystycznej.

Analiza otrzymanych wyników wykazała nadekspresję genu *ABCB1* w linii opornej na działanie cytostatyku (daunorubicyny) w porównaniu do linii wrażliwej na jej działanie. Traktowanie resweratrolem w stężeniu 30 μM skutkowało istotnym obniżeniem poziomu ekspresji genu *ABCB1* w porównaniu z komórkami nie poddanymi działaniu związku. Podobną zależność wykazano na poziomie białka. Działanie resweratrolem w stężeniach 30 μM i 50 μM spowodowało spadek poziomu P-glikoproteiny poniżej poziomu oznaczenia ilościowego w stosunku do komórek kontrolnych.

Wykazano także, że w linii komórkowej opornej na działanie daunorubicyny oprócz klasycznego mechanizmu oporności wielolekowej (nadekspresja P-glikoproteiny) istnieją także mechanizmy nietypowe, związane ze zwiększoną ekspresją genów *ANXA1* oraz *TXN*. Ekspresja genu *ANXA1* w komórkach opornych na działanie daunorubicyny jest istotnie wyższa w porównaniu do komórek wrażliwych na jej działanie. Traktowanie resweratrolem w stężeniu 30 μM i 50 μM skutkowało obniżeniem poziomu ekspresji genu *ANXA1* w porównaniu do komórek wrażliwych. Wraz ze wzrostem stężenia resweratrolu zaobserwowano wzrost poziomu Aneksyny I. Obecność tego białka w badanej linii komórkowej została potwierdzona za pomocą reakcji immunofluorescencji (IF).

Ekspresja genu *TXN* w komórkach opornych na działanie daunorubicyny jest wyższa w porównaniu do komórek wrażliwych. Obecność tioredoksyny w badanych komórkach potwierdzono za pomocą reakcji immunofluorescencji. Pod działaniem resweratrolu doszło do obniżenia poziomu ekspresji *TXN*. Zależność ta została także potwierdzona na poziomie białka.

W linii komórkowej opornej na działanie mitoksantronu odnotowano obecność nietypowych mechanizmów oporności wielolekowej, związanych z nadekspresją genu *TXN* w stosunku do komórek wrażliwych na działanie cytostatyku. Po zastosowaniu resweratrolu w stężeniu 30 μM zaobserwowano znaczące obniżenie poziomu ekspresji *TXN* w stosunku do komórek opornych, niepoddanych działaniu badanego związku. Zależności te potwierdziły eksperymenty na poziomie białka.

W pracy poglądowej (Katarzyna Ratajczak, Sylwia Borska. Cytotoxic and Proapoptotic Effects of Resveratrol in *In Vitro* Studies on Selected Types of Gastrointestinal Cancers. *Mol. 2021,* Vol. 26; s. 4350) został przedstawiony przegląd piśmiennictwa dotyczącego antyproliferacyjnego i proapoptotycznego działania resweratrolu w nowotworach przewodu pokarmowego (rak trzustki, żołądka, wątroby oraz jelit). Dodatkowo zwrócono także uwagę na

zjawisko MDR, będącej często przyczyną nieskuteczności klasycznej chemioterapii. Opisano rolę resweratrolu jako jednego z przedstawicieli związków naturalnych wykazujących właściwości plejotropowe, w tym przeciwnowotworowe, a także zdolność do „przełamywania" MDR. Przegląd piśmiennictwa dotyczącego badań w modelu *in vitro* dowodzi, że resweratrol działa na komórki wybranych nowotworów przewodu pokarmowego hamując ich proliferację i wykazując właściwości proapoptotyczne o różnym nasileniu. Jednocześnie substancja ta wykazuje zróżnicowane działanie w zależności od typu nowotworu, stężenia badanego związku, czasu inkubacji, w odniesieniu do efektu przeciwnowotworowego. W komórkach raka trzustki (PANC-1, AsPC-1, BxPC-3, Capan-2, CEPAC-1, MIA Paca-2, Hs766T) działanie antyproliferacyjne oraz proapoptotyczne obserwowane jest po 48 godzinach inkubacji w wyższych stężeniach resweratrolu (>100 μM). Działanie to polega między innymi na regulacji ekspresji białek pro- i antyapoptotycznych (Bax i Bcl-2). W przypadku komórek raka żołądka (MGC803, SGC-7901, BGC823, GES1) działanie to obserwowane jest w zakresie stężeń 0-400 μM i związane jest między ze wzrostem produkcji ROS (ang. *Reactive Oxygen Species*). Z kolei w komórkach raka wątroby (HepG2, Bel-7402, SMMC-7721, MHCC97-H) i jelita (HT-29, WiDr, HCA-17, SW480, HCT-116, Caco-2, CO-115, DLD1, HCT15) działanie antyproliferacyjne oraz proapoptotyczne obserwowane jest już w niższych stężeniach resweratrolu, w zakresach 0-100 μM i 0-50 μM. Efekty te są związane między innymi z regulacją ekspresji białek pro- i antyapoptotycznych.

Badania w trzeciej pracy z cyklu publikacji (Katarzyna Ratajczak, Natalia Glatzel-Plucińska, Katarzyna Ratajczak-Wielgomas, Katarzyna Nowińska, Sylwia Borska. Effect of Resveratrol Treatment on Human Pancreatic Cancer Cells through Alterations of Bcl-2 Family Members. *Mol. 2021,* Vol. 26; s. 6560) wykonano na trzech liniach nowotworowych trzustki: EPP85-181P, EPP85-181RNOV (komórki oporne na działanie mitoksantronu), AsPC-1 oraz prawidłowej linii trzustki H6c7. Za pomocą testu kolorymetrycznego (MTT) oraz metody cytometrii przepływowej (FACS) zbadano cytotoksyczne działanie resweratrolu. Następnie z wykorzystaniem metody FACS dokonano analizy wpływu resweratrolu na cykl komórkowy badanych komórek. Dokonano także oceny nasilenia apoptozy komórek spowodowanej działaniem resweratrolu metodą FACS oraz metodą TUNEL. Zmiany ekspresji genów związanych z procesem apoptozy (*BAX* i *BCL2*) zbadano z wykorzystaniem techniki real-time PCR. Metodę Western Blot wykorzystano do oceny zmian poziomu białek związanych z procesem apoptozy: Bax i Bcl-2.

Analiza wyników reakcji kolorymetrycznej MTT wykazała, że komórki linii EPP85-181RNOV pomimo oporności na mitoksantron są bardziej wrażliwe na działanie resweratrolu

w porównaniu do komórek EPP85-181P (linia wrażliwa na cytostatyk). Z kolei najmniejszą wrażliwość na działanie resweratrolu wykazywały komórki linii AsPC-1. Zależny od stężenia i czasu działania spadek żywotności komórek odnotowano także w prawidłowych komórkach trzustki (H6c7). Na postawie otrzymanych wyników wytypowano trzy stężenia resweratrolu: 25, 50 i 100 μM oraz czas inkubacji 48 godzin, stosowane w pozostałych eksperymentach. Badania rozkładu faz cyklu komórkowego wykazały, że resweratrol prowadzi do kumulacji komórek w fazie G0/G1 lub S, w zależności od typu komórek oraz stężenia związku. W komórkach nowotworowych EPP85-181P i EPP85-181RNOV hamowanie cyklu komórkowego w fazie S obserwowano przy niższych stężeniach związku (25 i 50 μM), natomiast przy wyższych stężeniach resweratrolu (100 μM) występowało hamowanie w fazie G0/G1. W przypadku komórek nowotworowych AsPC-1 nie wykazano istotnych statystycznie różnic w hamowaniu cyklu komórkowego pomiędzy badanymi stężeniami. Wystąpiły jednak istotne zmiany w rozkładzie komórek w określonych fazach cyklu komórkowego. W komórkach prawidłowych trzustki H6c7 działanie resweratrolem nie powodowało istotnego zatrzymania komórek w poszczególnych fazach cyklu.

Metoda TUNEL wykazała wzrost apoptozy we wszystkich badanych liniach komórkowych, a zmiany były zależne od zastosowanego stężenia związku. W linii komórkowej AsPC-1 zaobserwowano najmniejszy wzrost liczby komórek apoptotycznych. Z kolei linie komórkowe EPP85-181P oraz EPP85-181RNOV charakteryzował umiarkowany wzrost liczby komórek apoptotycznych. Największą liczbę komórek apoptotycznych odnotowano w linii komórkowej H6c7 przy najwyższym stężeniu resweratrolu (100 μM).

Reakcje immunocytochemiczne (IHC) mające na celu ocenę wpływu resweratrolu na białka związane z procesem apoptozy (Bax, Bcl-2, Kaspaza-3), wykazały zależny od stężenia spadek poziomu białka Bcl-2 oraz wzrost poziomu białek Bax i Kaspazy-3 we wszystkich badanych nowotworowych liniach komórkowych. W prawidłowej linii komórkowej trzustki resweratrol nie powodował istotnych zmian poziomu poszczególnych białek.

Badania wykonane metodą real-time PCR wykazały zależne od stężenia resweratrolu zmiany ekspresji genów związanych z procesem apoptozy (*BAX*, *BCL2*). W przypadku linii komórkowej EPP85-181P działanie resweratrolem spowodowało zmniejszenie ekspresji *BAX* i wzrost ekspresji *BCL2* (przy stężeniu 25μM) w porównaniu do komórek nietraktowanych ww. związkiem. Pomiędzy stężeniami 50 i 100 μM zaobserwowano spadek poziomu ekspresji *BCL2*. Z kolei w linii komórkowej EPP85-181RNOV resweratrol powodował zmniejszenie ekspresji *BAX* w sposób zależny od stężenia. Przy stężeniu 25 μM zaobserwowano spadek poziomu ekspresji *BCL2*, a pomiędzy stężeniami 50 i 100 μM odnotowano jego wzrost.

W przypadku linii komórkowej AsPC-1 uzyskano podobne zależności. W prawidłowej linii komórkowej trzustki H6c7 działanie resweratrolem skutkowało zwiększeniem poziomu ekspresji *BAX* przy stężeniu 25 μM oraz zmniejszeniem jego poziomu pomiędzy stężeniem 50 i 100 μM. Z kolei stężenie 25 μM prowadziło do obniżenia poziomu ekspresji *BCL2*, natomiast stężenia 50 i 100 μM powodowały jego wzrost.

Ocenę zdolności resweratrolu do modulacji białek związanych z procesem apoptozy przeprowadzono za pomocą metody Western Blot. W linii komórkowej EPP85-181P stężenie 25 μM powodowało wzrost poziomu proapoptotycznego białka Bax, natomiast pomiędzy wyższymi stężeniami (50 i 100 μM) doszło do jego obniżenia w porównaniu z komórkami nie poddanymi działaniu resweratrolu. W przypadku białka antyapoptotycznego Bcl-2 działanie resweratrolu w niższym stężeniu (25 μM) powodowało wzrost poziomu białka, podczas gdy wyższe stężenia powodowały jego spadek. W linii komórkowej EPP85-181RNOV, opornej na działanie cytostatyku, reswerarol w stężeniu 25 μM powodował wzrost poziomu białka Bax, a z kolei wyższe stężenia prowadziły do jego obniżenia i znajdowały się na podobnym poziomie. Poziom białka Bcl-2 przy stężeniu 25 μM był niższy w porównaniu do komórek nietraktowanych, a wyższe stężenia nie powodowały znaczących zmian. W kolejnej badanej linii komórkowej AsPC-1 przy stężeniu 25 μM zauważono niewielki spadek poziomu białka proapoptotycznego Bax w stosunku do komórek nie poddanych działaniu resweratrolu, a wyższe stężenia powodowały wzrost jego poziomu. Podobne zależności wykazano dla białka antyapoptotycznego Bcl-2. Stężenie 25 μM skutkowało obniżeniem poziomu białka, a wyższe stężenia prowadziły do jego wzrostu. W prawidłowej linii komórkowej trzustki H6c7 działanie resweratrolu nie powodowało istotnych zmian w poziomie białka proapoptotycznego Bax. Jedynie przy najwyższym jego stężeniu (100 μM) odnotowano jego wzrost. Z kolei w przypadku białka antyapoptotycznego Bcl-2 zaobserwowano wzrost jego poziomu, a zmiany były proporcjonalne do stężenia resweratrolu.

Podsumowując, badania w modelu *in vitro* na komórkach nowotworowych trzustki i żołądka wskazują na zależność pomiędzy efektem działania resweratrolu, a zastosowanym stężeniem i czasem inkubacji. Wyniki badań potwierdziły przeciwnowotworową aktywność resweratrolu poprzez wpływ na hamowanie proliferacji oraz indukowanie apoptozy. Mechanizmy działania obejmują zmianę ekspresji genów oraz poziomu białek związanych z cyklem komórkowym i apoptozą. Ponadto poprzez wpływ na geny i białka związane z opornością wielolekową, resweratrol może mieć istotne znaczenie w „przełamywaniu" tego zjawiska.

5. Summary

Despite advances in the field of prevention and early detection of neoplastic changes, cancer remains one of the leading causes of death worldwide. Pancreatic neoplasms are particularly dangerous due to the lack of characteristic symptoms allowing for diagnosis at an early stage of the disease development. Thus, late diagnosis translates into the inability to implement effective treatment.

The use of cytostatics in treating most types of cancer has the desired effect on cancer cells. However, this treatment is of low effectiveness in the case of pancreatic tumors, leading to numerous side effects simultaneously. Hence, compounds are sought that are toxic to neoplastic cells with low toxicity to normal cells simultaneously. Examples of such substances are polyphenolic compounds in many food products in our daily diet. One of the representatives of this group of compounds is resveratrol.

Resveratrol (3,5,4'-trihydroxystilbene) is a phytoalexin found in many plants, such as raspberries, blueberries, and black currants. Its main source is dark grapes. There are two stereoisomeric forms, *cis* and *trans*, the more active form being the *trans* form. Resveratrol exhibits several diverse biological activities, including anticancer activity. The antitumor activity of resveratrol consists, among other things, in inhibiting the proliferation of cells in particular phases of the cell cycle and inducing the process of apoptosis.

Research at work (Katarzyna Mieszała, Małgorzata Rudewicz, Agnieszka Gomułkiewicz, Katarzyna Ratajczak-Wielgomas, Jędrzej Grzegrzółka, Piotr Dzięgiel, Sylwia Borska. Expression of genes and proteins of multidrug resistance in gastric cancer cells treated with resveratrol. Oncol.Lett. 2018, Vol 15, pp. 5825-5832) was performed on three gastric cancer lines, sensitive (EPG85-257P) and resistant to cytostatics: daunorubicin (EPG85-257RDB) and mitoxantrone (EPG85-257RNOV). The changes in gene expression associated with multidrug resistance (MDR) as a result of using different concentrations of resveratrol were assessed using real-time PCR reactions in the tested cell lines. Then, the level of proteins encoded by these genes was determined using the Western Blot method. Using the immunofluorescence method, the presence of individual proteins in cell lines was confirmed. The results of the conducted research were subjected to statistical analysis.

The analysis of the obtained results showed the overexpression of the *ABCB1* gene in the line resistant to the cytostatics (daunorubicin) compared to the line sensitive to its action. Treatment with resveratrol at a concentration of 30 μM resulted in a significant reduction in the expression level of the *ABCB1* gene compared to untreated cells. A similar relationship was

demonstrated at the protein level. Treatment with resveratrol at concentrations of 30 μM and 50 μM resulted in a decrease in the level of P-glycoprotein below the quantification level of control cells.

It has also been shown that in the daunorubicin-resistant cell line, apart from the classic mechanism of multidrug resistance (P-glycoprotein overexpression), there are also atypical mechanisms related to increased expression of *ANXA1* and *TXN* genes. Expression of the *ANXA1* gene in daunorubicin-resistant cells is significantly higher than in daunorubicin-sensitive cells. Treatment with resveratrol at a concentration of 30 μM and 50 μM reduced the expression of the *ANXA1* gene compared to sensitive cells. As the concentration of resveratrol increased, an increase in the level of Annexin I was observed. The presence of this protein in the tested cell line was confirmed by the immunofluorescence (IF) reaction.

Expression of the *TXN* gene in daunorubicin-resistant cells is higher compared to sensitive cells. The presence of thioredoxin in the tested cells was confirmed by an immunofluorescence reaction. Under the action of resveratrol, the expression level of *TXN* was reduced. This relationship was also established on the protein level.

In the mitoxantrone-resistant cell line, the presence of atypical multi-drug resistance mechanisms related to the overexpression of the *TXN* gene in relation to cytostatic-sensitive cells has been reported. After applying resveratrol at a concentration of 30 μM, a significant reduction in the expression level of *TXN* was observed in resistant cells not exposed to the test compound. Experiments confirmed these relationships at the protein level.

The review (Katarzyna Ratajczak, Sylwia Borska. Cytotoxic and Proapoptotic Effects of Resveratrol in In Vitro Studies on Selected Types of Gastrointestinal Cancers. Mol. 2021, Vol. 26; p. 4350) presents a review of the literature on the antiproliferative and pro-apoptotic effects of resveratrol in gastrointestinal cancer (cancer of the pancreas, stomach, liver, and intestines). In addition, attention was also paid to the phenomenon of MDR, which is often the reason for the ineffectiveness of traditional chemotherapy. The role of resveratrol as one of the representatives of natural compounds demonstrating pleiotropic properties, including anticancer properties, as well as the ability to "break" MDR, has been described. A review of the literature on studies in an *in vitro* model proves that resveratrol acts on the cells of selected gastrointestinal neoplasms by inhibiting their proliferation and demonstrating proapoptotic properties of varying severity. At the same time, this substance exhibits various effects depending on the type of tumor, the concentration of the test compound, and incubation time, about the antitumor effect. In pancreatic cancer cells (PANC-1, AsPC-1, BxPC-3, Capan-2, CEPAC-1, MIA Paca-2, Hs766T), the antiproliferative and proapoptotic effect is observed after

48 hours of incubation in higher concentrations of resveratrol (>100 μM). This action is based, among other things, on regulating the expression of pro- and antiapoptotic proteins (Bax and Bcl-2). In the case of gastric cancer cells (MGC803, SGC-7901, BGC823, GES1), this effect is observed in the concentration range of 0-400 μM and is associated with an increase in the production of ROS (*Reactive Oxygen Species*). In turn, in liver cancer cells (HepG2, Bel-7402, SMMC-7721, MHCC97-H) and intestines (HT-29, WiDr, HCA-17, SW480, HCT-116, Caco-2, CO-115, DLD1, HCT15), antiproliferative and proapoptotic effects are already observed at lower concentrations of resveratrol, in the ranges of 0-100 μM and 0-50 μM. These effects are related to, among other things, the regulation of the expression of pro- and antiapoptotic proteins.

Research in the third work in the series of publications (Katarzyna Ratajczak, Natalia Glatzel-Plucińska, Katarzyna Ratajczak-Wielgomas, Katarzyna Nowińska, Sylwia Borska. Effect of Resveratrol Treatment on Human Pancreatic Cancer Cells through Alterations of Bcl-2 Family Members. Mol. 2021, Vol. 26; p. 6560) was performed on three pancreatic cancer lines: EPP85-181P, EPP85-181RNOV (mitoxantrone-resistant cells), AsPC-1, and the normal pancreatic line H6c7. The cytotoxic effects of resveratrol were investigated using the colorimetric test (MTT) and the flow cytometry (FACS) method. Then, using the FACS method, an analysis of the effect of resveratrol on the cell cycle of the studied cells was carried out. The intensity of cell apoptosis caused by resveratrol was also assessed using the FACS and the TUNEL methods. Changes in the expression of genes related to the apoptosis process (*BAX* and *BCL2*) were investigated using the real-time PCR technique. The Western Blot method was used to evaluate the changes in the level of proteins related to the apoptosis process: Bax and Bcl-2.

The analysis of the results of the MTT colorimetric reaction showed that the cells of the EPP85-181RNOV line, despite resistance to mitoxantrone, are more sensitive to the action of resveratrol compared to EPP85-181P cells (cytostatic-sensitive line). In turn, the AsPC-1 cells showed the lowest sensitivity to the effects of resveratrol. A concentration-dependent and time-dependent decrease in cell viability were also noted in normal pancreatic cells (H6c7). Based on the obtained results, three concentrations of resveratrol were selected: 25, 50, and 100 μM, and the incubation time of 48 hours was used in the remaining experiments. Cell cycle phase distribution studies have shown that resveratrol leads to cell accumulation in the G0/G1 or S phase, depending on the cell type and compound concentration. In tumor cells, EPP85-181P and EPP85-181RNOV, cell cycle inhibition in the S phase was observed at lower concentrations of the compound (25 and 50 μM). In comparison, inhibition in G0/G1 occurred at higher

concentrations of resveratrol (100 μM). In the case of AsPC-1 neoplastic cells, no statistically significant differences in cell cycle inhibition between the tested concentrations were found. However, there were substantial changes in cellular distribution at certain phases of the cell cycle. In normal H6c7 pancreatic cells, the action of resveratrol did not cause significant cell arrest at any stage of the cycle.

The TUNEL method showed an increase in apoptosis in all tested cell lines, and the changes depended on the concentration of the compound used. In the AsPC-1 cell line, the smallest growth in the number of apoptotic cells was observed. In turn, the EPP85-181P and EPP85-181RNOV cell lines were characterized by a moderate increase in the number of apoptotic cells. The highest number of apoptotic cells was recorded in the H6c7 cell line with the highest concentration of resveratrol (100 μM).

Immunocytochemical reactions (IHC) to assess the effect of resveratrol on apoptotic proteins (Bax, Bcl-2, Kaspaza-3) showed a concentration-dependent decrease in the level of Bcl-2 and an increase in the level of Bax and Kaspase-3 in all cancer cell lines tested. In a normal pancreatic cell line, resveratrol did not cause any significant changes in the levels of individual proteins.

Real-time PCR studies showed changes in the expression of genes related to the apoptosis process (*BAX*, *BCL2*) dependent on the concentration of resveratrol. In the case of the EPP85-181P cell line, treatment with resveratrol resulted in a decrease in *BAX* expression and an increase in *BCL2* expression (at a concentration of 25 μM) compared to untreated cells. Between concentrations of 50 and 100 μM, a decrease in the level of *BCL2* expression was observed. In contrast, in the EPP85-181RNOV cell line, resveratrol reduced *BAX* expression in a concentration-dependent manner. At a concentration of 25 μM, a decrease in the level of *BCL2* expression was observed, and an increase was noted between concentrations of 50 and 100 μM. Similar relationships were found in the case of the AsPC-1 cell line. In the normal H6c7 pancreatic cell line, treatment with resveratrol resulted in an increase in *BAX* expression levels at a concentration of 25 μM and a reduction in *BAX* levels between 50 and 100 μM. On the other hand, the concentration of 25 μM decreased the level of *BCL2* expression, while the concentrations of 50 and 100 μM caused its increase.

The ability of resveratrol to modulate proteins related to the apoptosis process was assessed using the Western Blot method. In the EPP85-181P cell line, the concentration of 25 μM increased the level of the proapoptotic protein Bax. In contrast, between the higher concentrations (50 and 100 μM), there was a reduction in it compared to cells not treated with resveratrol. In the case of the antiapoptotic protein Bcl-2, the action of resveratrol at a lower

concentration (25 μM) caused an increase in the level of the protein, while higher concentrations caused its decrease. In the cytostatic-resistant cell line EPP85-181RNOV, resveratrol at a concentration of 25 μM increased the level of the Bax protein, while higher concentrations led to a decrease in protein and were at a similar level. The level of Bcl-2 protein at the concentration of 25 μM was lower than the untreated cells, and the higher concentrations did not cause significant changes. In the following tested cell line, AsPC-1, at a concentration of 25 μM, a slight decrease in the proapoptotic protein Bax was observed about cells not treated with resveratrol, and higher concentrations increased its level. Similar relationships were demonstrated for the antiapoptotic protein Bcl-2. A concentration of 25 μM resulted in a decrease in the protein level, and higher concentrations led to an increase in protein. In the normal H6c7 pancreatic cell line, the action of resveratrol did not cause significant changes in the level of the proapoptotic protein Bax. Its increase was noted only at its highest concentration (100 μM). In turn, in the case of the antiapoptotic protein Bcl-2, an increase in its level was observed, and the changes were proportional to the concentration of resveratrol.

In conclusion, studies in an *in vitro* model on pancreatic and gastric cancer cells indicate a connection between the effect of resveratrol and the concentration used and the incubation duration. The study results confirmed the anticancer activity of resveratrol by inhibiting proliferation and inducing apoptosis. Mechanisms of action include altering gene expression and the level of associated proteins connected with the cell cycle and apoptosis. In addition, by affecting multidrug resistance-related genes and proteins, resveratrol may be important in ‘overcoming’ this phenomenon.

6. Publikacje

Publikacje będące podstawą pracy doktorskiej:

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Expression of genes and proteins of multidrug resistance in gastric cancer cells treated with resveratrol

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Abstract. Multidrug resistance (MDR) is a notable problem in the use of chemotherapy. Therefore, studies aimed at identifying substances capable of overcoming resistance of cancer cells are required. Examples of these compounds are polyphenols, including resveratrol, that exert a range of various biological activities. The aim of the present study was to demonstrate the effect of 3,5,4'-trihydroxy-*trans*-stilbene (resveratrol) on the expression of ATP binding cassette subfamily B member 1, Annexin A1 (*ANXA1*) and thioredoxin (*TXN*) genes, and the proteins encoded by these genes, which are associated with MDR. The experiments were performed in human gastric cancer cell lines EPG85-257RDB (RDB) and EPG85-257RNOV (RNOV), which are resistant to daunorubicin and mitoxantrone, respectively, in addition to EPG85-257P (control), which is sensitive to cytostatic drugs. Cells were treated with 30 or 50 μM resveratrol for 72 h and changes in the expression levels of the genes were analysed with the use of a reverse transcription-quantitative polymerase chain reaction. The cellular levels of P-glycoprotein (P-gp), ANXA1 and TXN were evaluated using immunofluorescence and western blot analysis. Resveratrol in both concentrations has been shown to have a statistically significant influence on expression of the mentioned genes, compared with untreated cells. In RDB cells, resveratrol reduced the expression level of all analyzed genes, compared with untreated cells. Similar results at the protein level were obtained for P-gp and TXN. In turn, in the RNOV cell line, resveratrol reduced TXN expression at mRNA and protein levels, compared with untreated cells. The results of the present study indicate that resveratrol may reduce the resistance of cancer cells by affecting the expression of a number of the genes and proteins associated with MDR.

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Abbreviations: P, human gastric carcinoma EPG85-257P cell line; RDB, human gastric carcinoma EPG85-257RDB cell line; RNOV, human gastric carcinoma EPG85-257RNOV cell line; R30, 30 μM resveratrol; P-gp, P-glycoprotein; GUSB, β-glucuronidase; POLR2A, RNA polymerase IIa

Key words: multidrug resistance, resveratrol, gastric cancer

Introduction

Resveratrol belongs to the stilbenoid group of polyphenols, possessing two phenol rings linked to each other by an ethylene bridge (1). In 1940, resveratrol was isolated from the root of *Veratrum grandiflorum* (1,2). A primary dietary source of resveratrol is red wine, as a very high resveratrol concentration is present in the skin of red grapes (50-100 μg/g) (3). In natural conditions, resveratrol is synthesized by plants in response to external environmental factors, including ultraviolet radiation and heavy metals (3,4). Resveratrol is identified in two isomeric forms, *cis* and *trans*-resveratrol (Fig. 1) (5,6).

The *trans* form is dominant in terms of its prevalence and biological activity (7). The hydrophobic nature of resveratrol considerably contributes to its limited bioavailability (8). Owing to the varied biological activity of resveratrol, it has been the subject of numerous studies aimed at revealing its health-enhancing properties, and its use in prevention and treatment of many diseases (1,3,5,9). The results of *in vitro* and *in vivo* studies have proved that resveratrol exhibits anticancer activity at all three stages of the oncogenic process: initiation, promotion and progression (10).

Neoplastic diseases are presently one of the main causes of global mortality. One of the most commonly used cancer treatment methods is chemotherapy (11); however, its effectiveness is substantially reduced by multidrug resistance (MDR) (12-14). MDR is defined as the insensitivity to therapeutic substances that are not associated by structure or mechanism of action (15,16). The classical mechanism of MDR is associated with the overexpression of the ATP binding cassette subfamily B member 1 (*ABCB1)* gene encoding P-glycoprotein (P-gp), which contributes to the

reduction of the effective drug concentration in the cell by transporting the drug out of the cell (14,15,17-20). In addition to the classical MDR mechanism associated with overexpression of P-gp, there are atypical mechanisms (21-23). Examples of these atypical mechanisms include the overexpression of Annexin A1 (ANXA1) and thioredoxin (TXN) proteins. The *ANXA1* gene encodes the ANXA1 protein, which reversibly binds Ca^{2+} ions and phospholipids of the plasma membrane (24). ANXA1 regulates cell growth, differentiation and apoptosis (25). Increased expression of *ANXA1* contributes to the formation of drug-filled vesicles, which are then exocytosed (26). The *TXN* gene encodes a protein involved in the process of the regulation of cellular redox state. In cancer cells, increased TXN expression results in an increase in proliferation and cell survival (27). Resveratrol is an example of a natural compound with the ability to overcome resistance to chemotherapy. *In vitro* studies on the sensitisation of cells to daunorubicin (DB) with the use of resveratrol, in cases of leukaemia, pancreatic and prostate cancer, it was revealed that resveratrol increased the apoptosis of those cells (28). The aim of the present study was to evaluate the effect of resveratrol on the expression level of *ABCB1*, *ANXA1* and *TXN* genes, which are associ- ated with the MDR phenomenon in DB- and mitoxantrone (MTX)-resistant human gastric cancer cells.

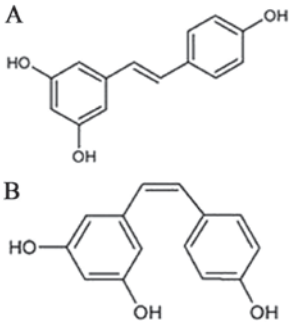

Figure 1. Structural formula of resveratrol isomeric forms. (A) *Trans*-resveratrol and (B) *cis*-resveratrol.

Materials and methods

Cell lines and culture conditions. In vitro studies were performed in three human gastric cancer cell lines that were either sensitive or resistant to cytostatic drugs. EPG85-257P (P) cell line is sensitive to DB and MTX, whereas EPG85-257RDB (RDB) and EPG85-257RNOV (RNOV)

cell lines are resistant to DB and MTX, respectively. Cell lines were obtained from the Institute of Pathology, Charité Campus Mitte, Humboldt University (Berlin, Germany). Cells were cultured in 75 cm^2 culture flasks (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Leibovitz's medium L-15 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), supple- mented with 10% foetal bovine serum, 1 mM L-glutamine,

6.25 mg/l fetuin, 80 IE/l insulin, 2.5 mg/l transferrin, 1 g/l glucose, 1.1 g/l $NaHCO_3$ and 1% minimal essential vitamins (all from Sigma-Aldrich; Merck KGaA) was used to culture all cells. Adequate resveratrol (Sigma-Aldrich; Merck KGaA) was added to the medium to maintain resistance of the cells,

2.5 µg/ml DB (Sigma-Aldrich; Merck KGaA) for RDB and

0.02 µg/ml MTX (Sigma-Aldrich; Merck KGaA) for the RNOV cell line.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Changes in the expression of *ABCB1*, *ANXA1* and *TXN* genes in P, RDB and RNOV cell lines were evaluated following the use of resveratrol at concentrations of 30 µM (R30) and 50 µM (R50). Resveratrol concentrations were chosen based on cytotoxicity studies for this compound (29). After 72 h incubation at 37˚C with resveratrol, the cells were trypsinized (37˚C for 7 min; Sigma-Aldrich; Merck KGaA) and the resulting residues were collected. RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) was used for RNA isolation according to the manufacturer's protocol. To remove genomic DNA, samples were digested using the RNase-Free DNase Set (Qiagen GmbH), according to the manufac- turer's protocol. Next, reverse transcriptase was performed using the High-Capacity cDNA Reverse Transcription kits (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Changes in gene expression were evaluated using RT-qPCR with the use of the 7900HT Fast Real Time PCR system, and SDS 2.3 and RQ Manager 1.2 software (Applied Biosystems; Thermo Fisher Scientific, Inc.). Glucuronidase-β (*GUSB)* and RNA poly- merase II subunit A (*POLR2A*) were used as reference genes. Relative expression of the genes in RDB and RNOV cells were assessed in comparison with the P cell line. The following sets of primers and TaqMan probes with FAM™ dye-labeled were used in the reactions: Hs00184500_m1 for *ABCB1*, Hs00167549_m1 for *ANXA1*, Hs01555212_g1 for *TXN*, Hs99999908_m1 for *GUSB* and Hs00172187_m1 for *POLR2A* (Applied Biosystems; Thermo Fisher Scientific, Inc.). Thermocycling reactions were conducted in triplicate under the following conditions: polymerase activation at 50˚C for 2 min, initial denaturation at 94˚C for 10 min, and 40 cycles of denaturation at 94˚C for 15 sec and annealing of the primers and probes and synthesis at 60˚C for 1 min. Relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (30). Three experimental replicates were performed.

Western blot analysis. Total cellular proteins were isolated from each of the tested cell lines at 4˚C for 10 min, using 200 µl/3 mln cells with radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% IGEPAL CA-630 and 0.5% sodium deoxycholate),
0.5 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail 10 µl/ml RIPA. The samples were agitated for 30 min at 4˚C, and centrifuged (4˚C for 12 min at 12,000 x g) and the supernatant was collected. Protein concentration was determined using a bicinchoninic acid assay with the use of Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). A suitable quantity of 4X Glo Lysis buffer (31) was added to the remaining supernatant and was denaturized at 95˚C for 10 min. Electrophoresis was performed by 10% SDS-PAGE at 140 V. Total cellular proteins were visual- ized with the Chemi Doc MP (Bio-Rad Laboratories, Inc., Hercules, CA, USA) apparatus. A total of 30 µg proteins (per lane) were transferred to polyvinylidene fluoride membrane (Immobilon-P; EMD Millipore, Billerica, MA, USA) in

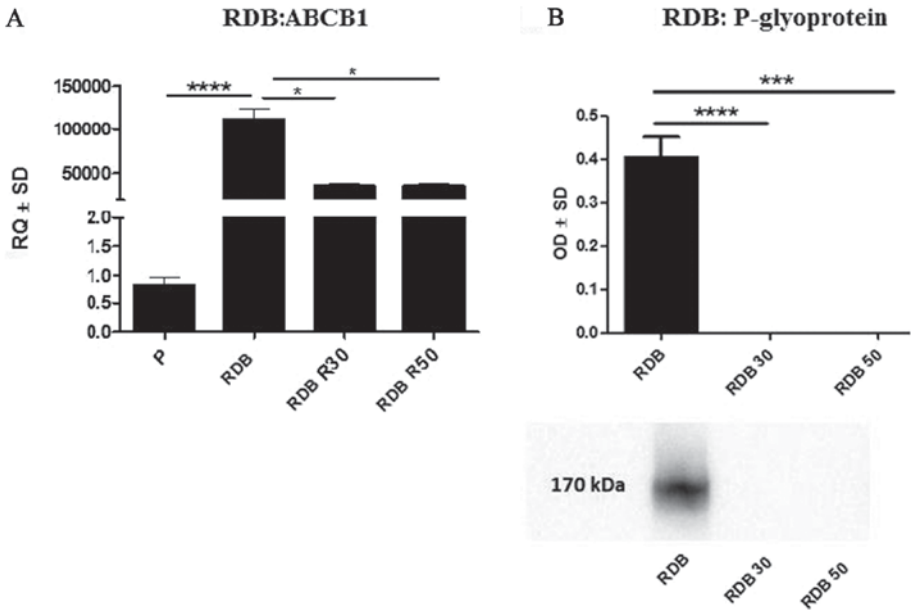

Figure 2. Effect of resveratrol on the levels of *ABCB1* and P-gp in a RDB cell line. Expression levels of (A) *ABCB1* gene and (B) P-gp protein in P and RDB cell lines. *P<0.05 and ****P<0.0001 with comparisons indicated by lines. *ABCB1*, ATP binding cassette subfamily B member 1; P-gp, P-glycoprotein; P, EPG85-257P cell line; RDB, EPG85-257RDB cell line; R30, 30 µM resveratrol; RQ, relative quantity; SD, standard deviation; OD, optical density.

transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol and 0.1% SDS, pH 9.2) by constant current at 250 mA for 1 h. Subsequent to washing the membranes in distilled water and 0.1% TBS-Tween-20 (TBST), they were blocked with 4% BSA (Sigma-Aldrich; Merck KGaA) in 0.1% TBST (room temperature for 1 h). Membranes were incubated with primary antibodies: Mouse anti-P-gp monoclonal antibody C219 (dilution 1:300; cat. no. ALX-801-002-C100; Enzo Life Sciences, Inc., Farmingsale, NY, USA), rabbit anti-ANXA1 polyclonal Ab H-65 (dilution 1:150; cat. no. sc-11387; Santa Cruz Biotechnology, Inc., Dallas, TX, US), rabbit anti-TXN monoclonal Ab (dilution 1:5,000; cat. no. LS-B7196; LifeSpan Biosciences, Inc., Seattle, WA, USA) at 4˚C for 12 h. Secondary anti-mouse and anti-rabbit antibodies were used at a dilution of 1:3,000 at room temperature for 1 h. (Cy™ 3 AffiniPure Donkey Anti-Rabbit IgG (H+L); cat. no. 711-025-152; and Peroxidase AffiniPure Donkey Anti-Mouse IgG (H+L); cat. no. 715-025-150; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). The measurements were taken with the Chemi Doc MP System apparatus, using Image Lab 5.0 Software (both from Bio-Rad Laboratories, Inc.). The level of protein expression was determined as the ratio between signal intensity for a given protein and signal intensity for total cellular proteins.

Immunofluorescence (IF). The cells for the IF reaction were cultured at a density of $1x10^4$ cells/well for RDB and $8x10^3$ cells/well for RNOV in 8-wells Milicell® EZ SLIDES (Sigma-Aldrich; Merck KGaA). Culturing was performed under the same conditions as for RT-qPCR. The cells were fixed in 4% paraformaldehyde for 12 min at room temperature. For membrane permeabilisation, 0.2% Triton X-100 was used (for 10 min at room temperature). The cells were incubated for 1 h at room temperature with the following primary antibodies: Mouse anti-P-gp monoclonal antibody C219 (dilution 1:100; cat. no. ALX-801-002-C100; Enzo Life Sciences, Inc.), rabbit anti-ANXA1 polyclonal antibody H-65 (dilution 1:100; cat. no. sc-11387; Santa Cruz Biotechnology, Inc.) and rabbit anti-TXN monoclonal antibody (dilution 1:100; cat. no. LS-B7196; LifeSpan Biosciences, Inc.). Proteins were detected with [tetramethylrhodamine (TRITC)-conjugated Affini Pure® donkey anti- rabbit Ig G (H+L)] and TRITC-conjugated AffiniPure® donkey anti-mouse IgG (H+L) secondary antibodies (cat. nos. 711-025-152 and 715-025-150; Jackson's ImmunoResearch Laboratories, Inc.), at a dilution of 1:2,000 (1 h incubation at 4˚C). Preparations were mounted on DAPI-containing media (Prolong Gold Antifode Reagent with DAPI; Thermo Fisher Scientific, Inc.). The analysis of protein expression was conducted using a fluorescent micro- scope (Olympus BX41; Olympus Corporation, Tokyo, Japan) at x40 magnification.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance for ranks (Kruskal-Wallis test) was performed using post-hoc Dunn's or Bonferroni tests. Data were presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of resveratrol on the level of expression of ABCB1 gene and P-gp protein in an RDB cell line. The untreated DB-resistant RDB cell line significantly overexpressed the *ABCB1* gene when compared with the P cell line, which is DB-sensitive (P<0.0001; Fig. 2A). The level of *ABCB1* gene expression was significantly reduced following the use of resveratrol at R30 concentration in comparison with expression in the untreated DB-resistant cell line (P<0.05). A similar

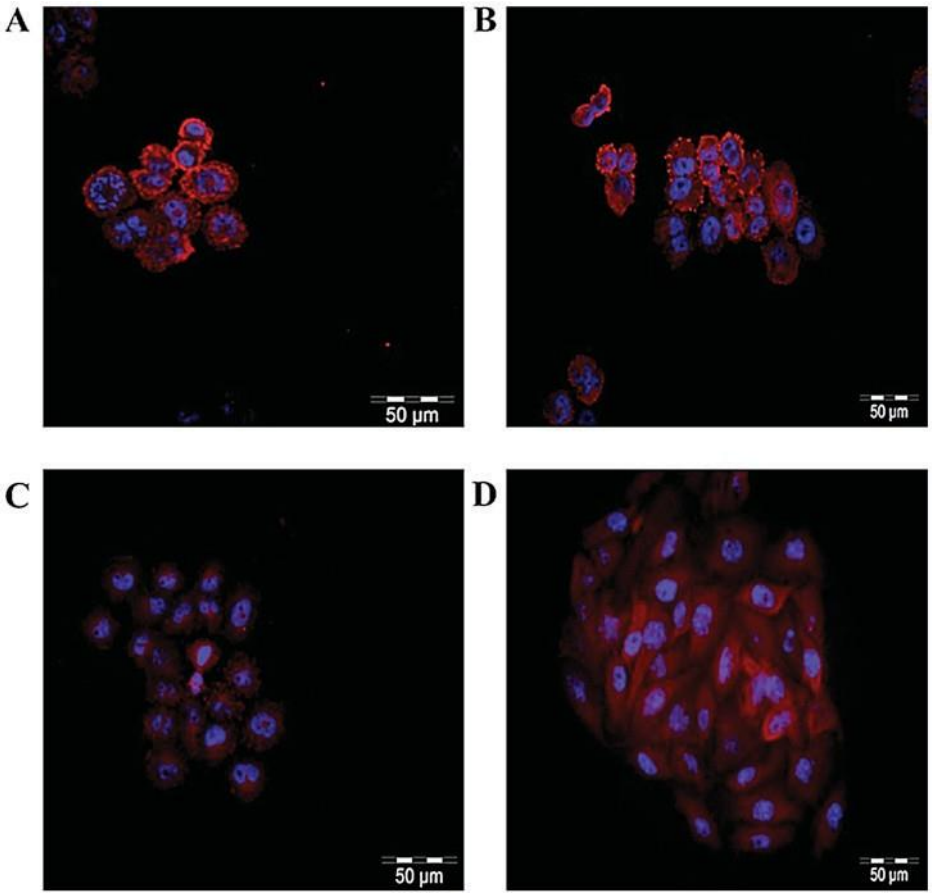

Figure 3. Immunofluorescence reactions confirming the presence of P-gp, ANXA1 and TXN in human gastric cancer cell lines. Presence of (A) membrane reaction, P-gp, primarily in the membrane; (B) ANXA1, primarily in the cytoplasm; (C) TXN, primarily in the cytoplasm, all in the EPG85-257RDB cell line; and (D) TXN, primarily in the cytoplasm in the EPG85-257RNOV cell line. P-gp, P-glycoprotein; SD, standard deviation; ANXA1, Annexin A1; TXN, thioredoxin.

association was revealed at the protein level with the use of western blot analysis. In the R30- and R50-treated groups, the protein level dropped below the quantitation level. Thus, there was a statistically significant difference between the two resveratrol-treated groups and the untreated group (P<0.0001; Fig. 2B).

The presence of individual proteins in cell lines was confirmed using IF. In the RDB cell line: P-gp (Fig. 3A); Annexin A1 (Fig. 3B); and thioredoxin (Fig. 3C), and in the RNOV cell line, thioredoxin (Fig. 3D).

Effect of resveratrol on the change of the expression of ANXA1 and TXN genes and proteins in the RDB cell line. It was revealed that in the RDB cell line, aside from the classical resistance mechanism (*ABCB1* gene overexpression), other atypical mechanisms are present that are associated with an increased expression of *ANXA1* and *TXN* genes Expression of the *ANXA1* gene in RDB cells is significantly higher than that in P cells (P<0.0001; Fig. 4A). R30 treatment resulted in the significant reduction of gene expression in the RDB cell line compared with the P cell line (P<0.001; Fig. 4A). R50 treatment resulted in the additional decrease in the expression of this gene, significantly when compared with control RDB cells (P<0.001) and non-significantly compared with R30-treated RDB cells (Fig. 4A). On the protein level, an increase in the expression of ANXA1 with increasing resveratrol concentration was revealed; however, this difference was not statistically significant (Fig. 4B). However, with respect to the *TXN* gene, significant overexpression was observed in the RDB cell line in comparison with the P cell line (P<0.001; Fig. 5A). Incubation with R30 resulted in the significant reduction of gene expression when compared with the control RDB cell line (P<0.001; Fig. 5A). Analysis of protein levels confirmed the results of RT-qPCR. Treating RDB cells with R30 resulted in a reduced level of protein expression when compared with RDB cells not treated with the polyphenol, with R50 resulting in the further reduction of TXN expression compared with non-treated cells and R30-treated cells (Fig. 5B).

Effect of resveratrol on the expression level of TXN mRNA and protein in the RNOV cell line. The presence of atypical MDR mechanisms associated with the overexpression of *TXN* was revealed in the RNOV cell line. Expression of this gene in the RNOV cell line was significantly higher in comparison with the P cell line (P<0.001; Fig. 6A). R30 significantly reduced the level of gene expression in compar- ison with the untreated RNOV cell line (P<0.0001; Fig. 6A). The same results were obtained at the protein level: The difference in expression levels between TXN in untreated RNOV cells and R50-treated RNOV cells was statistically significant (P<0.05; Fig. 6B).

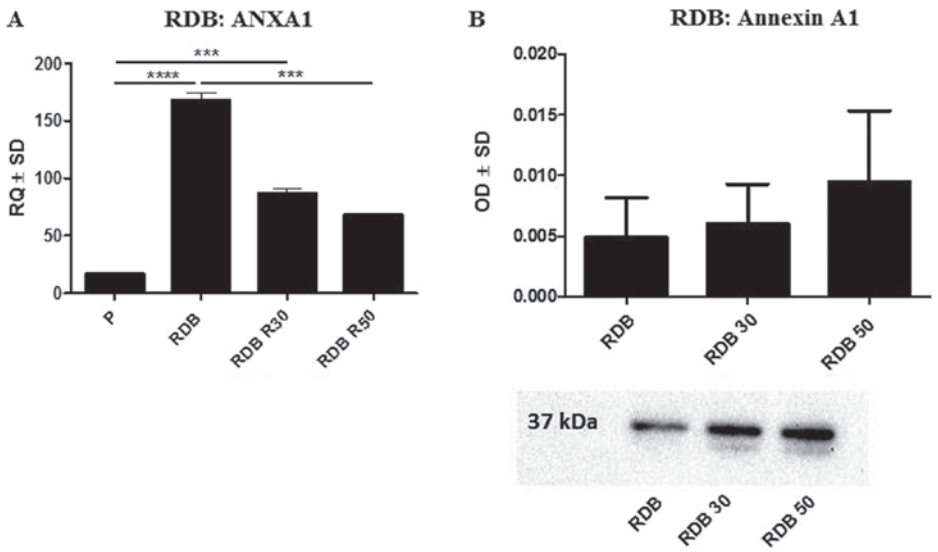

Figure 4. Effect of resveratrol on the expression levels of *ANXA1* and ANXA1. Expression levels of (A) *ANXA1* and (B) ANXA1 protein in P and RDB cell lines. ***P<0.001 and ****P<0.0001, with comparisons shown by lines. R30, 30 µM resveratrol concentration; R50, 50 µM resveratrol; P, EPG85-257P; RDB, EPG85-257RDB; *ANXA1*, Annexin A1; RQ, relative quantity; OD, optical density; SD, standard deviation.

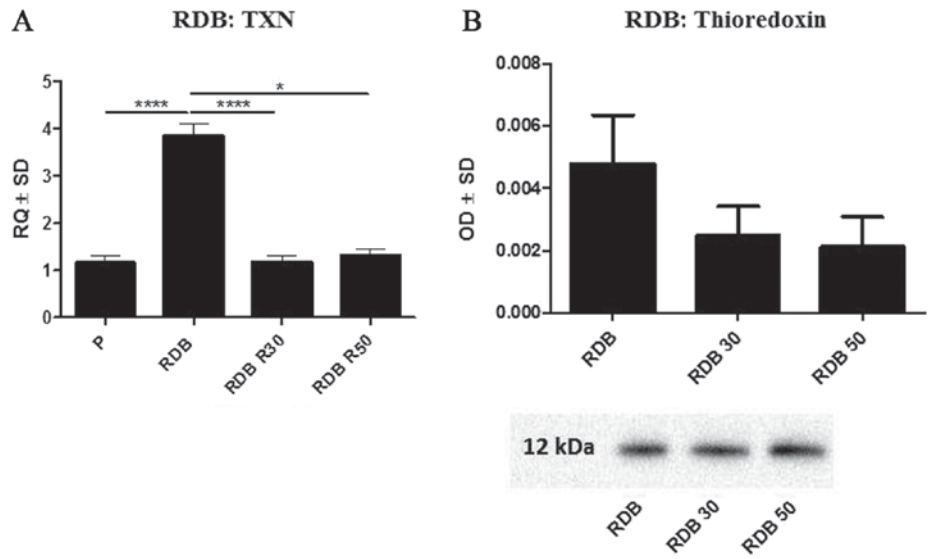

Figure 5. Effect of resveratrol on the expression levels of *TXN* and TXN in RDB cells. Expression levels of (A) *TXN* gene and (B) TXN protein in P and RDB cell lines. *P<0.05 and ****P<0.0001. R30, 30 µM resveratrol concentration; R50, 50 µM resveratrol; P, EPG85-257P; RDB, EPG85-257RDB; *TXN*, thioredoxin; RQ, relative quantity; OD, optical density; SD, standard deviation.

Discussion

MDR is a notable problem for chemotherapy and results in a lower chemotherapeutic efficacy (32). Drug-resistance in cancer cells develops as a result of numerous different mechanisms, of which understanding has improved owing to the use of novel research techniques. These mechanisms include the lowering of the intracellular drug concentration, activation of detoxification enzymes, changes in drug metabolism inside of the cell and the inhibition of cancer cell apoptosis (33,34). The most well-known and frequently described MDR mechanism is the increased expression of transport proteins at the membrane of cancer cells (35). Therefore, studies on substances that enable cancer cell sensitisation to cytostatics are necessary. Such compounds include polyphenols such as resveratrol, curcumin or quercetin (19,36). The effects of polyphenol compounds were studied in human leukaemia cell lines sensitive (K562) and resistant (K562/A02) to DB. In this study, it was revealed that curcumin contributes to the reduction of cell viability and to DNA damage. The effect of curcumin on cancer cell apoptosis was also reported (37). Resveratrol and its ability to overcome MDR were studied in pancreatic cancer cell lines EPP85-181 that were sensitive or resistant to cytostatics, in which the low level of type II topoisomerase expression was one of the causes of resistance. Resveratrol increased the expression of type II topoisomerse and therefore enhanced cells sensitivity to antracyclines (36).

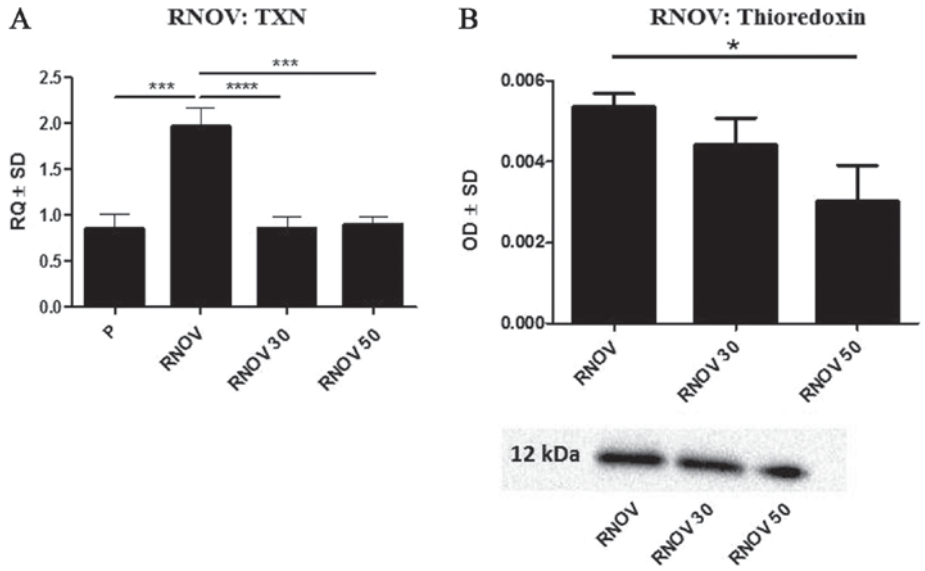

Figure 6. Effect of resveratrol on the expression levels of *TXN* and TXN in RNOV cell lines. Expression levels of (A) *TXN* gene and (B) TXN protein in P and RNOV cell lines. *P<0.05, ***P<0.001 and ****P<0.0001. R30, 30 µM resveratrol; R50, 50 µM resveratrol concentration; P, EPG85-257P; RNOV, EPG85-257RNOV; *TXN*, thioredoxin; RQ, relative quantity; OD, optical density; SD, standard deviation.

The present study attempted to demonstrate the effect of resveratrol on the expression of MDR-associated *ABCB1*, *ANXA1* and *TXN*, and the proteins encoded by these genes.

The ability of P-gp to actively remove drugs used for chemotherapy, including DB, results in a low intracel- lular concentration of these drugs and therefore treatment failure (14,38). The effect of resveratrol was studied in human colorectal cancer HCT116 cell lines that were sensitive or resistant to oxaliplatin (39). In this study, it was revealed that resveratrol treatment results in a reduction in *ABCB1* expression in iplatin-resistant cells in comparison with the expression in cisplatin-sensitive cells, by inhibiting nuclear factor-κB (NF-κB) and AMP-activated protein kinase-dependent activa- tion of cAMP response element binding protein. The effect of resveratrol on P-gp expression in the human epidermal carcinoma KBv200 cell line was additionally investigated, and it was demonstrated that resveratrol efficiently reduced the expression of this protein (40). The present study revealed the increased expression of the *ABCB1* gene and P-gp in the DB-resistant RDB cell line in comparison with a DB-sensitive cell line. Resveratrol significantly reduced P-gp expression at the mRNA and protein level in a concentration-dependent fashion. In the MTX-resistant RNOV cell line, no increased expression of P-gp or *ABCB1* was observed, indicating that an atypical resistance mechanism was being observed. A previous study demonstrated that, in cancer cells resistant to cytostatics the mechanism of action of curcumin is to inhibit P-gp in a competitive or allosteric manner (41).

Until now, to the best of our knowledge, the effect of resveratrol on *ANXA1* gene expression in cancer cells remained unstudied. A previous study examined the influ- ence of resveratrol on ANXA1 expression in an acute promyelocytic leukaemia HL-60 cell line, in which it was revealed that the protein level increased depending on the time of incubation with the polyphenol (42). ANXA1 inhibits proliferation and activates apoptosis by affecting the extracel- lular signal-regulated kinase signaling cascade, binding actin filaments and stimulating cyclin-dependent kinase 2 (43). The main pro-apoptotic mechanism of ANXA1 is most likely via caspase-3 activation (44,45). In addition to the classical MDR mechanism, atypical mechanisms are associated with increased *ANXA1* expression in the RDB cell line (46). In the present study, an increase in the expression of *ANXA1* in RDB cells compared with that in a DB-sensitive cell line was revealed. Treatment with resveratrol reduced the expression level of *ANXA1*. However, in MTX-resistant cells, high expression of *ANXA1* was not observed. At the protein level, treating cells with resveratrol resulted in an increase in ANXA1 expression in DB-resistant cells compared with cells that are DB-sensitive. These results are in line with those obtained by Li *et al* (42).

The results of studies performed using various types of cancer revealed that *TXN* gene expression is higher in cancer cells than in non-cancer cells (47). Using two-dimensional gel electrophoresis, *TXN* expression was observed in an MTX-resistant RNOV cell line (46). The high level of expres- sion of this gene may be associated with increased proliferation, resulting in cancer cell resistance to chemotherapy (48). These results are consistent with those of the present study, which found that *TXN* is overexpressed in RDB and RNOV cell lines when compared with their expression in drug-sensitive cell lines. Upon incubation of the cells with resveratrol, the level of expression of *TXN*, and that of the protein it encodes, was reduced.

The lowered expression of TXN may induce apoptosis; the key element is the TXN-TXN reductase system, which maintains a proper redox state (49). Deficiency of this protein affects compounds serving notable functions in the cancer transformation process. Amongst the most crucial compounds is the NF-κB transcription factor, which is responsible for the transcription of B-cell lymphoma 2 family genes; prod- ucts of this gene family additionally inhibit apoptosis (49). The decrease in TXN expression results in the inhibition of activation and pro-apoptotic action further apoptosis signal-regulating kinase-1 binding (50).

In summary, resveratrol is a compound that is able to overcome MDR in human gastric cancer cells. By affecting the expression of genes and proteins that are crucial for MDR, it may contribute to an increase in the efficiency of chemotherapy.

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Review

Cytotoxic and Proapoptotic Effects of Resveratrol in In Vitro Studies on Selected Types of Gastrointestinal Cancers

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Abstract: Cancer diseases are currently one of the greatest health challenges in clinical medicine worldwide. Classic methods of treatment often lead to numerous side effects, including the development of multidrug resistance. For this reason, increasing hope is being placed on compounds of natural origin, mainly due to their pleiotropic effect on different types of cells, protective effect on normal cells and toxic effect on cancerous ones. The most studied group are the polyphenolic compounds, which include resveratrol. The effectiveness of polyphenols in the treatment and prevention of many diseases, including cancer of various origins, has become the basis of many scientific studies. The anticancer effect of resveratrol has been demonstrated at all stages of the carcinogenesis process. Additionally, whether administered by itself or in combination with cytostatics, it may play a significant role in the process of reversing multidrug resistance. A review of the effects of resveratrol in in vitro conditions proves that it has a stronger or weaker antiproliferative and proapoptotic effect on the cells of certain neoplasms of the gastrointestinal tract. Despite the differences in the effect of this compound on different types of cancer, a similar tendency can be observed especially regarding the correlation between the concentration of the compound and the incubation time on the one hand and the antitumour effect on the other hand. The information included in this review may prove helpful in planning in vivo and clinical studies in the future.

Keywords: resveratrol; apoptosis; proliferation; multidrug resistance (MDR); anticancer therapy; gastrointestinal cancers

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1. Introduction

The progress in medicine in terms of prevention programs, diagnostic tests and therapeutic strategies, as well as the awareness of industrialized societies in relation to the importance of physical activity and proper nutrition, have contributed to a significant improvement in the quality of life and the average life expectancy. Unfortunately, this is connected with an increasing incidence of aging-associated diseases, including cancer.

Despite the implementation of cancer prevention, the incidence and mortality rates are constantly increasing everywhere in the world [1,2]. As a rule, only early detection of neoplastic changes allows for the introduction of effective treatment, significantly increasing the chances of complete remission and the patient's survival.

Gastrointestinal cancers, due to the lack of clear and characteristic symptoms, are most often diagnosed at a late stage of the development of the disease. The presence of metastases makes the treatment a long-term process which hardly ever brings the desired therapeutic effect. The factors influencing the formation and development of neoplastic diseases include, among others, lifestyle and diet. On the basis of numerous studies, it has been shown that the use of an appropriate and balanced diet rich in vegetables and fruit can contribute to reducing the risk of cancer [3–5]. For this reason, increasing attention is being paid to compounds of natural origin that have been present in medicine for hundreds of years. Paradoxically, the development of modern medicine has led to cancer's gradua

elimination by chemically synthesized compounds. In the case of chemotherapy, the drugs used show toxicity towards organs and often cause the emergence of resistance, which causes the lack of an adequate response to the treatment [6]. On the other hand, the substances used to reduce multidrug resistance (MDR) have additional side effects. MDR in the context of cancers diseases is an unfavourable phenomenon that contributes to the failure of the use of anticancer therapies. It is defined as the insensitivity of the tumour cells to groups of various therapeutic agents and develops as a result of using a single cytostatic drug [7]. The classic mechanism of MDR is related to the overexpression of the *ABCB1* gene, encoding the P-glycoprotein (P-gp) protein, through which the drug is transported outside the cell, leading to a reduction in the effective concentration of the drug in the cell [8,9]. In addition to the classic mechanism of multidrug resistance, there are also atypical mechanisms related to gene overexpression: *ANXA1* and *TXN* or topoisomerase II—an enzyme that plays an important role in replication, transcription and recombination, as well as in the construction and segregation of chromosomes. The *ANXA1* gene codes for the protein annexin I, which is involved in the regulation of cell growth, differentiation and apoptosis [10]. The *TXN* gene encodes the thioredoxin protein, which is involved in the regulation of the redox state of the cell. As an example of a breaking phenomenon of MDR by resveratrol, studies were conducted on stomach cancer cell lines sensitive and resistant to the action of suitable cytostatics: EPG85-257P line (sensitive to both cytostatics), EPG85-257RDB line (resistant to daunorubicin) and EPG85-257RNOV line (resistant to mitoxantrone). The action of resveratrol led to the sensitization of cancer cells to the action of chemotherapeutic agents by influencing the expression of genes (*ABCB1*, *ANXA1*, *TXN)* and proteins (P-gp, annexin I, thioredoxin) related to the MDR process [11]. The multidirectional effect of resveratrol on the mechanisms of MDR was also demonstrated in pancreatic cancer cell lines: EPP85-181P (sensitive to cytostatics), EPP85-181RDB (resistant to daunorubicin) and EPP85-181RNOV (resistant to mitoxantrone). In EPP85-181RDB cells, resveratrol reduced the level of P-gp expression, while in EPP85-181RNOV cells, resveratrol increased the expression of topoisomerase II [12]. Hence, increasing hope is being placed on compounds of natural origin such as plant polyphenols, which, when administered in a monitored manner, are not toxic to healthy organs. This is evidenced by numerous scientific studies that document the anticarcinogenic properties of polyphenols, which are important in the prevention and treatment of cancer diseases [13–17]. Natural substances are a rich source of compounds with various biological properties, such as antibacterial, anti-inflammatory and anticarcinogenic properties, thus offering more comprehensive therapeutic effects than individual drugs [18,19]. Naturally occurring polyphenolic compounds are secondary metabolites produced by plants in order to protect them against stress factors from the natural environment, such as UV radiation and viral and fungal infections [20,21].

One of the plant polyphenols more often studied is resveratrol. Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a representative of stilbenes, which are small-molecule compounds containing two aromatic rings in their structure. Resveratrol was first isolated in 1940 from the roots of white hellebore (*Veratrum grandiflorum*) [15]. Depending on the attachment position of individual functional groups and their mutual position in relation to each other and in relation to the double bond, these compounds exist in two stereoisomeric forms: *cis*-resveratrol and *trans*-resveratrol (Figure 1) [22]. Resveratrol is found in both isomeric forms. However, the steric point of view, the *trans* isomer is more stable than the *cis* isomer. In addition, the *trans*-resveratrol form is better researched and shows greater activity compared with the *cis* form. Moreover, *trans*-resveratrol is more biologically active, for example, in its antioxidant and anticancer properties [23–25]. The *cis* and *trans* isomers coexist in plants and wine. However, the *cis*-resveratrol isomer has never been found in grape extract [26]. The *cis* form is formed by isomerization through the action of UV radiation, artificial light, high pH on the *trans* form or during the fermentation of grape skins (Figure 2) [27,28].

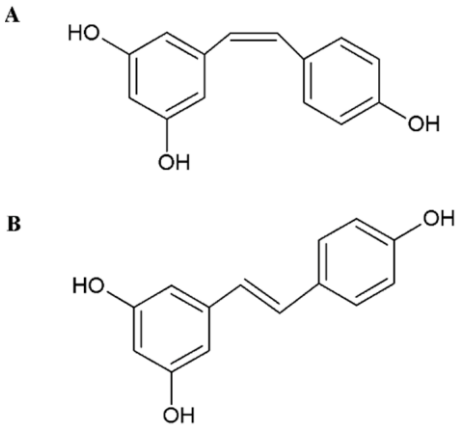

Figure 1. Chemical structure of *cis*-(**A**) and *trans*-resveratrol (**B**).

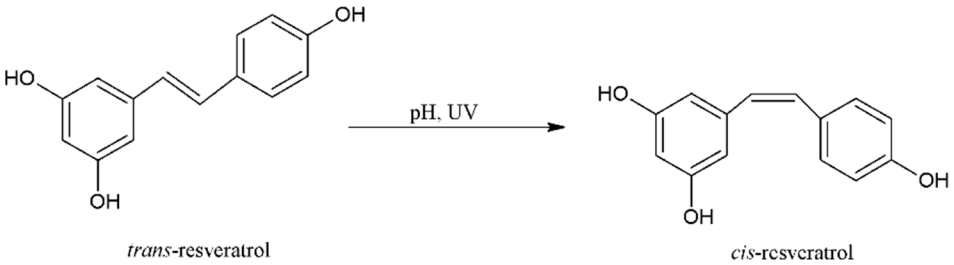

Figure 2. Isomerisation of resveratrol.

Since then, this compound has been extracted from many other naturally occurring plants included in a varied diet, including bilberries, blueberries and peanuts [29,30]. However, its main source is the skin of dark grapes (50–100 µg/g) [31,32].

Resveratrol is a phytoalexin, a compound produced by plants in response to the attack of pathogens and as a result of UV radiation and the action of heavy metals [33,34]. For this reason this compound exhibits several diverse biological activities (Figure 3) [35]. In France, despite limited physical activity, high consumption of animal fats and substantial consumption of red wine, a low mortality rate from cardiovascular disease is observed. This phenomenon is known as the French paradox. The association of the presence of resveratrol in red wine with its cardioprotective effect resulted in a great interest in this compound [36]. The anti-inflammatory effect of resveratrol is due to its ability to inhibit the synthesis and secretion of inflammatory mediators [37]. Resveratrol also has a neuroprotective effect on the brain. These properties are used in the treatment of many neurodegenerative diseases, such as Alzheimer's disease or Parkinson's disease [38]. The antioxidant effect of resveratrol is related to the inhibition of the formation of free radicals and the removal of those already formed from the body [39]. In our review, we focus on the antitumour activity of resveratrol.

The antitumour activity of resveratrol was first described in 1997 by Jang et al. [40–42]. Due to the risk associated with traditional methods of cancer treatment, the use of this compound in cancer patients is particularly important, something that has been confirmed by numerous studies on the relationship between resveratrol and the protection against the side effects of chemotherapy [32]. The in vitro studies conducted so far have proven that resveratrol exhibits antitumour activity at all stages of the carcinogenesis process [43]. However, the mechanism of action of this compound is still not fully understood. Moreover, the effect of resveratrol varies depending on factors such as the type of cancer, the concentration and the duration of the action. The healing properties of resveratrol can be observed in many types of cancer, including breast, skin, intestine, prostate and lung cancers [44–47]. Additionally, resveratrol participates in the process of overcoming multidrug resistance by changing the expression of the genes and proteins related to the MDR phenomenon and by sensitizing neoplastic cells to the action of chemotherapy agents [11], [48].

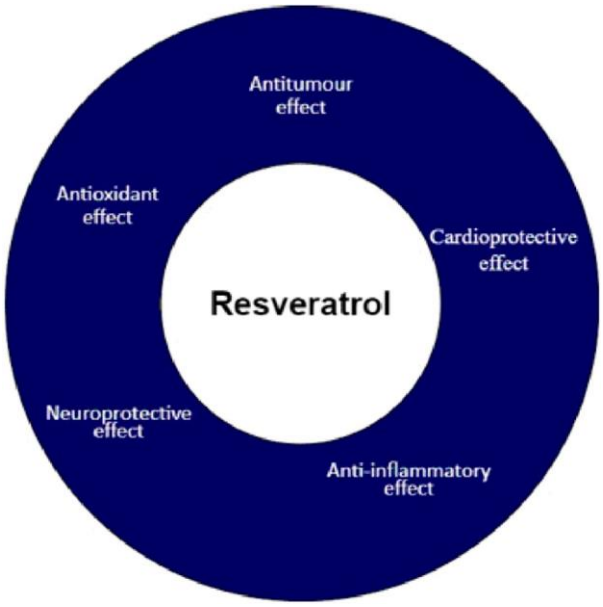

Figure 3. Potential clinical properties of resveratrol.

The main goal of this review is to present the results of in vitro studies on the antiproliferative and proapoptotic effects of resveratrol on human gastrointestinal cancers.

2. Pancreatic Cancer

Pancreatic cancer is the ninth most common cancer in women and the tenth most common cancer in men [49,50]. The highest number of cases is recorded among the elderly [51,52]. Pancreatic cancer is difficult to diagnose due to the lack of characteristic symptoms [53]. Moreover, the lack of appropriate and cost-effective screening tests allowing for early detection of this cancer leads to its detection in the advanced stage of the disease. Currently, the most feasible treatment option for pancreatic cancer is surgery performed at an early stage of the disease [54]. Late diagnosis significantly affects poor prognosis in patients due to metastases and the development of MDR, which is the cause of chemotherapy failure [55,56]. Additionally, the selection of an appropriate treatment depends on the patient's physical condition, including the presence of other diseases [51].

2.1. Antiproliferative Effect

In the case of pancreatic cancers, the results of numerous in vitro studies indicate the potential antiproliferative effect of resveratrol, depending on the dose and the time of incubation with the compound.

In a study on two pancreatic cancer cell lines, PANC-1 and AsPC-1, Xian-Zhong Ding's team showed that after 48 and 72 h of incubation with a resveratrol solution at various concentrations, there was a statistically significant change in cell viability. These changes were dependent on the time and concentration of the compound tested. The greatest effects were observed at a concentration of 100 µM [57]. However, these studies did not show the effect of this compound on normal pancreatic cells.

The studies of Jing Cui's team also showed an inhibition of cancer cell survival dependent on the concentration and time of the effect of resveratrol. The tests were performed on the PANC-1, BxPC-3 and AsPC-1 pancreatic cancer lines, which were treated with resveratrol in a concentration range from 0 to 200 µM/L at 24, 48 and 72 h [58]. Studies on the effects of resveratrol in the same cell lines made by scientists at the Tongji University also showed its inhibitory effect on cell proliferation. The cells were incubated with resveratrol at concentrations ranging from 25 to 250 µM/L at 24, 48 and 72 h [59].

Another neoplastic model of pancreatic cancer—the Capan-2 line—was also used in order to study the effect of resveratrol on cell survival. After 24 h of exposure of the cells

to resveratrol (10, 50, 100 µM), a concentration-dependent decrease in cell viability was observed [60]. The studies did not take into account the effect of the compound on normal pancreatic cells.

The effect of resveratrol on cell survival was also studied by P. Liu's team. Three pancreatic tumour lines were used in the study: PANC-1, CFPAC-1 and MIA PaCa-2, as well as normal pancreatic cells derived from tissue collected from a patient during surgery. The cells of the lines tested were exposed for 72 h to various concentrations of resveratrol. An increased cell growth inhibition was observed to take place with an increasing compound concentration. Compared with neoplastic cells, normal pancreatic cells showed greater resistance to the cytotoxic effect of resveratrol [61]. The cell lines differed from one another in their sensitivity to the effects of polyphenol. Lines PANC-1 and MIA PaCa-2 were the most sensitive to the effects of resveratrol. The AsPC-1 line showed moderate sensitivity to the action of the compound. However, the lowest effects of the action of the compound were recorded for the Hs766T line [62].

Most studies confirm that the higher the dose of resveratrol, the stronger its antiproliferative effect on cells of various pancreatic cancer lines. At the same time, such a strong effect is not observed in normal pancreatic cells, which may translate into resveratrol being an effective and safe treatment option in the future.

2.2. Proapoptotic Effect

Apoptosis is a complex physiological process that follows a specific pattern that significantly influences the proper functioning of the organism. This process leads to the removal of unnecessary and damaged cells that could potentially pose a threat to the organism (e.g., cancer cells) [63]. Due to its essential importance in the tumorigenesis process, apoptosis is a topic that has been frequently explored in research.

For pancreatic cancer, resveratrol has been shown to be proapoptotic in many cases. In the studies of Xian-Zhong Ding's team, the ability of resveratrol to induce apoptosis was examined by using flow cytometry. The pancreatic tumour cell lines PANC-1 and AsPC-1 were subjected to 72 h of incubation with 100 µM/L resveratrol. After this time, the induction of the apoptotic process was 27% and 34% for PANC-1 and AsPC-1, respectively [57].

Similar tests were performed by Jing Cui et al. The cells of three pancreatic cancer lines (PANC-1, AsPC-1 and BxPC-3) were incubated in resveratrol for 48 h at concentrations ranging from 0 to 200 µM/L. The analysis of the results showed that the induction of the apoptotic process was 26.20% for the PANC-1 cell line. A similar value was found for the BxPC-3 line: 22.08%. As for the AsPC-1 line, the percentage increase in apoptotic cells was only observed at a concentration above 150 µM/L [58]. The team at Tongji University obtained similar results for the same cell models. During a 24 h incubation with resveratrol at concentrations ranging from 0 to 200 µM, it was shown that the percentage of apoptotic cells at a concentration of 100 µM was 16.2%, 18.21% and 22.26% for the PANC-1, AsPC-1 and BxPC-3 lines, respectively [58].

Studies on resveratrol's ability to induce the apoptotic process performed on the Capan-2 cancer cell line showed that the number of apoptotic cells after 24 h of incubation with 100 µM/L resveratrol solution increased significantly (from 12.92% to 21.31%) compared with cells not treated with the compound [60].

The effect of resveratrol on apoptosis was also demonstrated by P. Liu's team. Research shows that microRNAs can act as oncogenes or tumour suppressors; hence, they play an important role in the process of cancer initiation and progression [64]. One of the most frequently expressed miRNAs in tumours is miR-21. According to data in the literature, miR-21 influences the level of Bcl-2, a key apoptotic regulator [65]. Three pancreatic tumour lines were used in the studies: PANC-1, CEPAC-1 and MIA PaCa-2. Cells were treated with resveratrol at a concentration of 50 µM for 24 h. A real-time PCR was then performed to check for changes in miR-21 expression following the treatment of the cells with the compound. It turned out that there was a statistically significant reduction in the level of

miR-21 against the control. The inhibition of the miR-21 expression resulted in a reduction of the Bcl-2 protein expression [61].

The effect of resveratrol on the activation of the executioner caspase-3, which is normally involved in typical apoptosis, was also studied. The tests were carried out on four pancreatic cell lines: PANC-1, MIA PaCa-2, Hs 766T and AsPC-1. The cells were incubated for 48 h with resveratrol (0–40 µM). Caspase-3 activity, which was measured with the help of a fluorometer, was the lowest for the Hs 766T line, while the highest activity was observed for the PANC-1 and the MIA PaCa-2 lines. For the AsPC-1 line, caspase-3 activity was intermediate in relation to the remaining cell lines. These studies did not include a reference to a normal pancreatic cell line [62].

In conclusion, resveratrol is a proapoptotic compound in human pancreatic cancer, and it acts through many different pathways involved in this process, especially at high doses.

3. Stomach Cancer

Despite the observed reduction in the incidence of stomach cancer, it remains one of the most common neoplasms, especially among men [66]. Similar to pancreatic cancer, stomach cancer is typically diagnosed at a late stage due to the lack of early characteristic symptoms [67]. A late diagnosis often limits the use of effective treatments, something that is associated with a poor prognosis. The causes of stomach cancer are not clearly defined. However, it is known that in the case of gastrointestinal cancers, the type of diet is an important factor [67]. Epidemiological studies have shown that the risk of developing the disease is significantly reduced when the diet includes fruits and vegetables containing large amounts of polyphenols [66–68].

3.1. Antiproliferative Effect

Separate reports from recent years indicate that resveratrol is a compound that is also used in the treatment of gastric cancer [69].

Xiaoping et al. analysed in vitro the effect of resveratrol on the survival of human gastric cancer cells as well as its potential mechanism of action. For this purpose, MGC-803 cells were exposed to different concentrations of resveratrol for 24 and 48 h. In this experiment, resveratrol was shown to inhibit cell growth depending on the dose and the duration of the effect. Accordingly, further experiments were performed to evaluate the effect of the compound on the cell cycle. The cells were incubated with resveratrol (50 and 100 µM, 24 h). The analysis showed that the treatment with resveratrol led to the inhibition of the cycle in the G0/G1 phase [70]. Other studies also showed an inhibitory effect of resveratrol on the proliferation of MGC-803 gastric cancer cells. In this case, the treatment performed with concentrations of 50 µM, 75 µM and 100 µM for 24, 48 and 72 h resulted in a time- and dose-dependent decreased cell viability. These changes were statistically significant [71].

Studies on the effect of resveratrol on the inhibition of cell growth were also carried out on the gastric cancer model SGC-7901 cell line. A 48 h cell incubation with resveratrol (0–200 µM) resulted in an inhibition of cell proliferation proportional to an increasing concentration of the compound [72]. Similar studies also performed on the SGC-7901 cell line showed that a 24 h incubation with a resveratrol solution (25 µM and 50 µM) also resulted in a concentration-dependent and statistically significant reduction in cell viability compared with untreated cells [73]. Concentration- and time-dependent inhibition of cell viability in the same cell line was demonstrated by treating it with a resveratrol solution (0–400 µM) for 24, 36 and 48 h [74].

Further studies on the effect of resveratrol on neoplastic cells in gastric cancer were carried out on two cancer cell lines: the aforementioned SGC-7901 line and the BGC-823 line, as well as the benign epithelial cell line GES-1. The cell lines were exposed to various resveratrol concentrations ranging from 0 to 400 µM for 24, 48 and 72 h. A significant reduction in viability was observed in all cell lines tested. These changes were dose- and time-dependent [75].

The proliferative activity of gastric cancer cells is significantly inhibited by resveratrol. The effect of the compound's action is stronger when the concentration is higher and the exposure time is longer.

3.2. Proapoptotic Effect

Studies on the effect of resveratrol on the induction of the apoptotic process were carried out on a gastric cancer cell model: the SGC-7901 cell line. In this experiment, cells were incubated for 48 h with the compound in a concentration ranging from 0 to 200 µM. It was shown that the effect of resveratrol results in an increase in the production of ROS (reactive oxygen species), which according to the literature are involved in the apoptotic process [72]. Additionally, the action of superoxide dismutase and catalase reduced the proapoptotic effects of resveratrol [76]. Yang et al. showed in their studies (also performed on the SGC-7901 cell line) that after 24 h of incubation with a resveratrol solution, the number of apoptotic cells increased significantly in a dose-dependent manner [73]. The same relationship was demonstrated in studies in which cells from the SGC-7901 line were exposed to resveratrol for 24 h at 0, 50, 200 and 400 µM concentrations [74].

As in pancreatic cancer, resveratrol also has a proapoptotic effect in gastric cancer, and the effect observed is stronger when the concentration of the compound is higher. However, there is less documented research on this tumour model.

4. Liver Cancer

Liver cancer is also one of the most common cancers worldwide. It is diagnosed more often in men than in women [77]. Despite the improvement of conventional treat- ment methods, including surgery, chemotherapy and radiotherapy, liver cancer remains a difficult cancer to treat [77]. Similar to other tumours of the gastrointestinal tract, it is typically first diagnosed at an advanced stage, which usually translates into high mortality among patients [77,78]. Resveratrol has an inhibitory effect on liver cancer, which has been confirmed by the results of, among others, in vitro studies.

4.1. Antiproliferative Effect

The effect of resveratrol on cell viability was also studied in a liver cell model: the HepG2 line. In the experiment, cells were exposed to resveratrol (0–100 µM) for 24 and 48 h. The results of an MTT test showed a concentration- and time-dependent reduction of the viability in the cell line tested [79]. P.-L. Kuo's team obtained similar test results. In this case, the study model consisted of two cell lines: HepG2–p53(+) and Hep3B–p53( ). In the experiments, the cells were treated with resveratrol in concentrations of 1, 5, 10 and 20 µg/mL. The cell incubation times with the compound were 12, 24, 48 and 72 h. In the case of the HepG2 line, treatment with resveratrol resulted in a statistically significant reduction in cell viability. These changes were dependent on the concentration and the duration of the action of the compound. For the Hep3B line, no statistically significant changes in cell viability were found [80]. Additionally, an analysis of the changes in the cell cycle after incubation with resveratrol was performed for the HepG2 line. It was shown that at concentrations of 10 and 20 µg/mL during a 24 h incubation, cells accumulate in the G1 phase. The most visible changes were observed at a concentration of 20 µg/mL. The increase in G1 cell population resulted in a decrease in G2 cell population. However, in the S phase, no significant changes in the population of HepG2 cells were observed [81]. In subsequent studies on the effect of resveratrol on cell proliferation, in addition to the HepG2 line, the Bel-7402 and SMMC-7721 cell lines as well as the normal liver cell line HL-7702 were used. The studies showed that a 24 h incubation with the compound (0–200 µM) resulted in the inhibition of the tumour cells compared with normal cells. This effect was seen at resveratrol concentrations higher than 80 µM. These results indicate that resveratrol reduces cell viability depending on the dose [81]. In an experiment performed on the human liver cancer cell line MHCC97-H, a time- and concentration-dependent effect of resveratrol on the inhibition of the proliferation of the cells tested was observed. This effect

could be seen during a 24 h and 48 h incubation of the cells with the compound (0, 20, 60 and 100 µM) [82].

In conclusion, the tests performed on liver cancer cell models indicate the antiproliferative properties of resveratrol, primarily the inhibition of the cell cycle. The results are dependent on the concentration and time of the incubation with the compound, as well as the tissue origin of the cells tested.

4.2. Proapoptotic Effect

The mechanism of apoptotic induction by resveratrol in liver cancer cells in vitro has not been fully understood yet. However, it is known that the effect depends on the type of cancer lines studied, among which there are differences in the response to the effect of the compound.

In the case of HepG2 cells, P.-L. Kuo's team showed that resveratrol inhibits cell growth and induces the apoptotic process. The level of p53 protein in the HepG2 and Hep3B cell lines was analysed after incubation with resveratrol. In the HepG2 cell line, the expression level of this protein increased along with the increase in resveratrol concentration, while in the Hep3B cell line, no significant changes in p53 expression level were observed. These results provide evidence that, in this case, the proapoptotic effect of resveratrol is mediated by the p53 protein [80]. Proteins from the Bcl-2 family participate in the regulation of the apoptotic process [83–85]. Bax is a proapoptotic protein, while Bcl-2 is one of the antiapoptotic proteins [84]. The confirmation of the proapoptotic effect of resveratrol in the HepG2 cell line is the increase in the expression of the Bax protein along with the increase in resveratrol concentration. Such a relationship was not observed for the Bcl-2 protein [80].

In other studies performed on the HepG2 cell line, the most pronounced effect on the induction of apoptosis was observed (by flow cytometry) at a concentration of 50 µM during a 48 h incubation with the compound [85].

The TUNEL method was used to evaluate the effect of resveratrol on the induction of apoptosis in the liver cancer cell lines HepG2, Bel-7402 and SMMC-7721. After 24 h of incubation with 100 µM resveratrol solution, it was observed that resveratrol increased apoptosis in relation to untreated cells. Moreover, it was shown that resveratrol also influences the apoptotic proteins Bcl-2 (antiapoptotic) and Bax (proapoptotic), which leads to a decrease in the Bcl-2/Bax ratio [81].

The results of all the studies described above suggest that resveratrol may play a significant role in the apoptosis of liver cancer cells by participating in the regulation of the expression of cycle-suppressor and proapoptotic proteins.

5. Intestinal Cancer

Intestinal cancer is a common cancer characterised by a high mortality rate all over the world [86]. It most often affects people between 65 and 74 years of age [87,88]. However, in recent years, an increase in the incidence of the disease has been observed among younger people. In the pathogenesis of this cancer, in addition to genetic and epigenetic factors, environmental factors and poor eating habits also play an important role [89,90]. Many studies indicate that family history of intestinal cancer increases the risk of developing the disease. Therefore, it is recommended that such people be screened first [91]. The use of a high-fat diet and eating too much red meat combined with little physical activity contribute to an increased risk of this type of cancer [91]. Early detection of neoplastic changes during screening tests allows for the implementation of the appropriate treatment, which in most cases brings the desired therapeutic effects.

5.1. Antiproliferative Effect

Studies carried out on the intestinal cancer lines HT-29 and WiDr have shown that the effect of resveratrol leads to an increasingly stronger inhibition of cell proliferation as the concentration of the compound increases [92].

In another experiment, the intestinal adenocarcinoma cell lines HCA-17, SW480 and HT-29 were used. The cell lines were exposed to various concentrations of resveratrol. A concentration- and time-dependent inhibition of the number of cells was demonstrated in all the lines tested [93].

Another team's studies on the HT-29 cell line showed varying resveratrol effects depending on the concentration after 48 h of incubation with the compound. The MTT test showed that lower concentrations of resveratrol (1 and 10 µM) stimulated cell growth, while higher concentrations (25 and 50 µM) inhibited cell proliferation [94]. By extending the duration of the incubation to 96 h, a significant cell growth inhibition was observed, especially at a concentration of 50 µM. The two-phase effect of the compound was not observed in HCT-116 colon cancer cells. In this cell line, treatment with resveratrol resulted in a concentration-dependent reduction in cell viability [95].

The anticancer properties of resveratrol have also been shown in in vitro studies in which the research model was the HCT-116 and Caco-2 human intestinal cancer cell lines. A proliferation inhibition dependent on resveratrol concentration was observed in both cell lines [95].

In another study performed on the intestinal cancer cell lines HCT-116, CO-115 and SW480, after a 24 h incubation with the compound at concentrations ranging from 0 to 50 µM, it was observed that cell viability decreased as the concentration of the compound increased [96].

The effect of resveratrol on the proliferation of neoplastic intestinal cells was also observed in the DLD1 and HCT15 cell lines. Treatment with resveratrol at concentrations ranging from 0 to 40 µM for 24, 48 and 72 h resulted in a dose- and time-dependent cell growth inhibition [97].

In intestinal cancers, resveratrol may act bi-directionally, depending on the concentration. Higher concentrations of the compound result in a significant inhibition of proliferation in particular cell systems, while lower concentrations can stimulate cell growth.

5.2. Proapoptotic Effect

Cyclooxygenase 2 (COX-2) overexpression is associated with physiological and pathological processes in the body, including the formation and progression of neoplasms [98,99]. One study compared the levels of COX-2 expression. In the cancer lines HCA-17, SW480 and HT-29, COX-2 was significantly higher compared with the normal cell line CCD-18Co. After 72 h of treatment with resveratrol, a concentration-dependent decrease in COX-2 expression was observed in the intestinal cancer lines. The inhibition of COX-2 expression by resveratrol is frequently associated with an induction of the apoptotic process [93]. After a 24 h incubation of the HT-29 cells with low concentrations of resveratrol (1 and 25 µM), no increase in the number of apoptotic cells was observed. This effect was only visible when higher concentrations of the compound were used [94]. Similar results were noted in studies on the HCT-116, CO-115 and SW480 cell lines. A 24 h incubation with different concentrations of resveratrol (0–50 µM) induced programmed cell death [96].

In intestinal cancer, resveratrol also has proapoptotic properties. This effect is most pronounced when higher concentrations of the compound are used.

6. Discussion

Cancer is a complex disease of various aetiologies, and the use of conventional treatments in most cases is associated with numerous side effects. Hence, the current scientific research focuses largely on the search for new therapeutic strategies, especially ones based on compounds of natural origin. High hopes are placed on the possibility of using these compounds in clinical conditions. The most often studied compounds are the polyphenolic compounds, including resveratrol. Due to the diverse range of biological effects, it is considered a promising anticancer agent.

Based on the available scientific literature, in this study we have documented the antitumour effect of resveratrol, especially its role in inhibiting cell proliferation and inducing the apoptotic process.

In vitro studies conducted over the last few decades show that resveratrol can be antiproliferative (decreased cell viability, inhibition of the cell cycle) and proapoptotic in various types of cancer.

In this work, we focused on the effects of resveratrol in cancers of the gastrointestinal tract (pancreatic, stomach, liver and intestinal cancers).

When analysing the scientific reports presented in our study, the effect of resveratrol is strongly dependent on the type of cancer, the concentration of the compound and the duration of its action (Table 1). In the studies examining the effects of resveratrol on pancreatic cancer cells, changes in cell proliferation are seen to occur with higher concentrations of resveratrol (>100 µM), and the most pronounced changes take place after at least 48 h of incubation with the compound.

Table 1. List of different cancer cell lines in each type of cancer.

Type of Cancer	Cell Lines	The Most Effective Concentration of Resveratrol (_M)	Antitumor Activity of Resveratrol	Mechanism of Action of Resveratrol	Article
Pancreatic cancer	PANC-1	>100	antiproliferative proapoptotic	Hedgehog signalling pathway	[57–59,61,62]
	AsPC-1				[57–59,62]
	BxPC-3			up-regulation of Bax protein	[58,59]
	Capan-2			inhibition of Bcl-2 expression by downgrading miR-21 expression	[60]
	CFPAC-1				[61]
	MIA Paca-2			activation of FOXO transcription factors	[61,62]
	Hs766T				[62]
Stomach cancer	MGC803	0–400	antiproliferative	increase in ROS production	[70,71]
	SGC-7901		antiproliferative proapoptotic	PTEN/PI3K/Akt pathway inhibition of Wnt signal pathway	[72–76]
	BGC823		antiproliferative	inhibition of MALAT1-mediated epithelial-to-mesenchymal transition	[75]
	GES1				
Liver cancer	HepG2	0–100	antiproliferative proapoptotic	up-regulation of Bax protein	[79–81,85]
	Bel-7402				[81]
	SMMC-7721		antiproliferative	down-regulation of Bcl-2 protein	[82]
	MHCC97-H				
Intestinal cancer	HT-29	0–50	antiproliferative proapoptotic	inhibition of COX-2 expression down-regulation of high telomerase activity (TLMA)	[92–94]
	WiDr				[92]
	HCA-17		antiproliferative		[93]
	SW480		antiproliferative proapoptotic		[93,96]
	HCT-116				[94–96]
	Caco-2		antiproliferative		[95]
	CO-115		antiproliferative proapoptotic		[96]
	DLD1				[97]
	HCT15		antiproliferative		

The effect of resveratrol in stomach cancer (inhibition of cell proliferation) is also specific to certain cells. Changes in cell survival are observed in a wide range of concentrations (0–400 µM). As happens in pancreatic cancer, resveratrol exerts an inhibitory effect on stomach cancer cells in a time-dependent manner.

In liver cancer, the effect of resveratrol on cell proliferation also shows a relationship between the concentration and the duration of the effect. However, in this neoplasm, changes are observed at lower concentrations of the compound (0–100 μM).

A similar relationship between the concentration and the duration of the action of the compound and cell survival can be observed in intestinal cancer. Studies evaluating the antiproliferative properties of resveratrol showed a notable inhibition of cell proliferation in intestinal cancer at significantly lower concentrations of the compound (0–50 μM).

Many of the in vitro studies presented indicate that the antitumor activity of resveratrol may be largely due to the induction of the apoptotic process. Translating the promising results of these in vitro studies into clinical studies is challenging due to the poor pharmacokinetic parameters of the compound—low bioavailability, poor water solubility and fast metabolism [100]—hence, the importance of increasing the bioavailability of resveratrol. For this purpose, delivery systems are being developed to enable compound absorption and an increase in plasma concentration. The methods to increase the bioavailability of resveratrol include, among others, the encapsulation of liposomes or lipid nanocarriers and the development of micelles and emulsions [100,101].

7. Conclusions

In in vitro studies, resveratrol elicits a weaker or stronger response depending on the type of cancer. However, in each case there is a correlation between the concentration and time of the action and the effectiveness of the compound. The promising results of the in vitro studies presented in this paper, as well as in many others, emphasize the need for further experiments, especially in vivo, on the effect of resveratrol to determine the therapeutic doses of the compound and the exact mechanisms of its action in relation to different types of cancer.

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Effect of Resveratrol Treatment on Human Pancreatic Cancer Cells through Alterations of Bcl-2 Family Members

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Abstract: Pancreatic cancers are among of the most lethal types of neoplasms, and are mostly detected at an advanced stage. Conventional treatment methods such as chemotherapy or radiotherapy often do not bring the desired therapeutic effects. For this reason, natural compounds are increasingly being used as adjuvants in cancer therapy. Polyphenolic compounds, including resveratrol, are of particular interest. The aim of this study is to analyze the antiproliferative and pro-apoptotic mechanisms of resveratrol on human pancreatic cells. The study was carried out on three human pancreatic cancer cell lines: EPP85-181P, EPP85-181RNOV (mitoxantrone-resistant cells) and AsPC-1, as well as the normal pancreatic cell line H6c7. The cytotoxicity of resveratrol in the tested cell lines was assessed by the colorimetric method (MTT) and the flow cytometry method. Three selected concentrations of the compound (25, 50 and 100 µM) were tested in the experiments during a 48-h incubation. TUNEL and Comet assays, flow cytometry, immunocytochemistry, confocal microscopy, real-time PCR and Western Blot analyses were used to evaluate the pleiotropic effect of resveratrol. The results indicate that resveratrol is likely to be anticarcinogenic by inhibiting human pancreatic cancer cell proliferation. In addition, it affects the levels of Bcl-2 pro- and anti-apoptotic proteins. However, it should be emphasized that the activity of resveratrol was specific for each of the tested cell lines, and the most statistically significant changes were observed in the mitoxantrone-resistant cells.

Keywords: resveratrol; Bax; Bcl-2; Caspase-3; apoptosis; multidrug resistance; pancreatic cancer

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1. Introduction

Despite developments in the field of early cancer detection, the number of new cases is increasing at an alarming rate [1]. Pancreatic cancers, classified as one of the most aggressive malignant neoplasms in humans, pose a particular problem [2–4].

Cancer treatment focuses mainly on surgery, radiotherapy, and chemotherapy, depending on the type and severity of the disease at the time of diagnosis. In the case of pancreatic cancer, surgery is the most effective treatment [5]. However, due to the diagnosis of this disease in its late stage of development, only 15–20% of patients qualify for surgical removal of the tumor [6]. At the time of diagnosis, the vast majority of patients have numerous metastases, disqualifying them from surgery [5,6]. In turn, the use of chemotherapeutic agents has led to the development of acquired multidrug resistance [7,8].

The abovementioned methods of treatment often turn out to be ineffective, especially if the cancer is diagnosed at an advanced stage. For this reason, chemoprevention and the search for alternative treatment methods showing no or minimal side effects are becoming more and more important [8]. High hopes are placed on compounds of natural origin, including polyphenols, which are characterized by a wide range of biological activities. Many plant-derived compounds participate in the neoplastic process by affecting cell survival, inhibiting angiogenesis or inducing apoptosis [9]. Apoptosis, as a complex

physiological process following a specific pattern, has a significant effect on the proper functioning of the body, leading to the removal of unnecessary and damaged cells that could pose a threat to the body over time (e.g., cancer cells). Proteins from the Bcl-2 family are involved in the process of apoptosis. Within them, we can distinguish two functional groups: one which has an inhibitory effect on apoptosis (e.g., Bcl-2) and one which influences the promotion of the apoptosis process (e.g., Bax). Maintaining the balance between pro- and anti-apoptotic proteins is essential for cell survival [10].

An example of a compound exhibiting anticancer properties is resveratrol. Resveratrol (3,5,4^{0}-trihydroxystilbene) is a polyphenol belonging to the stilbene group, found in many plant foods such as grapes, blueberries, peanuts, tea and dark chocolate [11,12]. However, its main source is red wine, due to the fact that its highest concentration can be found in the skin of red grapes (50–100 µg/g) [13]. Resveratrol exists in the form of *cis* and *trans* isomers (Figure 1). The *trans* isomer is used for research due to its greater activity compared to the *cis* one [14–16].

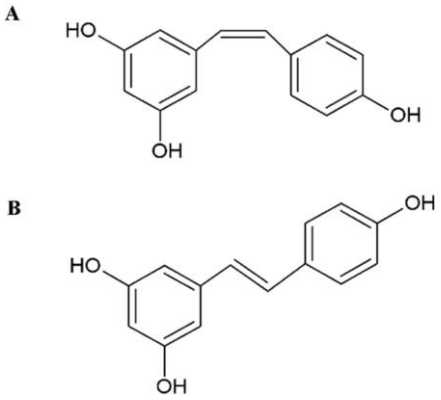

Figure 1. Chemical structures of resveratrol isomers: (**A**) *cis*-resveratrol, (**B**) *trans*-resveratrol.

Resveratrol is a phytoalexin that is produced in very small amounts by plants in response to the harmful effects of environmental factors, such as excessive UV radiation, exposure to heavy metals or fungal infections [11,17–19]. This compound is characterized by a wide range of biological activities, including antitumor activity [20]. Numerous scientific studies have shown that resveratrol exhibits antitumor activity at all stages of the carcinogenesis process in various types of neoplasms, including pancreatic neoplasm [3,21–23]. In addition, it also participates in overcoming the phenomenon of multidrug resistance (MDR), which has been demonstrated in research on cell models of gastric and pancreatic cancer [24,25].

The aim of our research is to demonstrate the potential antiproliferative and pro-apoptotic effects of *trans*-resveratrol on normal cells and various types of pancreatic cancer cells in vitro. So far, in vitro studies on the effects of resveratrol on members of the Bcl-2 family in pancreatic cancer cells have not been compared with studies on normal cells. It was also important to include cells resistant to cytostatics in the presented comparative studies in this field.

2. Results

2.1. Assessment of the Effect of Resveratrol on Cell Viability

Cell viability was analyzed with the MTT colorimetric assay. For this purpose, the cells of the tested lines were treated with increasing concentrations of resveratrol (0, 5, 10, 25, 50, 100, 150, 200 µM) for 24 h, 48 h and 72 h (37 °C, 5% CO_2). It was observed that the exposure of pancreatic cell lines to resveratrol inhibited proliferation in a concentration- and time-dependent manner compared to untreated cells (Figure 2A–D). EPP85-181RNOV cells, despite their resistance to mitoxantrone, turned out to be more sensitive to the action of resveratrol compared to mitoxantrone-sensitive cells (EPP85-181P line). AsPC-1 cells

were the least sensitive to the effects of resveratrol. A concentration- and time-dependent decrease in cell viability was also observed in normal H6c7 pancreatic cells. Based on the analysis of the results obtained, concentrations of 25 µM, 50 µM and 100 µM, as well as an incubation time of 48 h, were selected for further experiments on all the tested cell lines.

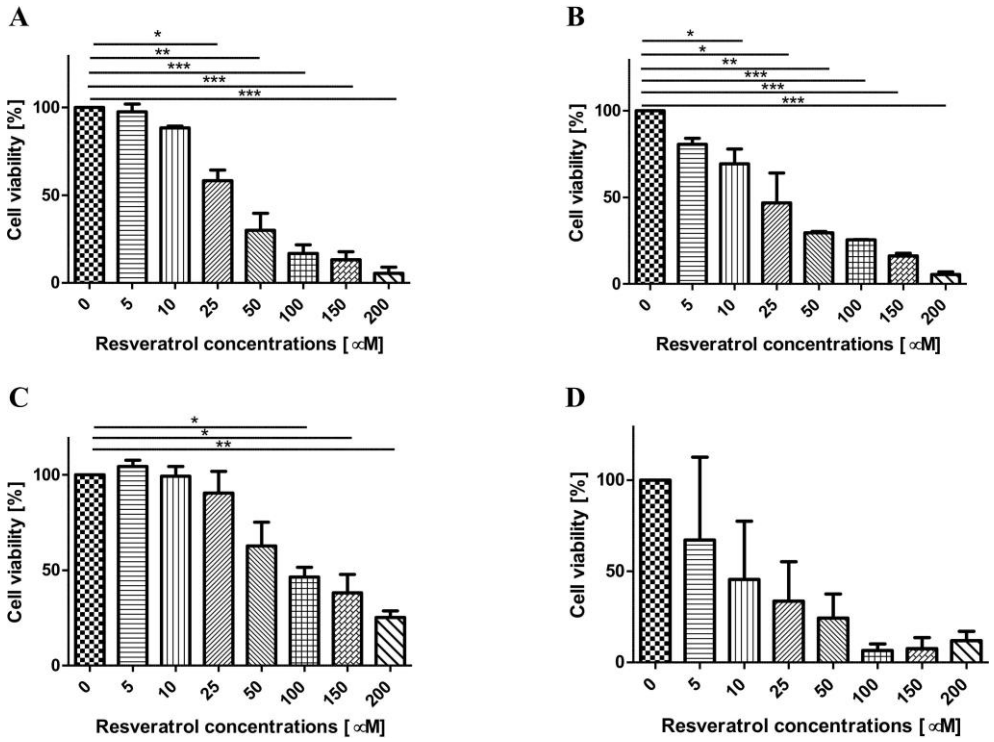

Figure 2. Effect of resveratrol on the proliferation of human cancer and normal pancreatic cells. Cells were treated with various concentrations of resveratrol for 48 h. Cell viability was assessed by MTT assays. (**A**) EPP85-181P cell line, (**B**) EPP85-181RNOV cell line, (**C**) AsPC-1 cell line, (**D**) H6c7 cell line. Values are expressed as mean ± SD, (n = 3), * $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$.

2.2. Analysis of the Effect of Resveratrol on the Cell Cycle of Pancreatic Cells Using Flow Cytometry (FACS)

The distribution of the cell cycle phases was observed using flow cytometry. Treatment with resveratrol resulted in cell accumulation in the G0/G1 or S phase, depending on the type of cells and the concentration of the compound. In the EPP85-181P cell line, after 48 h of exposure to resveratrol at a concentration of 50 µM, the cell cycle was inhibited in the S phase. However, at a higher concentration (100 µM), the cycle was inhibited in the G1 phase (Figure 3A). Similar relationships were observed in the EPP85-181RNOV line, in which the cycle was already inhibited in the S phase at concentrations of 25 and 50 µM, although more pronounced changes could be seen at a concentration of 50 µM. Cycle inhibition in the G1 phase was recorded at 100 µM (Figure 3B). In the case of the AsPC-1 cell line, there were no statistically significant differences in the inhibition of the cycle between concentrations in the range of 0–100 µM, but there was a significant change in the distribution of cells in the various phases of the cell cycle (Figure 3C). The effect of various concentrations of resveratrol in the range of 0–100 µM on normal pancreatic cells H6c7 did not change cell distribution throughout the phases of the cell cycle (Figure 3D) (Table 1).

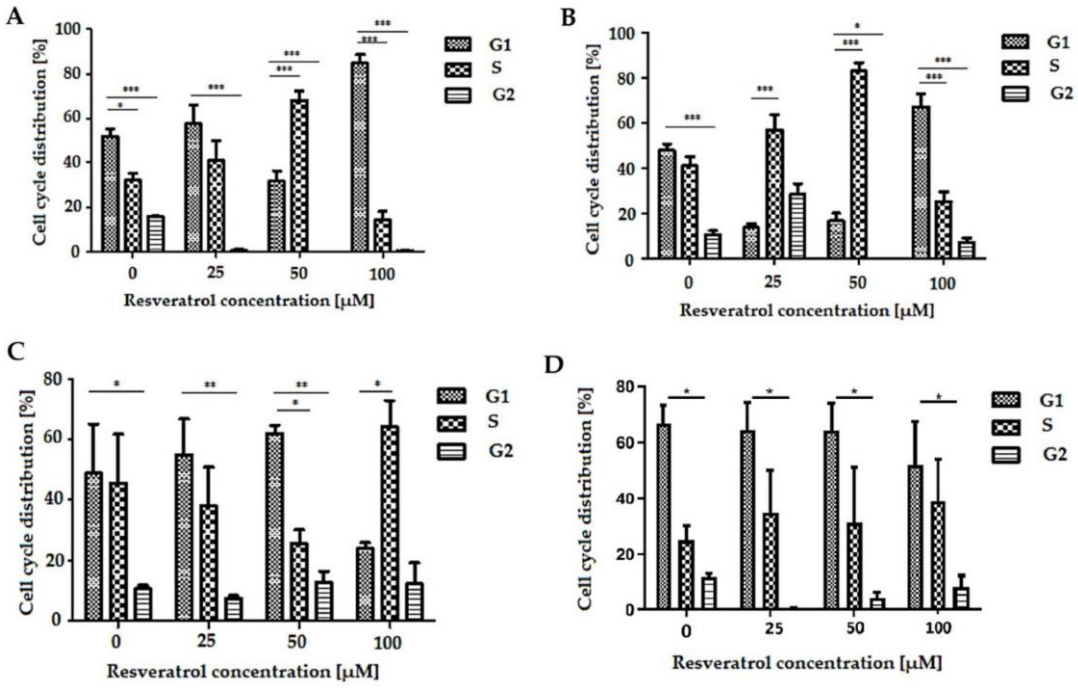

Figure 3. Cell distribution in particular phases of the cell cycle. (**A**) EPP85-181P cell line, (**B**) EPP85-181RNOV cell line, (**C**) AsPC-1 cell line, (**D**) H6c7 cell line, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 1. Change in cell cycle phase distribution after 48-h treatment with resveratrol (0–100 μM) on EPP85-181P, EPP85-181RNOV, AsPC-1 and H6c7 cells. Results are presented as mean of triplicate measurements.

Cell Line	Resveratrol Concentration [μM]	Percentage of Cell Breakdown in Different Phases of the Cell Cycle [%]		
		G1	S	G2
EPP85-181P	0	51.99 ± SD 5.47	32.28 ± SD 5.66	15.73 ± SD 0.44
	25	57.66 ± SD 14.31	4.40 ± SD 15.06	0.61 ± SD 1.05
	50	31.91 ± SD 7.54	68.09 ± SD 7.54	0.00 ± SD 0.0
	100	84.90 ± SD 6.42	14.78 ± SD 6.75	0.32 ± SD 0.56
EPP85-181RNOV	0	47.80 ± SD 5.37	41.70 ± SD7.10	10.50 ± SD 2.85
	25	13.61 ± SD 3.75	57.99 ± SD 11.56	28.40 ± SD 8.07
	50	16.52 ± SD 6.75	83.48 ± SD 6.75	0.00 ± SD 0.00
	100	67.32 ± SD 10.70	25.57 ± SD 7.74	7.11 ± SD 3.00
AsPC-1	0	48.85 ± SD 28.16	45.60 ± SD 28.29	10.52 ± SD 2.16
	25	54.77 ± SD 20.86	38.05 ± SD 22.19	7.18 ± SD 1.83
	50	62.02 ± SD 4.52	25.45 ± SD 8.65	12.53 ± SD 6.55
	100	23.78 ± SD 3.35	64.20 ± SD 15.36	12.02 ± SD 12.36
H6c7	0	66.68 ± SD 6.96	25.41 ± SD 5.86	11.87 ± SD 1.21
	25	64.40 ± SD 10.11	35.37 ± SD 10.41	0.23 ± SD 0.40
	50	64.33 ± SD 9.98	31.22 ± SD 10.86	4.45 ± SD 0.98
	100	51.84 ± SD 15.81	39.89 ± SD 15.80	8.27 ± SD 4.09

2.3. Analysis of the Percentage of Apoptotic Cells after 48 h of Incubation with Resveratrol Solution Measured by Flow Cytometry (FACS)

Flow cytometry analysis was performed to assess the ability of resveratrol to induce apoptosis in pancreatic cells. The tested cells were treated with various concentrations of resveratrol (0, 25, 50 and 100 μM) for 48 h. The obtained results indicated that resver- atrol could induce apoptosis in a concentration-dependent manner (Table 2). The most prominent changes in early apoptosis markers were noted in the EPP85-181P cell line. In

contrast, the smallest increase in the percentage of apoptotic cells was observed in the AsPC-1 cell line. The EPP85-181RNOV and H6c7 cell lines showed a moderate increase in the percentage of cells in the early phase of apoptosis (Figure 4).

Table 2. Apoptosis percentage in neoplastic and normal pancreatic cells induced by resveratrol. Results are presented as mean of triplicate measurements.

Resveratrol Concentration [μM]	Early Apoptosis Percentage [%]			
	EPP85-181P	**EPP85-181RNOV**	**AsPC-1**	**H6c7**
0	7.18 ± SD 1.62	4.04 ± SD 1.59	6.08 ± SD 1.38	2.06 ± SD 0.57
25	10.13 ± SD 2.24	4.92 ± SD 0.79	4.55 ± SD 1.85	6.94 ± SD 1.87
50	18.90 ± SD 1.35	10.40 ± SD 1.27	4.21 ± SD 1.82	10.73 ± SD 3.00
100	21.00 ± SD 2.40	11.64 ± SD 2.13	5.82 ± SD 1.24	36.73 ± SD 8.91

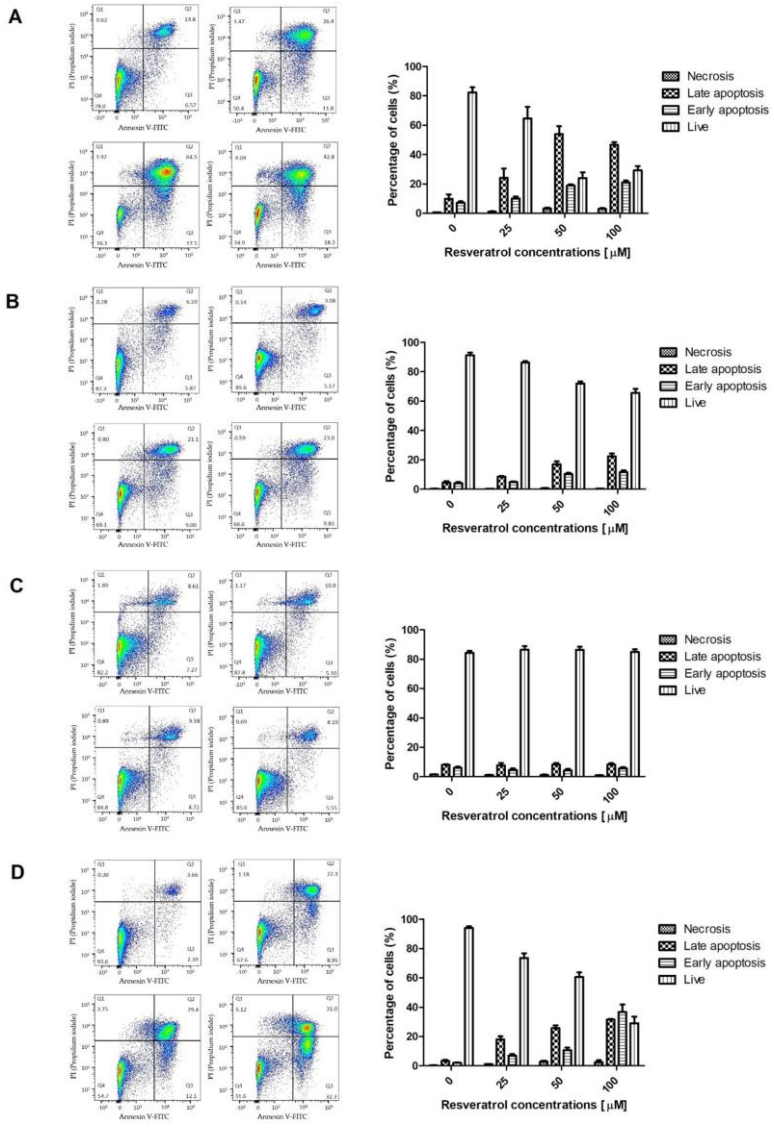

Figure 4. Analysis of apoptotic cells after incubation with resveratrol using flow cytometry. (**A**) EPP85-181P cell line, (**B**) EPP85-181RNOV cell line, (**C**) AsPC-1 cell line and (**D**) H6c7 cell line.

2.4. Analysis of the Percentage of Apoptotic Cells after 48 h of Incubation with Resveratrol Solution Measured Using the TUNEL Assay

The TUNEL reaction showed an increase in apoptosis in all of the cell groups after an incubation of 48 h with various concentrations of resveratrol (Figure 5). The increase in apoptosis in the tested cell lines was consistent with the increase in the concentration of the compound. The smallest increase in the number of apoptotic cells was noted in the AsPC-1 cell line. The EPP85-181P and EPP85-181RNOV cell lines were characterized by a moderate increase in the number of apoptotic cells. The highest number of apoptotic cells was observed in the H6c7 line, but only at the highest resveratrol concentration (100 µM). In vitro experiments have shown that resveratrol can induce apoptosis in pancreatic cells.

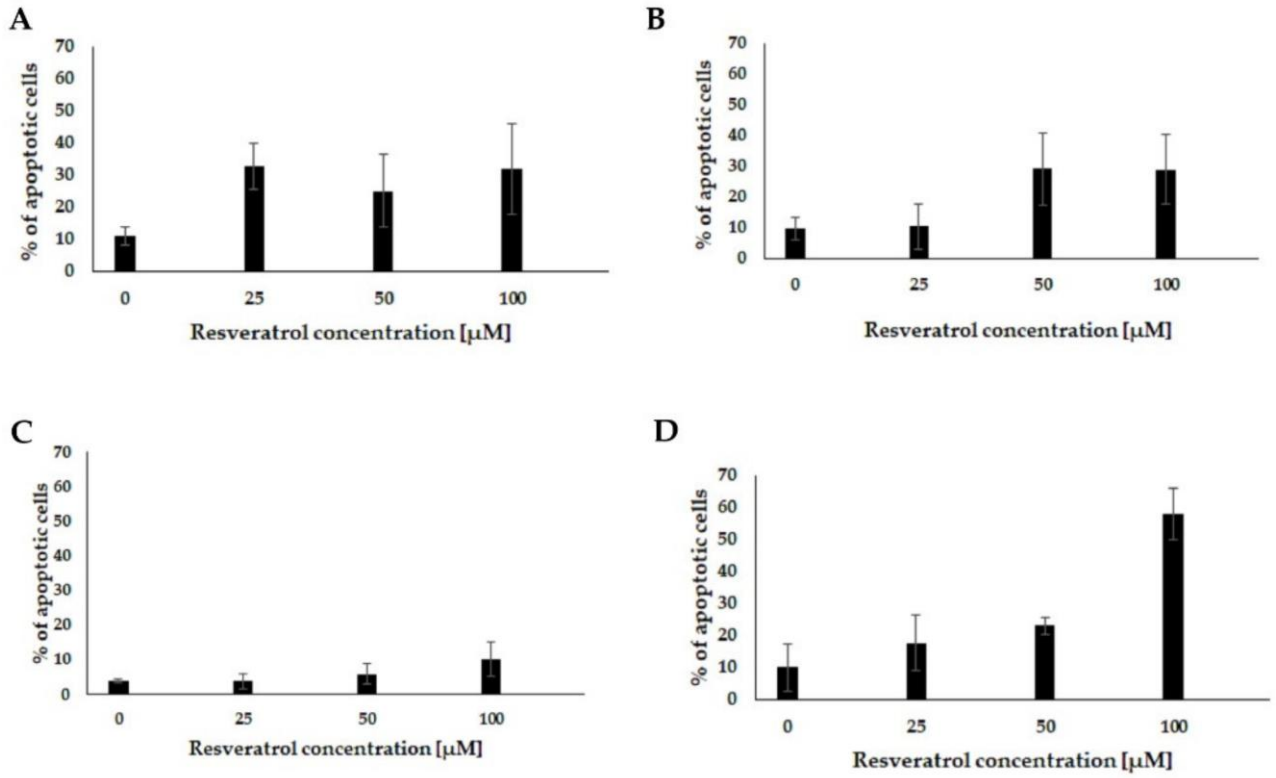

Figure 5. Detection of apoptotic cells using the TUNEL method. The number of apoptotic cells increases with the concentration of resveratrol in all cell lines: (**A**) EPP85-181P cell line, (**B**) EPP85-181RNOV cell line, (**C**) AsPC-1 cell line and (**D**) H6c7 cell line.

2.5. Detection of DNA Damage by Comet Assay

The Comet assay performed under neutral, nondenaturing conditions detects breaks in the double-stranded DNA chain, thus allowing the detection of apoptosis. Using this method, the percentage of apoptotic cells was estimated after 48 h of incubating individual cells with various concentrations of resveratrol. In all the cell lines tested, an increase in the number of damaged cells was observed after exposure to resveratrol. Moreover, this effect was most pronounced in cytostatic-resistant cells (EPP85-181RNOV). The EPP85-181P and H6c7 cell lines showed moderate sensitivity to the action of the compound. The lowest sensitivity was noted in the AsPC-1 cell line (Figure 6).

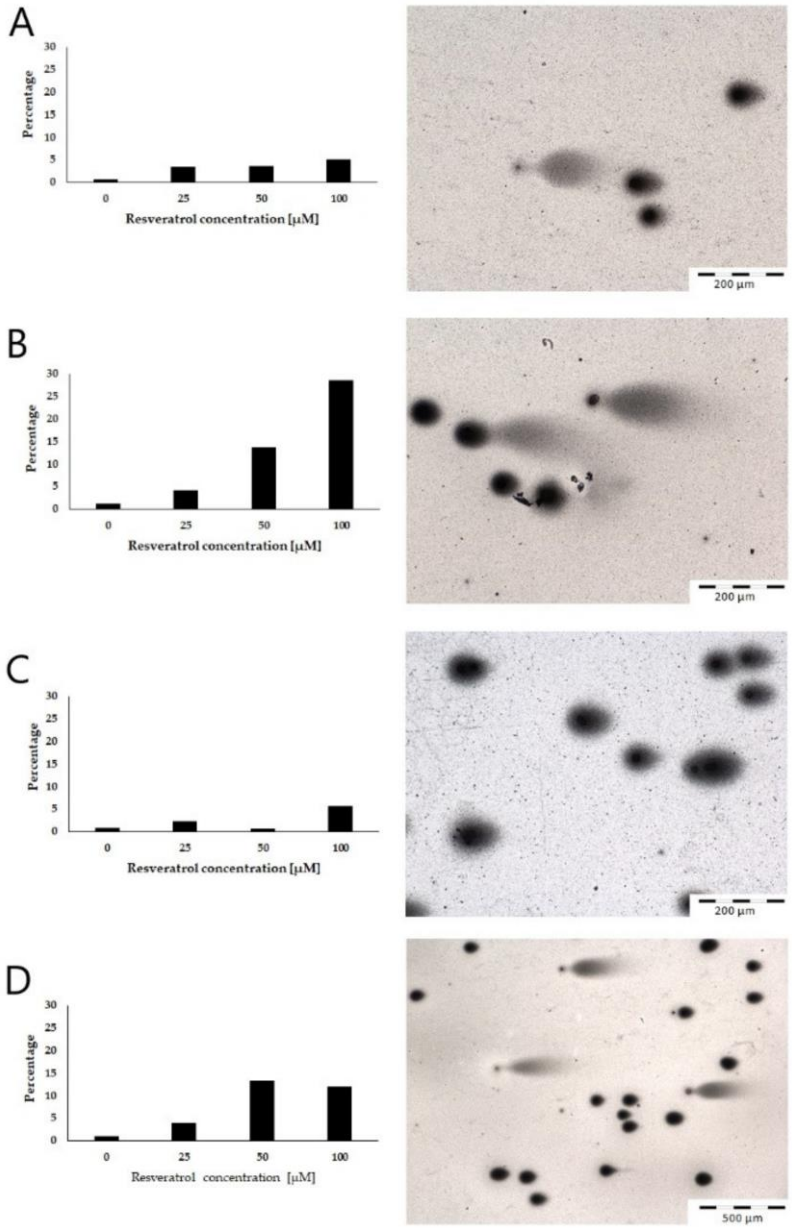

Figure 6. Percentage of nuclei with DNA damage in pancreatic cell lines after 48 h of incubation with different concentrations of resveratrol: (**A**) EPP85-181P cell line, (**B**) EPP85-181RNOV cell line, (**C**) AsPC-1 cell line and (**D**) H6c7 cell line.

2.6. Immunocytochemical Analysis of Resveratrol's Ability to Induce Apoptosis in Human Pancreatic Cells

To assess the ability of resveratrol to induce apoptotic in the tested cell lines, immunocytochemical reactions were performed; on this basis, the impact of the compound on the level of the Bcl-2, Bax and Caspase-3 proteins (which are related to the apoptosis process) was assessed (Figure 7). In the case of the anti-apoptotic protein Bcl-2, a significant decrease in the level of protein was observed after treatment with resveratrol in all of the tested cancer lines (Figure 7A–C). These changes were dependent on the concentration of the compound. The smallest changes were observed in the AsPC-1 cell line (Figure 7C). In turn, treatment with resveratrol caused a significant increase in the level of the Bax and Caspase-3 proteins, with changes depending on the concentration of the compound (Figure 7A–C). In the normal pancreatic cell line H6c7, resveratrol did not cause significant changes in the level of individual proteins.

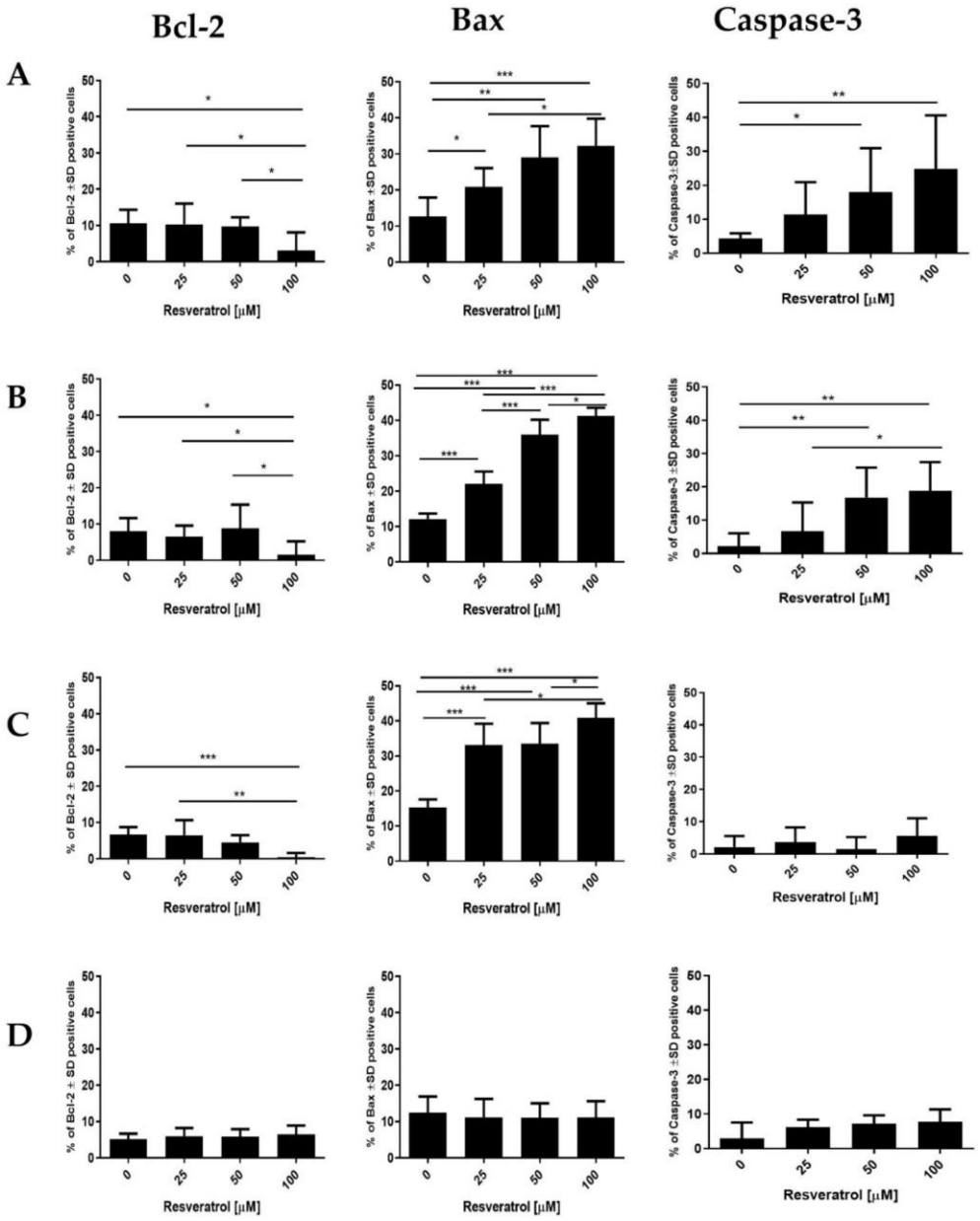

Figure 7. Analysis of resveratrol's ability to induce apoptosis in human pancreatic cells by immunocytochemistry. In the tested cell lines, the effect of resveratrol on the expression level of the Bcl-2, Bax and Caspase-3 proteins (related to the apoptotic process) was assessed. The cells of each cell line were exposed to 48 h of treatment with various concentrations of resveratrol. (**A**) EPP85-181P cell line, (**B**) EPP85-181RNOV cell line, (**C**) AsPC-1 cell line, (**D**) H6c7 cell line; * $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$.

2.7. Changes in the Expression Level of Genes Encoding Proteins of Bcl-2 Family in Pancreatic Cells by Real-Time PCR

The effect of resveratrol on the changes in the expression of the genes encoding proteins related to the apoptotic process was determined by real-time PCR. The analysis of mRNA expression showed that after 48 h of incubation of the cells with various concentrations of resveratrol, concentration-dependent changes in the expression of the *BAX* and *BCL2* genes appeared. In the case of the EPP85-181P cell line, a reduction in *BAX* expression was observed after treatment with resveratrol compared to untreated cells. In contrast, the level of *BCL2* expression after treatment with resveratrol increased at a concentration of

25 µM compared to untreated cells. Between concentrations of 50 and 100 µM, a decrease in the level of *BCL2* expression was observed. In the EPP85-181RNOV cell line, resveratrol reduced *BAX* expression in a concentration-dependent manner. On the other hand, a concentration of 25 µM caused a decrease in the level of *BCL2* expression, while an increase was observed between 50 and 100 µM. Similar results were obtained with the AsPC-1 cell line. The effect of resveratrol resulted in a concentration-dependent decrease in *BAX* expression and an increase in *BCL2* expression. In the H6c7 cell line, resveratrol increased the level of *BAX* expression at a concentration of 25 µM, while decreasing it between 50 and 100 µM. In the case of *BCL2*, a concentration of 25 µM caused a decrease in its expression, while concentrations of 50 and 100 µM led to an increase (left panel of Figure 8).

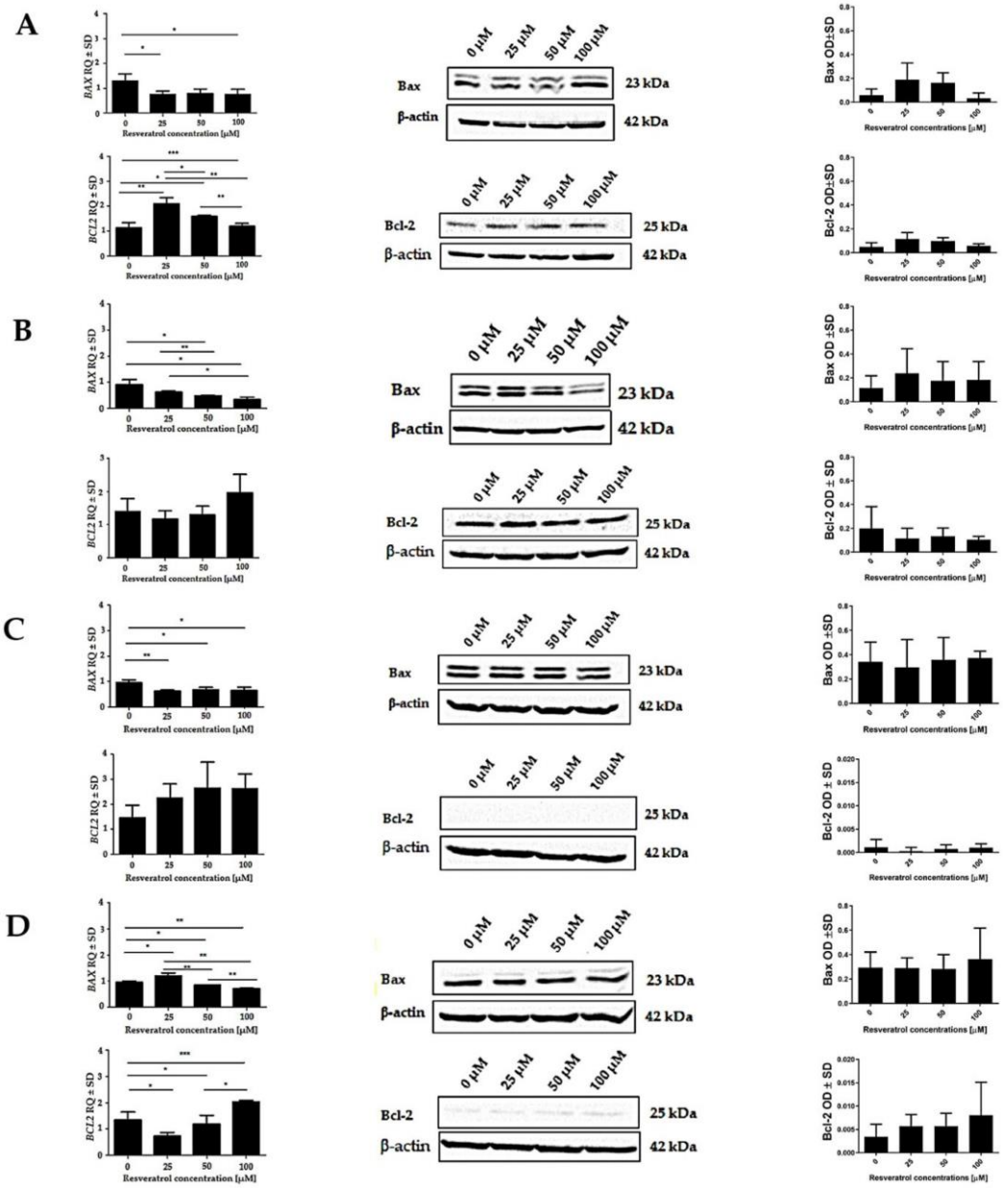

Figure 8. Effect of resveratrol on changes in the expression level of genes and proteins related to the apoptotic process assessed by real-time PCR (left panel) and Western Blot (middle and left panels). The analysis was performed on human pancreatic cell lines after 48 h of exposure to various concentrations of resveratrol. (**A**) EPP85-181P cell line, (**B**) EPP85-181RNOV cell line, (**C**) AsPC-1 cell line and (**D**) H6c7 cell line; * $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$.

2.8. Changes in the Level of Bcl-2 Proteins in Pancreatic Cells (WB)

Western Blot analysis was performed to assess the possible modulation of apoptotic proteins by resveratrol. The changes in the level of Bax and Bcl-2 proteins were examined after 48 h of incubation of human pancreatic cells with various concentrations of resveratrol. After treatment with resveratrol at a concentration of 25 μM, EPP85-181P cells showed a higher level of the pro-apoptotic protein Bax compared to untreated cells. Between 50 and 100 μM, a concentration-dependent decrease in the level of this protein was observed. In the case of the anti-apoptotic protein Bcl-2, an increase in the level was initially observed (25 μM), while higher concentrations of the compound caused a decrease in the level of the protein.

In the cell line showing resistance to mitoxantrone (EPP85-181RNOV), exposure to resveratrol at a concentration of 25 μM resulted in an increase in the level of the Bax protein compared to untreated cells. The effect of higher concentrations led to a decrease in the level of Bax in comparison to its level at 25 μM. However, in this case, the changes in the 50 and 100 μM concentrations were at a similar level. The level of the Bcl-2 protein at a concentration of 25 μM was lower compared to that in cells not treated with resveratrol, and no significant differences in the level of protein were observed with subsequent concentrations.

In the AsPC-1 cell line, slight changes in the level of the Bax protein were observed. There was a slight decrease in the level at a concentration of 25 μM in relation to cells not treated with resveratrol, and between 50 and 100 μM, an increase in the level of expression took place. A similar relationship was observed for the Bcl-2 protein. The action of resveratrol at a concentration of 25 μM reduced the level of protein, while at higher concentrations, an increase in Bcl-2 level was noted.

In the normal H6c7 pancreatic cell line, no significant changes in the level of the Bax protein were observed after treatment with resveratrol. Only at the highest concentration (100 μM), an increase in the level of this protein was noted. In the case of the anti-apoptotic protein Bcl-2, there was an increase in its level in the treated cells, with the changes being proportional to the concentration of the compound (middle and right panels of Figure 8).

2.9. Cell Morphological Changes Induced by Resveratrol

A 48-h treatment with resveratrol induced morphological changes in all the tested cell lines. A decrease in cell density and a change in cellular shape and size were observed (Figure 9).

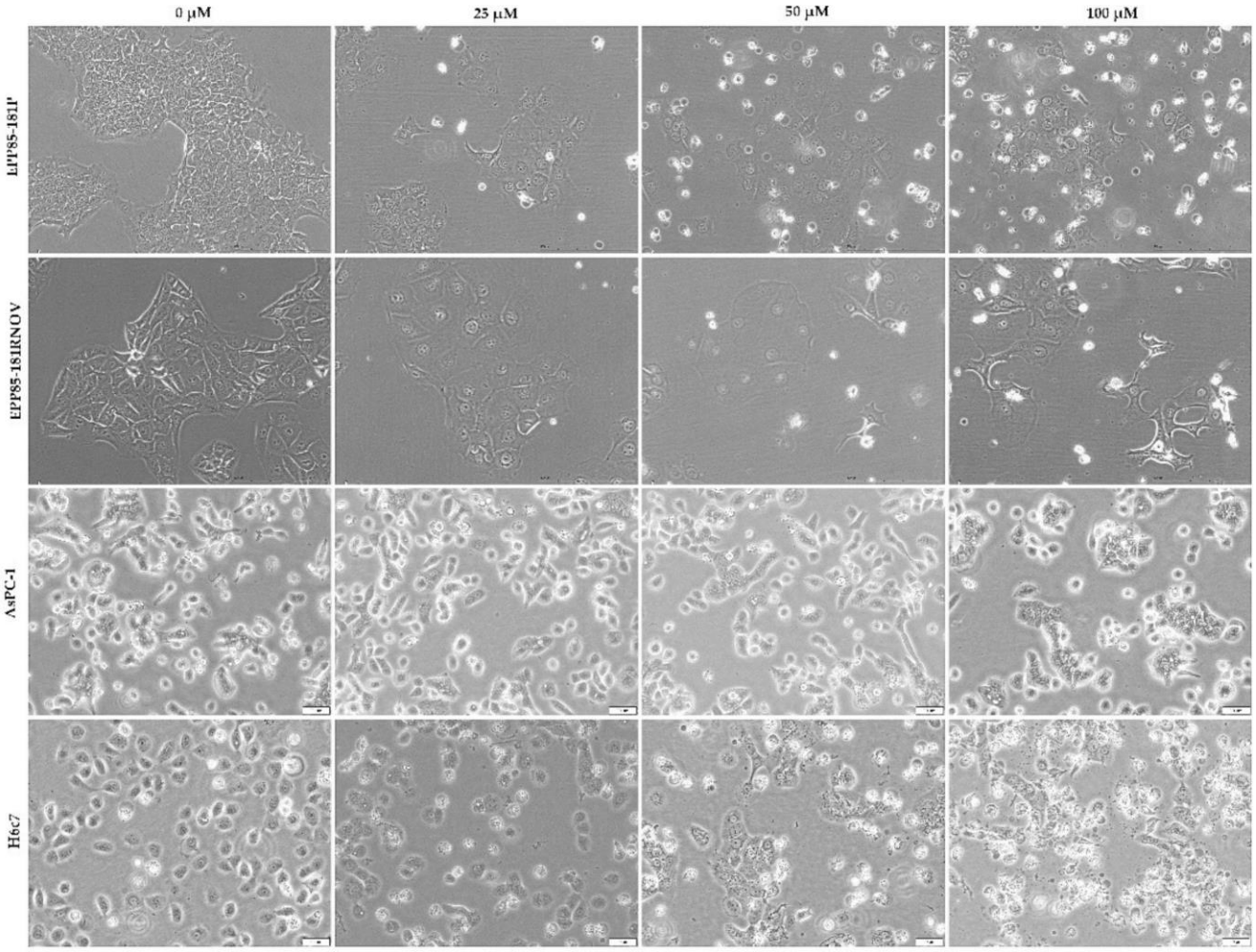

Figure 9. Resveratrol induces morphological changes in EPP85-181P, EPP85-181RNOV, AsPC-1 and H6c7 cells after 48 h incubation with different concentrations of the compound. As the concentration increases, a smaller number of cells can be observed compared to the control, as well as many cells detached (iridescent) from the substrate.

2.10. Bax and Bcl-2 Expression Levels by Confocal Microscopy

To visualize the effect of resveratrol on the level of the Bax and Bcl-2 proteins, an immunofluorescence reaction was performed on the AsPC-1 tumor cell line. The analysis of the results using a confocal microscope showed that 48-h treatment with resveratrol (100 μM) resulted in an increase in the level of the pro-apoptotic protein Bax in relation to untreated cells. In the case of the anti-apoptotic protein Bcl-2, the level was reduced compared to untreated cells (Figure 10).

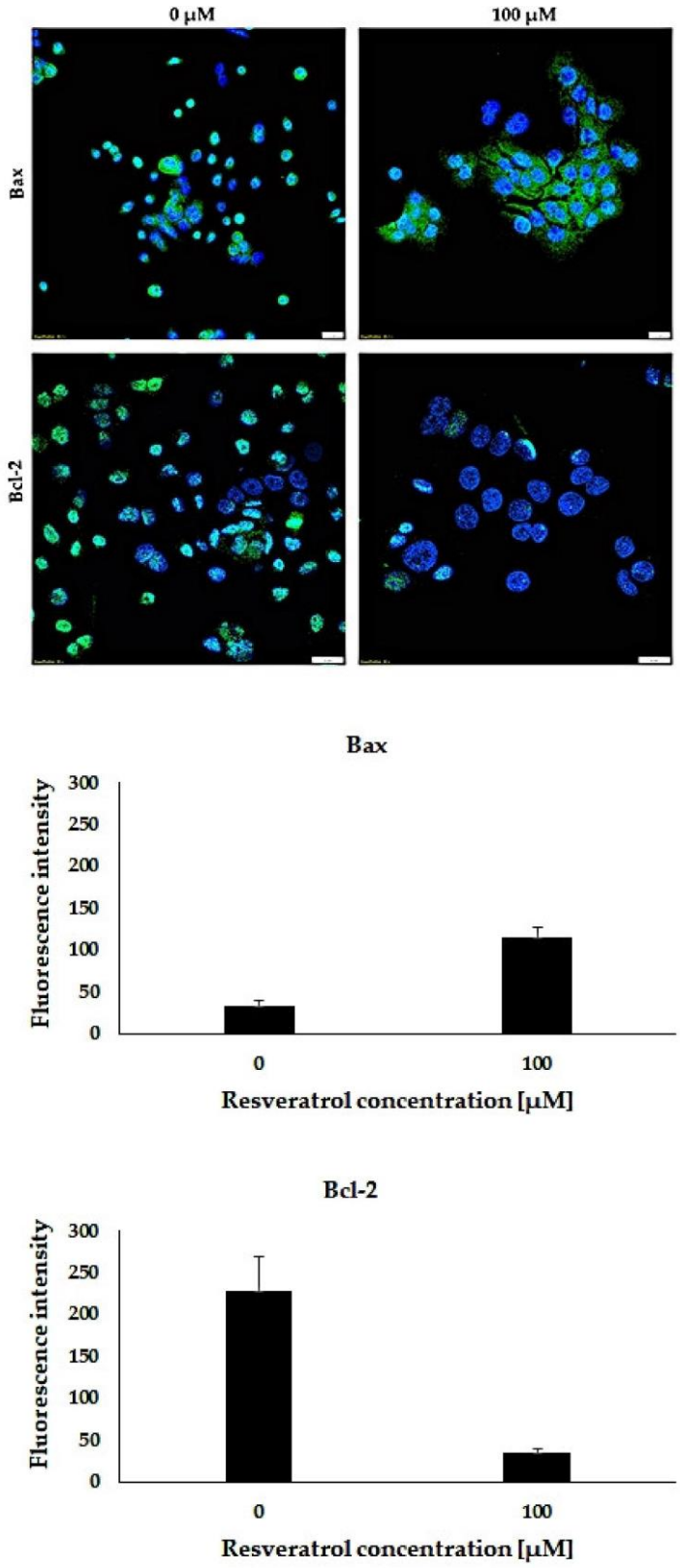

Figure 10. Confocal images showing changes in the level of the Bax and Bcl-2 proteins in the pancreatic cancer cell line AsPC-1 after treatment with 100 μM of resveratrol. Magnification 60×, scale = 20 μm.

3. Discussion

Cancer remains one of the leading causes of death worldwide. Pancreatic neoplasms pose a particular problem due to their late diagnosis because of the lack of early and characteristic symptoms [26]. Hence, there is an increasingly urgent need for compounds (especially of natural origin) that show anticancer activity while protecting normal cells [27]. An example of such a bioactive compound is resveratrol [28]. Even though it has been the subject of many studies, its mechanism of action is not fully understood and requires further research. However, it is known that resveratrol has a pleiotropic effect, and its action depends on many factors (concentration, duration of action, type of cell line).

The present study shows that resveratrol can significantly inhibit the proliferation of pancreatic cells, in a manner dependent both on the duration of the effect and the concentration of the compound. At the same time, changes in the distribution of neoplastic cells between different phases of the cell cycle were also noticed under the influence of different concentrations of resveratrol.

In one of the studies that analyzed the effect of resveratrol on the proliferation of the pancreatic cancer cells PANC-1 and AsPC-1, significant changes in cell survival were observed after incubations longer than 24 h and at a higher concentration of the compound (100 µM) [3]. Moreover, significant changes in AsPC-1 cell survival appeared only at higher resveratrol concentrations ($\geq$100 µM). The studies conducted by Cui et al. showed that the action of resveratrol inhibits the proliferation of the pancreatic neoplastic cell lines PANC-1, BxPC-3 and AsPC-1, and the changes depend on the concentration and duration of the compound's effect. There were differences in the sensitivity of cells to resveratrol between individual cell lines, with AsPC-1 being the least sensitive (concentration > 100 µM), which was consistent with our observations [29]. Another research team demonstrated the antiproliferative effect of resveratrol on the pancreatic cancer cells MIA PaCa-2, AsPC-1, PANC-1 and Hs766T. In this case, a 48-h incubation with various concentrations of resveratrol resulted in a concentration-dependent inhibition of cell growth. The cell lines differed in their sensitivity to the effects of resveratrol. As in previous studies, the AsPC-1 cell line was one of the least susceptible to the effects of resveratrol [30]. In their studies, Liu et al. assessed the effect of resveratrol on the proliferation of neoplastic (PANC-1, CFPAC-1 and MIA PaCa-2) and normal pancreatic cells (Pancreatic Duct Cells). Cells were exposed to various concentrations of resveratrol (10, 50 and 100 µM) for 72 h. Resveratrol was shown to have a concentration-dependent inhibitory effect on cell viability, which is consistent with our observations. Compared to neoplastic cells (PANC-1, CFPAC-1 and MIA PaCa-2), normal pancreatic duct cells showed greater resistance to the cytotoxic effect of resveratrol [31]. In our study, on the other hand, the normal pancreatic cell line H6c7 showed less tolerance to the compound.

The cell cycle is a basic process common to all living organisms, essential for reproduction and growth. It comprises two main phases: the interphase (G1, S and G2 phases) and mitosis (M) [32]. It was found that resveratrol influences the cell cycle by reducing the number of cells in the G1/S and S/G2 phases, which leads to the inhibition of cell proliferation [33,34]. The analysis of the cell cycle in the tumor lines EPP85-181P and EPP85-181RNOV showed that lower concentrations of resveratrol (25 and 50 µM) increase the number of cells in the S/G2 phase. In the mitoxantrone-resistant line, this effect was stronger. On the other hand, at a higher concentration (100 µM), a greater accumulation of cells in the G1/S phase was observed. These results are consistent with previous studies performed on the EPP85-181P and EPP85-181RNOV cell lines, treated for 72 h with two concentrations of resveratrol (30 and 50 µM) [25]. In the EPP85-181P and EPP85-181RNOV lines, at a concentration of 50 µM, an increase in the number of cells in the S/G2 phase was observed, while in the EPP85-181RNOV line, these changes were visible at a lower concentration (30 µM) [25]. In our study, no significant changes in the distribution of the different phases of the cell cycle were observed in the AsPC-1 line after 48 h of incubation with resveratrol (0–100 µM). In contrast, the team of Cui et al. showed an increase in cell

accumulation in the S phase of the cell cycle. However, these changes were observed after 72 h of incubation with resveratrol at a concentration of 100 µM [3].

Apoptosis is a process involving the activation, expression, and regulation of a wide range of genes with a consequent programmed cell death. This process is aimed at ensuring and maintaining a stable internal environment by removing unwanted and abnormal cells from the body [35]. In neoplastic diseases, the balance between cell division and death is usually disturbed [36]. Therefore, understanding the process of apoptosis may prove helpful not only in assessing the pathogenesis of cancer, but also in developing a treatment strategy.

Along with the effect on the growth and changes in the distribution of cells among the various phases of the cell cycle under the action of resveratrol, the participation of the compound in the process of apoptotic induction has also been observed. In our studies, we have shown that resveratrol induces apoptosis in pancreatic cells, and that the observed changes are concentration dependent. These observations are consistent with research conducted by Roy et al., who showed a similar dependence on the neoplastic pancreatic cell lines PANC-1, MIA PaCa-2, Hs766T and AsPC-1. Additionally, the intensity of the changes depends on the pancreatic cell line. Both in our study and in the work of Roy et al., the AsPC-1 cell line was characterized by its low sensitivity to the action of resveratrol [30]. Furthermore, Cui et al. showed that, out of the three pancreatic cancer lines (PANC-1, AsPC-1 and BxPC-3) incubated for 48 h with various concentrations of resveratrol (0–200 µM), the AsPC-1 line showed the lowest susceptibility to the effect of the compound at lower concentrations. Significant changes appeared only at concentrations over 150 µM [29]. The team of Zhou et al. investigated the ability of resveratrol to induce apoptosis on the pancreatic cancer cell models Capan-1, Capan-2, BxPC-3, MIA PaCa-2 and Colo357. After 24 h of exposure of the cells to resveratrol at a concentration of 200 µM, Capan-1, MIA PaCa-2 and BxPC-3 turned out to be less sensitive to the compound compared to the other two, i.e., Capan-2 and Colo357. The same study also showed a slight effect of resveratrol on the induction of apoptosis in normal pancreatic cells (HPDE—Human Pancreatic Duct Epithelial Cell Line) [37]. In our study, we observed apoptotic changes in the normal pancreatic cell line, but these were most pronounced only at the highest concentration of resveratrol (100 µM). It is also worth noting that we analyzed the effect of the compound after a longer duration of the effect (48 h). The Comet assay results showed DNA damage typical of apoptosis, the trend was similar to that of the TUNEL method.

To better understand the effect of resveratrol on the inhibition of cell growth and the induction of the apoptotic process, Western Blot analyses were performed. Proteins from the Bcl-2 family play a key role in the control of the apoptotic execution process. Among the members of this family, there are pro-apoptotic proteins (e.g., Bid, Bax) and proteins that inhibit this process (e.g., Bcl-2). Thanks to these two opposite types of regulatory proteins, it is possible to maintain the homeostasis of the processes of this type of programmed cell death [38,39].

In studies performed on the PANC-1 and MIA PaCa-2 cell lines, 24-h incubation with different concentrations of resveratrol (0, 50, 10, 150 and 200 µM) resulted in an increase in the level of the Bax protein and a decrease in the level of the Bcl-2 protein [40]. The studies by Yang et al. on the Capan-2 cell line showed that a 24-h incubation of the cells with resveratrol at a concentration of 100 µM led to a significant increase in the level of the Bax protein [41]. An increase in the level of the Bax protein and a decrease in the level of the Bcl-2 protein (in direct proportion to the increase in resveratrol concentration 50, 200 and 400 µM) were also demonstrated in the gastric tumor cell line SGC-7901 after 24 h of incubation [42]. The team of Cui et al., in their studies performed on three pancreatic cell lines (PANC-1, BxPC-3, AsPC-1) exposed for 48 h to different concentrations of resveratrol (0–200 µM), observed changes in the level of pro- and anti-apoptotic proteins. In two of the analyzed cell lines (PANC-1 and BxPC-3), a concentration-dependent increase in the expression level of the pro-apoptotic protein (Bax) and a decrease in the level of the anti-apoptotic protein (Bcl-2) were observed. In the case of the AsPC-1 cell line, no significant

changes in the level of the Bax protein were observed. However, there were changes in the level of the Bcl-2 protein, which were most visible at the highest concentration of resveratrol used [29]. Similarly, in our study, we did not observe significant changes in the level of the Bax protein in the AsPC-1 line after a 48-h incubation with different concentrations of the compound (0, 25, 50 and 100 µM). On the other hand, the most visible changes in the level of the Bcl-2 protein level were noticed at higher concentrations (50 and 100 µM). Immunofluorescence also allowed us to obtain confirmation of changes in the level of these proteins in cells that had been incubated with resveratrol. Confocal microscopy analysis showed an increase in the level of the Bax protein and a decrease in the Bcl-2 protein level in AsPC-1 cells after a 48-h incubation with resveratrol (100 µM).

Furthermore, the analysis of the real-time PCR reaction showed that a 48-h incubation of cells with resveratrol (0, 25, 50 and 100 µM) led to a decrease in the expression of the gene encoding the pro-apoptotic protein Bax (*BAX*) and an increase in the expression of the gene encoding the anti-apoptotic protein Bcl-2 (*BCL2*). This was confirmed by the results obtained by a research team that carried out similar studies on the pancreatic cancer cell line Panc 2.03. In that study, a real-time PCR reaction was performed after 12 and 24 h of exposure to resveratrol at a concentration of 40 µg/mL. The reaction showed an increase in the mRNA expression level of *BAX* and a decrease in the mRNA expression level of *BCL2*. Moreover, Western Blot studies performed on the same cell line after 48-h exposure to resveratrol (10, 20, 40 and 80 µg/mL) confirmed the same relationship we observed in the experiments carried out after the same incubation time [43]. It follows that 48 h is the optimal time to study Bcl-2 and Bax level changes.

4. Materials and Methods

4.1. Cell Lines and Culture Conditions

In vitro studies were carried out on three human pancreatic cancer cell lines: EPP85-181P, EPP85-181RNOV (cell lines were obtained from Institute of Pathology, Charité Campus Mitte, Humboldt University Berlin, Berlin, Germany) and AsPC-1 (ATCC, Manassas, VA, USA), as well as the normal pancreatic line H6c7 (Kerafast, Inc., Boston, MA, USA). The EPP85-181P cell line is sensitive to the action of cytostatics, while the EPP85-181RNOV line is resistant to the action of mitoxantrone. The appropriate culture media were selected for the cultivation of individual cell lines. Lines EPP85-181P and EPP85-181RNOV were grown in Leibovitz's L-15 medium (Sigma, St. Louis, MO, USA) enriched with the following supplements: 10% Fetal Bovine Serum (FBS), 1 mM L-glutamine, 6.25 mg/L fetuin, 80 IE/L insulin, 2.5 mg/L transferrin, 1 g/L glucose, 1.1 g/L $NaHCO_3$ and 1% minimal essential vitamins (Sigma, St. Louis, MO, USA). Mitoxantrone was present in the culture of EPP85-181RNOV cells at a dose of 0.02 µg/mL. RPMI-1640 medium (Gibco Life Technologies, Paisley, Scotland, UK) containing 10% FBS (Sigma, St. Louis, MO, USA) was used to culture AsPC-1 cells. The H6c7 cell line was grown in Keratinocyte serum- free medium SFM (Gibco Life Technologies, Paisley, Scotland, UK) supplemented with bovine pituitary extract (25 µg/mL) and recombinant human epidermal growth factor (0.25 ng/mL). Additionally, the media were supplemented with a 1% penicillin solution and streptomycin (Sigma, St. Louis, MO, USA). Cells were grown in monolayers in 75 cm^2 culture flasks (Thermo Scientific, Roskilde, Denmark) which were placed in an incubator (37 °C, 5% CO_2). A solution of 0.25% trypsin-ethylene diamine tetraacetic acid (Sigma, St. Louis, MO, USA) was used to pass the cells of the pancreatic cancer lines. The passage of normal pancreatic cells was performed with the TrypLETM Express Enzyme solution (Gibco Life Technologies, Paisley, Scotland, UK).

4.2. MTT Assay

The effect of resveratrol on cell proliferation was investigated using the MTT colorimetric assay [44]. The cells of the tested lines were plated in 96-well plates 24 h before the start of the experiment in the following amounts: EPP85-181P and AsPC-1 cells—5 × 10^3 cells/well, EPP85-181RNOV cells—2.5 × 10^3 cells/well and H6c7 cells—

1.2 × 10^4 cells/well. Dimethyl sulfoxide (DMSO) was used as an initial solvent. Resveratrol was subsequently dissolved in culture media for cell treatment. The cells were then treated with a solution of resveratrol at various concentrations (0, 5, 10, 25, 50, 100, 150 and 200 μM) at three-time regimens: 24, 48 and 72 h (37 °C, 5% CO_2). After incubation under the set conditions, cells were treated with a solution of MTT (3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide) (0.5 mg/mL) for 4 h (37 °C, 5% CO_2). The MTT cytotoxicity test is based on the color reaction of a tetrazole salt and the assessment of the mitochondrial activity of the cells. As a result of the reduction of the substrate in the mitochondria of living cells, a water-insoluble purple formazan compound is formed depending on the viability of the cells. After the formazan crystals were thoroughly dissolved in dimethylsulfoxide (DMSO), the absorbance was measured for each sample at 570 nm using a microplate reader (Infinite 200 Pro, TECAN, Männedorf, Switzerland). The experiment was repeated independently three times.

4.3. Cell Cycle Analysis, Flow Cytometry (FACS)

The distribution of the cell cycle phases following resveratrol treatment was assessed using the flow cytometric method. Twenty-four hours before the start of the experiment, the cell lines were plated in the following amounts: EPP85-181P—1.2 × 10^5 cells/well, EPP85-181RNOV—1.8 × 10^5 cells/well, AsPC-1—4.6 × 10^5 cells/well and H6c7—4.6 × 10^5 cells/ well. They were then placed in an incubator (37 °C, 5% CO_2). After this time, cells were treated with resveratrol at concentrations of 0, 25, 50 and 100 μM for 48 h (37 °C, 5% CO_2). Afterwards, they were trypsinized and centrifuged in fresh culture medium (1050 rpm, 5 min), and then rinsed twice in ice-cold Phosphate Buffered Saline (PBS) and fixed in cold 70% ethanol overnight at 4 °C. After that, cells were centrifuged (1050 rpm, 5 min, 4 °C) and rinsed twice in PBS. The samples were stained with an FxCycle™ PI/RNase Staining Solution kit (Life Technologies, Carlsbad, CA, USA) and incubated for 30 min at 37 °C in the dark. Propidium iodide fluorescence was measured using a BD FACSCanto II flow cytometer on channel 630/22 (Beckton Dickinson, Franklin Lakes, NJ, USA). Data from at least 20,000 events per sample were collected and calculated using the ModFit LTTM software, version 4.0.5 (Verity Software House, Inc., Topsham, ME, USA). The experiment was carried out in three independent laboratory replications.

4.4. Apoptosis, Flow Cytometry Method (FACS)

The flow cytometry method was used to study the intensity of the apoptotic induction under the effect of resveratrol. The cells of the tested lines (EPP85-181P, EPP85-181RNOV, AsPC-1 and H6c7) were cultured in 25 cm^2 flasks for 24 h (37 °C, 5% CO_2). After this time, cells were treated with resveratrol at concentrations of 0, 25, 50 and 100 μM for 48 h (37 °C, 5% CO_2). Subsequently, they were trypsinized and then centrifuged (1050 rpm, 5 min) in fresh culture medium. Cells were then rinsed twice in PBS solution and cen- trifuged afterwards (1050 rpm, 5 min). Cells were diluted to 1 × 10^6 cells/mL and stained with the FITC Annexin V Apoptosis Detection Kit II (Beckton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Data from at least 10,000 events were collected for each sample. The results obtained were further analyzed with the FlowJo 10.5 software (FlowJo, Asham, OR, USA). The experiment was carried out in three independent laboratory replications.

4.5. Apoptosis, TUNEL Assay

The ability of resveratrol to induce the apoptotic process was also detected by using the TUNEL assay. The cells of the tested lines were plated on Millicell® EZ SLIDES eight-well glass slides (Merck Millipore, Gernsheim, Germany) in the following amounts: EPP85-181P—1 × 10^4 cells/well, EPP85-181RNOV—1 × 10^4 cells/well, AsPC-1—1.5 × 10^4 cells/ well and H6c7—1.5 × 10^4 cells/well. After 24 h, cells were treated with resveratrol at concentrations of 0, 25, 50 and 100 μM for 48 h (37 °C, 5% CO_2). After this time, cells were fixed in cold methanol-acetone (1:1) for 10 min at 4 °C and then dried. Apoptosis was

detected with the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Merck Millipore, Gernsheim, Germany) according to the manufacturer's instructions. Cells were rinsed with PBS solution (pH 7.4), then incubated with Proteinase K (5 min, room temperature) and rinsed again with PBS solution. Endogenous peroxidase blocking was done by incubation in 3% H_2O_2 in PBS (5 min, room temperature). Next, cells were rinsed again with PBS solution. Cells were then incubated, first with pre-incubation buffer (10 min, room temperature), then with incubation buffer (1 h, 37 °C). The reaction was stopped by adding a stop buffer (10 min, room temperature). Cells were then incubated with antidigoxigenin antibodies (30 min, room temperature). To visualize the nuclei of the apoptotic cells, cells were incubated with diaminobenzidine (DAB, 5 min, room temperature). Contrast staining with hematoxylin was performed. The expression of the nuclei of the apoptotic cells was assessed using a BX-41 light microscope (Olympus, Tokyo, Japan).

4.6. DNA-Damages Visualisation, Comet Assay

The detection of apoptosis-related DNA damage was assessed by using a neutral Comet assay. Cell lines EPP85-181P, EPP85-181RNOV, AsPC-1 and H6c7 were cultured in 25 cm^2 flasks for 24 h (37 °C, 5% CO_2). Afterwards, they were treated with resveratrol at concentrations of 0, 25, 50 and 100 µM for 48 h (37 °C, 5% CO_2). Cells were trypsinized, then centrifuged (1050 rpm, 5 min) in fresh culture medium and later rinsed twice in PBS and centrifuged (1050 rpm, 5 min). The method described by Collins [45] was used to detect DNA damage. Portions of the cells (min. 1 × 104) treated with the specified concentrations of the analyses compound for 48 h were combined with low melting point agarose (type VII) and transferred onto a glass slide precoated with high melting point agarose (type I). Then, the slides were placed in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% Triton X-100, pH 10) at 40 °C for 60 min. Afterwards, the slides were rinsed in electrophoresis buffer (TBE) for 30 min at 40 °C. Then, electrophoresis was carried out at a voltage of 1.0 V/cm, 490 mA intensity for 20 min at 40 °C. Staining was carried out with the silver method. 80–100 nuclei were counted on each slide. DNA damage was assessed, assigning each nucleus to the appropriate category: apoptosis, indirect damage, or no damage.

4.7. Immunocytochemistry (ICC)

To assess the ability of resveratrol to induce the apoptotic process, an ICC reaction was performed. The cells of the tested lines were plated on Millicell EZ SLIDES eight-well glass slides (Merck Millipore, Gernsheim, Germany) in the following amounts: EPP85-181P—1 × 10^4 cells/well, EPP85-181RNOV—1 × 10^4 cells/well, AsPC-1—1.5 × 10^4 cells/well and H6c7—1.5 × 10^4 cells/well. 24 h later, cells were treated with resveratrol at concentrations of 0, 25, 50 and 100 µM for 48 h. After this time, cells were fixed with methanol-acetone (1:1) for 10 min at 4 °C. The ICC reaction was performed on an Autostainer Link48 (Dako, Glostrup, Denmark). The following primary antibodies were used: Bcl-2 (Dako, Glostrup, Denmark), Bax (Santa Cruz Biotechnology, Dallas, TX, USA) and activated Caspase-3 (Cell Signaling Technology, Boston, MA, USA). Slides were first incubated with primary antibodies against Bcl-2 (ready-to-use), Bax (1:25) and activated Caspase-3 (1:400) for 20 min at room temperature, followed by 20 min with EnVision FLEX/HRP (Dako, Glostrup, Denmark). In the next step, the slides were incubated for 10 min with 3,3'-diaminobenzidine (DAB, Dako. Glostrup, Denmark). The slides were counterstained with EnVision FLEX Hematoxylin (Dako, Glostrup, Denmark) and sealed with coverslips in a mounting medium. The ICC reaction was assessed using a BX-41 light microscope (Olympus, Tokyo, Japan).

4.8. Real-Time PCR

An assessment of the changes in the *BAX* and *BCL2* gene expressions was performed after treatment with resveratrol at concentrations of 0, 25, 50 and 100 µM on the cell lines EPP85-181P, EPP85-181RNOV, AsPC-1 and H6c7. After 48 h of incubation with the compound, cells were trypsinized, as described in the Cell Lines and Culture Conditions

section above. RNA was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The samples were digested with RNase DNset (Qiagen, Hilden, Germany) to remove genomic DNA. The concentration and quality of the isolated RNA was measured on a NanoDrop 1000 spectrophotometer (Thermo-Fischer Waltham, MA, USA). A reverse transcription reaction was then performed using the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). The assessment of the changes in the gene expression was performed by real-time PCR using a 7900HT Fast Real Time PCR System thermocycler with SDS 2.3 and RQ Manager 1.2 software (Applied Biosystems, Foster City, CA, USA). Primers *BAX* (Hs00180269_m1 BAX) and *BCL2* (Hs00608023_m1 BCL2) were obtained from Applied Biosystems (Foster City, CA, USA). *GUSB* (beta glucuronidase—Hs99999908_m1 GUSB, Applied Biosystems, Foster City, CA, USA) was used as a reference gene. The reaction was performed in triplicate under the following conditions: polymerase activation at 50 °C for 2 min, initial denaturation at 94 °C for 10 min, 40 cycles including denaturation at 94 °C for 15 s, annealing of primers and probes as well as synthesis at 60 °C for 1 min. The results were analyzed based on the expression of the GUSB reference gene. The relative expression (RQ) of *BCL2* and *BAX* mRNA was calculated using the ΔΔCt method ($RQ = 2^{-\Delta\Delta Ct}$).

4.9. Western Blot

The Western Blot (WB) method was used to study the effect of resveratrol on the changes in the level of the proteins related to the process of apoptosis. The cells of the tested lines (EPP85-181P, EPP85-181RNOV, AsPC-1 and H6c7) were treated with various concentrations of resveratrol (0, 25, 50 and 100 μM) for 48 h. Total cellular protein was isolated from the cell lines tested. The procedure was performed at 4 °C using RIPA lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% IGEPAL CA-630, 0.5% sodium deoxycholate) with the addition of PMSF (2.5 μL/mL RIPA) and an inhibitor cocktail (2 μL/200 μL RIPA). The samples were incubated for 20 min on ice with vortexing every 5 min. After this time, the samples were centrifuged (4 °C, 12 min, 12,000 ×*g*). The supernatant was transferred to clean tubes and frozen at −80 °C. Protein concentration was determined by the BCA method using the Pierce BCA Protein Assay kit (Thermo Fischer Scientific, Waltham MA, USA). Protein samples were loaded in GLB (4×) and denatured (95 °C, 10 min). Total protein (50 μg) was separated by SDS-PAGE in a 12% polyacrylamide gel (Bio-Rad, Hercules, CA, USA) at a voltage of 140 V. Subsequently, wet transfer was performed onto nitrocellulose membranes (Millipore, Billerica, MA, USA) in buffer (48 mM Tris, 39 mM glycine, 20% methanol, 0.1% SDS, pH 9.2) at 100 V for one hour. After transfer, the membranes were rinsed with distilled water then 0.1% TBST solution. After blocking for 1 h at room temperature (Bax: 5% milk in 0.1% TBST; Bcl-2: 4% BSA in 0.1% TBST), the membranes were incubated overnight at 4 °C with specific primary antibodies: mouse anti-Bax (sc-7480; 1:200; Santa Cruz Biotechnology, Dallas, TX, USA) and mouse anti-Bcl-2 (124, 1:200, Novus Biologicals, Littleton, CO, USA). Additionally, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (715-035-152; Jackson ImmunoResearch, Cambridgeshire, UK) at a dilution of 1:3000 (1 h, room temperature). After incubation with the secondary antibodies, the membranes were rinsed and then treated with a Luminata Classico chemiluminescent substrate (Merck KGaA, Darmstad, Germany). As an internal control, β-actin was used to normalize the amount of individual proteins levels. β-actin was detected with primary rabbit antihuman β-actin antibody (4970; Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:2000 (overnight incubation at 4 °C) and horseradish peroxidase conjugated secondary antibody (711-035-152; Jackson ImmunoResearch, Cambridgeshire, UK) at a dilution of 1:3000 (1 h, room temperature). The visualization was made with the ChemiDoc Imaging System with the ImageLab software (Bio-Rad Laboratories, Marnes-la-Coquette, France). A densitometry analysis of the results obtained was performed with the ImageLab software (Bio-Rad Laboratories, Marnes-la-Coquette, France).

4.10. Confocal Microscopy

For immunofluorescence, AsPC-1 cells were plated (1.5×10^4 cells/well) on Millicell® EZ SLIDES eight-well glass slides (Merck Millipore, Gernsheim, Germany). After 24 h, cells were treated with resveratrol (0 and 100 μM) for 48 h. Cells were then fixed in 4% paraformaldehyde (12 min, room temperature). The membranes were permeabilized with 0.2% Triton X-100 (10 min, room temperature). Nonspecific binding sites were blocked with 3% BSA in PBS (1 h, room temperature). Cells were incubated overnight at 4 °C with primary antibodies: Bax (1:25 dilution, Santa Cruz Biotechnology, Dallas, TX, USA) in 3% BSA/PBS and Bcl-2 (ready-to-use, Dako, Glostrup, Denmark). Protein detection was performed with Alexa Fluor 488 conjugated antibody secondary antimouse (dilution 1:2000, Abcam, Cambridge, UK, Cat# ab150113, RRID: AB_2756499), incubation 1 h, temp. 4 °C. The slides were sealed in a medium containing DAPI (Invitrogen, Carlsbad, CA, USA). The analysis of the proteins levels was performed using a Fluoview FV3000 confocal microscope (Olympus, Tokyo, Japan, RRID: SCR_017015) with the cellSens software (Olympus, Tokyo, Japan, RRID: SCR_016238).

4.11. Statistical Analysis

The experiments were performed in three independent laboratory replications. The unpaired t-test was used to compare two groups of data. The one-way ANOVA with post hoc analysis using the Dunn's or Bonferroni multiple comparison tests were used to compare 3 or more groups. Statistical analysis was performed using the Prism 5.0 software (Graphpad Software, Inc., La Jolla, CA, USA). The differences were regarded as significant when $p < 0.05$.

5. Conclusions

The results of our research show the antitumor potential of resveratrol in terms of antiproliferative and pro-apoptotic effects on human pancreatic cells by changing the expression of proteins related to the apoptotic process. At the same time, resveratrol has been shown to have a stronger effect on cancer cells than on normal cells, which is extremely important in the context of possibly using this compound not only in the prevention, but also in the treatment, of pancreatic tumors while protecting normal tissues. Additionally, each cell line is characterized by a different sensitivity to the effect of the compound, which confirms the validity of separate studies for different types of cancer. The action of resveratrol may also be important in the process of overcoming multidrug resistance (MDR) due to the induction of larger changes in cytostatic-resistant cancer cells compared to cytostatic-sensitive cells.

The results of our in vitro study are the basis for planning the direction of further in vivo and clinical research. Due to the low bioavailability of orally administered resveratrol, it is necessary to determine the appropriate dosage and method of administration of the compound, e.g., intravenous (IV) injection or encapsulation.

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7. Podsumowanie i wnioski

Powyższa praca przedstawia analizę wyników badań w modelu *in vitro,* mającą na celu określenie działania cytotoksycznego i proapoptotycznego resweratrolu na wybrane nowotwory (komórki nowotworowe) przewodu pokarmowego. Dostępne wyniki badań *in vitro* wskazują, że działanie resweratrolu jest różne, silniejsze bądź słabsze w zależności od rodzaju nowotworu, co tym samym potwierdza słuszność prowadzenia odrębnych badań dla różnych typów tych schorzeń. Można jednak zaobserwować zależność, pomiędzy efektem działania, a zastosowanym stężeniem i czasem inkubacji z resweratrolem. Analiza wyników przeprowadzonych badań wskazuje na to, że resweratrol może w znacznym stopniu hamować proliferację komórek nowotworowych trzustki, a obserwowany efekt jest zależny od zastosowanego stężenia tego związku oraz czasu inkubacji z nim. Dodatkowo widoczne są także zależne od stężenia resweratrolu zmiany w rozkładzie komórek nowotworowych w różnych fazach cyklu komórkowego. Oprócz wpływu na wzrost i rozkład komórek w poszczególnych fazach cyklu, resweratrol wykazuje także zdolność do indukcji procesu apoptozy poprzez zmiany ekspresji białek proapoptotycznych (Bax) oraz antyapoptotycznych (Bcl-2) w sposób zależny od stężenia. Poprzez wywieranie większego wpływu na komórki oporne na działanie cytostatyków w porównaniu do komórek wrażliwych, resweratrol może mieć znaczenie w przełamywaniu zjawiska oporności wielolekowej (MDR).

Wnioski:

1) Resweratrol wykazuje potencjał przeciwnowotworowy w zakresie działania antyproliferacyjnego i proapoptotycznego na komórki różnych typów raka trzustki i żołądka.
2) Wpływ resweratrolu na komórki nowotworowe jest silniejszy niż na komórki prawidłowe, co wiąże się ze zmniejszeniem negatywnych skutków terapii.
3) Resweratrol może uczestniczyć w przełamywaniu zjawiska MDR poprzez indukowanie zmian w ekspresji genów oraz białek w komórkach opornych na działanie cytostatyków.

8. Załączniki

8.1.Oświadczenia współautorów publikacji stanowiących podstawę pracy doktorskiej.

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Oświadczenie o współautorstwie

Oświadczam, że w pracy: **Katarzyna Mieszała, Małgorzata Rudewicz, Agnieszka Gomułkiewicz, Katarzyna Ratajczak-Wielgomas, Jędrzej Grzegrzółka, Piotr Dzięgiel, Sylwia Borska. Expression of genes and proteins of multidrug resistance in gastric cancer cells treated with resveratrol. Oncol. Lett. 2018, Vol. 15; s. 5825-5832**, mój udział polegał na wykonaniu badań metodą Western Blot oraz ocenie otrzymanych wyników.
Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej Katarzyny Ratajczak, pt.: „Działanie cytotoksyczne i proapoptotyczne resweratrolu na wybrane nowotwory przewodu pokarmowego w badaniach *in vitro*".

Małgorzata Rudewicz

Wrocław, 05.05.2022 r.

Dr Agnieszka Gomułkiewicz
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Oświadczenie

Oświadczam, że w pracy: **Katarzyna Mieszała, Małgorzata Rudewicz, Agnieszka Gomułkiewicz, Katarzyna Ratajczak-Wielgomas, Jędrzej Grzegrzółka, Piotr Dzięgiel, Sylwia Borska. Expression of genes and proteins of multidrug resistance in gastric cancer cells treated with resveratrol. Oncol. Lett. 2018, Vol. 15; s. 5825-5832**, mój udział polegał na wykonaniu badań metodą real-time PCR oraz ocenie otrzymanych wyników.

Agnieszka Gomułkiewicz

Wrocław, 05.05.2022 r.

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Oświadczenie

Oświadczam, że w pracy pt. **„Expression of genes and proteins of multidrug resistance in gastric cancer cells treated with resveratrol" Katarzyna Mieszała, Małgorzata Rudewicz, Agnieszka Gomułkiewicz, Katarzyna Ratajczak-Wielgomas, Jędrzej Grzegrzółka, Piotr Dzięgiel, Sylwia Borska. Oncol. Lett. 2018, Vol. 15; s. 5825-5832**, mój udział polegał na ocenie reakcji immunofluorescencyjnej (IF).

Wrocław, 05.05.2022 r.

Dr n. med. Jędrzej Grzegrzółka
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Oświadczenie

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Wrocław, 05.05.2022 r.

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Oświadczenie

Oświadczam, że w pracy: **Katarzyna Mieszała, Małgorzata Rudewicz, Agnieszka Gomułkiewicz, Katarzyna Ratajczak-Wielgomas, Jędrzej Grzegrzółka, Piotr Dzięgiel, Sylwia Borska. Expression of genes and proteins of multidrug resistance in gastric cancer cells treated with resveratrol. Oncol. Lett. 2018, Vol. 15; s. 5825-5832**, mój udział polegał na konsultacji i opiece merytorycznej oraz korekcie pracy przed złożeniem do druku.

Sylwia Borska

Wrocław, 10.05.2022 r.

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Oświadczenie

Oświadczam, że w pracy: **Katarzyna Ratajczak, Sylwia Borska. Cytotoxic and Proapoptotic Effects of Resveratrol in *In Vitro* Studies on Selected Types of Gastrointestinal Cancers. Mol. 2021, Vol. 26; s. 4350**, mój udział polegał na opracowaniu koncepcji pracy, zebraniu literatury, napisaniu manuskryptu oraz sporządzeniu rycin.

Katarzyna Ratajczak

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Oświadczenie

Oświadczam, że w pracy: **Katarzyna Ratajczak, Natalia Glatzel-Plucińska, Katarzyna Ratajczak-Wielgomas, Katarzyna Nowińska, Sylwia Borska. Effect of Resveratrol Treatment on Human Pancreatic Cancer Cells through Alterations of Bcl-2 Family Members. Mol. 2021, Vol. 26; s. 6560**, mój udział polegał na opracowaniu koncepcji oraz metodologii pracy, wykonaniu badań metodą: MTT, TUNEL, kometową, Western Blot oraz Real-time PCR oraz wykonaniu reakcji immunocytochemicznych, graficznym przedstawieniu wyników, zebraniu literatury, napisaniu manuskryptu oraz korekcie pracy przed złożeniem do druku.

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Oświadczenie

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Oświadczenie

Oświadczam, że w pracy pt:" **Effect of Resveratrol Treatment on Human Pancreatic Cancer Cells through Alterations of Bcl-2 Family Members"Katarzyna Ratajczak, Natalia Glatzel-Plucińska, Katarzyna Ratajczak-Wielgomas, Katarzyna Nowińska, Sylwia Borska. Mol. 2021, Vol. 26; s. 6560**, mój udział polegał na wykonaniu reakcji i ocenie intensywności fluorescencji przy pomocy mikroskopu konfokalnego.

Wrocław, 22.05.2022 r.

Dr Katarzyna Nowińska
Zakład Histologii i Embriologii
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Oświadczenie

Oświadczam, że w pracy: **Katarzyna Ratajczak, Natalia Glatzel-Plucińska, Katarzyna Ratajczak-Wielgomas, Katarzyna Nowińska, Sylwia Borska. Effect of Resveratrol Treatment on Human Pancreatic Cancer Cells through Alterations of Bcl-2 Family Members. Mol. 2021, Vol. 26; s. 6560**, mój udział polegał na wykonaniu analizy statystycznej oraz sporządzeniu wykresów.

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Wrocław, 22.05.2022 r.

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Sylwia Borska

8.2.Dorobek naukowy.

Prace oryginalne:

1. Marcin Mączyński, Sylwia Borska, **Katarzyna Mieszała**, Maja Kocięba, Ewa Zaczyńska, Iwona Kochanowska, Michał Zimecki: *Synthesis, immunosuppressive properties, and mechanism of action of a new isoxazole derivative.*
   Molecules 2018, Vol. 23 no. 7, art. 1545.

2. **Katarzyna Mieszała**, Małgorzata Rudewicz, Agnieszka Gomułkiewicz, Katarzyna Ratajczak-Wielgomas, Jędrzej Grzegrzółka, Piotr Dzięgiel, Sylwia Borska: *Expression of genes and proteins of multidrug resistance in gastric cancer cells treated with resveratrol.*
   Oncol. Lett. 2018, Vol. 15 no. 4, s: 5825-5832.

3. Katarzyna Ratajczak-Wielgomas, Krzysztof Kassolik, Jędrzej Grzegrzółka, Tomasz Halski, Aleksandra Piotrowska, **Katarzyna Mieszała**, Iwona Wilk, Marzenna Podhorska-Okołów, Piotr Dzięgiel: *Effects of massage on the expression of proangiogenic markers in rat skin.*
   Folia Histochem. Cytobiol. 2018, Vol. 56 no. 2, s:83-91.

4. Sylwia Cyboran-Mikołajczyk, Katarzyna Solarska-Ściuk, **Katarzyna Mieszała**, Natalia Glatzel-Plucińska, Karolina Matczak, Halina Kleszczyńska: *The impact of O-glycosylation on cyanidin interaction with RBCs and HMEC-1 cells – structure – activity relationships.*
   Int. J. Mol. Sci. 2019, Vol. 20 no. 8, art. 1928.

5. Jędrzej Grzegrzółka, Mateusz Olbromski, Agnieszka Gomułkiewicz, Aleksandra Piotrowska, Natalia Glatzel-Plucińska, **Katarzyna Ratajczak**, Patrycja Sputa-Grzegrzółka, Adam Rzechonek, Bożena Weryńska, Marzena Podhorska-Okołów, Piotr Dzięgiel: *Role of tesmin expression in non-small cel lung cancer.*
   Oncol. Lett. 2021, Vol. 21 no. 48.

Doniesienia zjazdowe:

1. Sylwia Borska, **Katarzyna Mieszała**, Agnieszka Gomułkiewicz, Piotr Dzięgiel: *Zmiany ekspresji genów związanych z opornością na chemioterapię w komórkach raka żołądka pod wpływem trans-resweratrolu w badaniach in vitro,* XLIX Sympozjum Polskiego Towarzystwa Histochemików i Cytochemików „Współczesne techniki mikroskopowe i molekularne w biologii i medycynie". Międzyzdroje, 9-12 września 2015.

2. **Katarzyna Mieszała**, Agnieszka Gomułkiewicz, Katarzyna Ratajczak-Wielgomas, Jędrzej Grzegrzółka, Piotr Dzięgiel, Sylwia Borska: *Wpływ resweratrolu na ekspresję genów ABCB1, ANXA1, TXN w komórkach ludzkiego raka żołądka wykazujących oporność wielolekową,* VII Sympozjum „Współczesna myśl techniczna w naukach medycznych i biologicznych". Wrocław, 24-25 czerwca 2016.

3. **Katarzyna Mieszała**, Agnieszka Gomułkiewicz, Katarzyna Ratajczak-Wielgomas, Jędrzej Grzegrzółka, Piotr Dzięgiel, Sylwia Borska: *Rola resweratrolu jako modulatora oporności wielolekowej komórek raka żołądka*, 50 Sympozjum Polskiego Towarzystwa Histochemików i Cytochemików „Od przeszłości do teraźniejszości …". Wojanów, 5-8 września 2016.

4. Katarzyna Ratajczak-Wielgomas, Krzysztof Kassolik, Jędrzej Grzegrzółka, Tomasz Halski, Aleksandra Piotrowska, **Katarzyna Mieszała**, Iwona Wilk, Marzenna Podhorska-Okołów, Piotr Dzięgiel, Waldemar Andrzejewski: *Wpływ masażu na ekspresję markerów angiogenezy w skórze,* X Międzynarodowe Dni Fizjoterapii „Jakość i styl życia w świetle nowoczesnej rehabilitacji". Wrocław, 23-25.11.2017.

5. **Katarzyna Mieszała**, Natalia Glatzel-Plucińska, Jędrzej Grzegrzółka, Piotr Dzięgiel, Sylwia Borska: *Rola resweratrolu w regulacji ekspresji Sirt1 w komórkach nowotworowych raka trzustki,* 51 Sympozjum Polskiego

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6. **Katarzyna Mieszała**, Piotr Dzięgiel, Sylwia Borska: *Regulacja eksprsji Sirt1 pod wpływem resweratrolu w komórkach nowotworowych – badania wstępne,* VIII Sympozjum „Współczesna myśl techniczna w naukach medycznych i biologicznych". Wrocław, 23-24 czerwca 2017.

7. Waldemar Andrzejewski, Katarzyna Ratajczak-Wielgomas, Krzysztof Kassolik, Jędrzej Grzegrzółka, Tomasz Halski, Aleksandra Piotrowska, **Katarzyna Mieszała**, Iwona Wilk, Marzenna Podhorska-Okołów, Piotr Dzięgiel: *Masaż jako czynnik inicjujący zmiany strukturalne w skórze*, XIV Konferencja Polskiego Towarzystwa Fizjoterapii „Perspektywy rozwoju fizjoterapii". Pabianice, 7-8 grudnia 2018.

8. **Katarzyna Mieszała**, Łucja Cwynar-Zając, Natalia Glatzel-Plucińska, Katarzyna Nowińska, Piotr Dzięgiel, Sylwia Borska: *Działanie cytotoksyczne resweratrolu na wybrane komórki prawidłowe i nowotworowe trzustki w badaniach in vitro*, IX Sympozjum „Współczesna myśl techniczna w naukach medycznych i biologicznych". Wrocław, 22-23 czerwca 2018.

9. Katarzyna Haczkiewicz, Aleksandra Piotrowska, Aleksandra Partyńska, **Katarzyna Mieszała**, Michał Kulus, Piotr Dzięgiel, Marzenna Podhorska-Okołów: *Autofagia w raku gruczołu piersiowego – badania wstępne,* 52. Zjazd Naukowy Polskiego Towarzystwa Histochemików i Cytochemików „Immunohistochemia i biologia molekularna w morfologii". Białystok, 13-16 września 2018.

10. **Katarzyna Mieszała**, Małgorzata Drąg-Zalesińska, Łucja Cwynar-Zając, Natalia Glatzel-Plucińska, Katarzyna Nowińska, Piotr Dzięgiel, Marzenna Podhorska-Okołów: *Antyproliferacyjne i proapoptotyczne działanie resweratrolu na komórki ludzkich linii nowotworowych i prawidłowych trzustki*, 52. Zjazd Naukowy Polskiego Towarzystwa Histochemików i Cytochemików

„Immunohistochemia i biologia molekularna w morfologii". Białystok, 13-16 września 2018.

11. Katarzyna Solarska-Ściuk, Sylwia Cyboran-Mikołajczyk, **Katarzyna Mieszała**, Natalia Glatzel-Plucińska, Halina Kleszczyńska: *The effect of O-glycosylation on cyanidin interaction with HMEC-1 cells – structure-activity relationships,* 3rd Wroclaw Scientific Meetings. Wrocław, 1-2 marzec 2019.

12. K. Solarska-Ściuk, K. Adah, S. Cyboran-Mikołajczyk, **K. Mieszała,** D. Bonarska-Kujawa, M. Fijałkowski: *Effect of biogenic and pyrogenic SiO_2 NPs on the viability and the level of reactive oxygen species,* 44th FEBS Congress „From molecules to living systems". Kraków, 6-11lipiec 2019.

13. Jędrzej Grzegrzółka, Agnieszka Gomułkiewicz, Aleksandra Piotrowska, Natalia Glatzel-Plucińska, Mateusz Olbromski, **Katarzyna Mieszała**, Patrycja Sputa-Grzegrzółka, Adam Rzechonek, Marzenna Podhorska-Okołów, Piotr Dzięgiel: *Correlation of tesmin, MCM5 and MCM7 expression in non-small cell lung cancer,* 53rd Sympozjum of the Polish Society for Histochemistry and Cytochemistry „From ultrastructure to in vivo imaging: progress in microscopial techniques". Gdańsk, 15-18 września 2019.

14. Alicja Kmiecik, Jędrzej Grzegrzółka, Aleksandra Piotrowska, Katarzyna Ratajczak-Wielgomas, **Katarzyna Ratajczak**, Hanna Romanowicz, Beata Smolarz, Piotr Dzięgiel: *Expression of nucleobindin-2 in breast cancer cells,* 53rd Sympozjum of the Polish Society for Histochemistry and Cytochemistry „From ultrastructure to in vivo imaging: progress in microscopial techniques". Gdańsk, 15-18 września 2019.