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„Ocena interakcji genotoksycznych szczepów *E.coli* z nabłonkiem jelita grubego i wpływu pektyn na aktywność przeciwnowotworową irynotekanu”

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STRESZCZENIE

Skuteczne leczenie chorób nowotworowych stanowi ważny problem kliniczny o dużym znaczeniu społecznym. Rak jelita grubego (CRC) jest coraz częściej spotykanym nowotworem złośliwym przewodu pokarmowego. Spośród rozpoznawanych nowotworów złośliwych, pod względem śmiertelności zajmuje drugie miejsce po raku sutka u kobiet i płuc u mężczyzn. Dotyczy nie tylko osób starszych, nierzadko diagnozowany jest także u osób młodych z wieloletnim wywiadem w kierunku obecności stanów przednowotworowych. Około 10% przypadków CRC rozwija się z powodu zespołów dziedzicznych, takich jak rodzinna polipowatość gruczolakowata i zespół Lyncha. Większe ryzyko zachorowania na CRC obserwuje się również u pacjentów z nieswoistymi zapaleniami jelit, takimi jak wrzodziejące zapalenie jelita grubego i choroba Leśniowskiego-Crohna. Dodatkowo na progresję CRC może wpływać mikrobiota jelita grubego, która jest znacznie zmieniona u chorych na raka w porównaniu z osobami zdrowymi. Uważa się, że zmiany w mikrobiocie odgrywają ważną rolę nie tylko w utrzymaniu homeostazy i integralności jelit, ale także w modulacji reakcji zapalnych. Cytotoksyczne szczepy *E. coli* są nadreprezentowane u pacjentów cierpiących na CRC. Skład i aktywność enzymatyczna mikrobioty jelita grubego może w istotny sposób wpływać na skuteczność chemioterapii. Z drugiej strony, leki chemioterapeutyczne mogą wpływać na mikrobiotę jelitową. Irynotekan jest szeroko stosowanym chemioterapeutykiem w leczeniu raka jelita grubego. Jednak jego rozkład w jelitach z powrotem do aktywnej formy leku przez bakteryjną β-glukuronidazę (GUS) stanowi główną przyczynę obserwowanej toksyczności jelitowej irynotekanu. Leczenie wspomagające mające na celu złagodzenie niepożądanych objawów jest zatem równie ważne jak sama chemioterapia. Celem projektu była ocena interakcji genotoksycznych szczepów *E. coli* z nabłonkiem jelita grubego i wpływu pektyn na aktywność przeciwnowotworową irynotekanu.

Pektyny to heterogeniczna rodzina polisacharydów, stanowiąca około 35-50 % suchej masy ścian komórek roślin wyższych dwuliściennych i jednoliściennych. W diecie człowieka pektyny stanowią główny składnik błonnika pokarmowego – tzw. błonnik rozpuszczalny o bardzo różnorodnej aktywności biologicznej. Cechują się zdolnością do obniżania poziomu lipidów, cholesterolu i glukozy w surowicy krwi. Pektyny pełnią również funkcję czynników immunomodulujących. Udowodniony został także korzystny wpływ obecności pektyn w diecie na mikroflorę przewodu pokarmowego, jak również ich właściwości antykancerogenne. Charakteryzują się dużą zdolnością adhezji do błony śluzowej jelita, tworząc w ten sposób barierę ochronną przed kolonizacją przez oportunistyczną mikroflorę. Prawie 90% pektyny podanej doustnie dociera do okrężnicy. Pektyna w świetle jelita tworzy sprzyjające środowisko dla prawidłowego mikrobiomu i stanowi ważne źródło jego składników odżywczych.

Przemysłowo pektyny ekstrahuje się z macierzy roślinnej poprzez wymywanie słabymi kwasami mineralnymi w wysokiej temperaturze. Taka procedura powoduje jednak, że rozgałęzione struktury pektyn, takie jak ramnogalakturonian I czy ramnogalakturonian II w znacznej części ulegają zniszczeniu. Uważa się, że to właśnie te regiony są kluczowe dla prebiotycznych, immunomodulacyjnych i przeciwnowotworowych właściwości pektyn.

Pektyny badane w niniejszym projekcie zostały enzymatycznie wyekstrahowane z wytłoków jabłkowych przy użyciu endo- celulazy i endoksylanazy syntetyzowanych przez workowce z gatunku *Trichoderma viride*. Oba enzymy z dużą selektywnością hydrolizują wiązania β-1,4 glikozydowe odpowiednio w łańcuchach celulozy i ksylanu dlatego mogą współdziałać w uwalnianiu pektyny ze ścian komórkowych. Dzięki ich użyciu solo i w kooperacji otrzymano trzy unikalne preparaty pektyn jabłkowych nazwane PC, PX i PCX. Właściwości przeciwnowotworowe pektyn jabłkowych badano w panelu linii komórkowych raka okrężnicy (HCT116, Caco-2 i HT-29). Nowotwory jelita grubego są molekularnie niejednorodne i można je podzielić na klinicznie istotne podtypy związane z rokowaniem pacjenta i odpowiedzią na leczenie. HT-29, HCT116 i Caco-2 to modelowe linie komórkowe raka okrężnicy powszechnie stosowane w badaniach. Różnią się pochodzeniem płciowym i statusem mutacji. Toksyczność badanych pektyn została również oceniona wobec komórek nienowotworowych linii FHC (ludzki nabłonek okrężnicy płodu).

Dowiedziono, że nowe, enzymatycznie wyekstrahowane pektyny jabłkowe mogą być obiecującymi kandydatami jako uzupełnienie terapii irynotekanem. Uzyskane pektyny same w sobie zmniejszały żywotność komórek raka jelita grubego, indukowały apoptozę i zwiększały wewnątrzkomórkową produkcję reaktywnych form tlenu. Ponadto nasilały cytotoksyczne i proapoptotyczne działanie irynotekanu. Wyniki badań pozwoliły na stwierdzenie, że w molekularnym mechanizmie działania przeciwzapalnego i przeciwnowotworowego wyizolowanych pektyn jabłkowych biorą udział zarówno: Gal-3 jak i TLR4.

Ponadto pektyny inhibitowały aktywność β-glukuronidazy wytwarzanej przez szczepy *E. coli*-komensalnej mikroflory jelitowej. Dodatkowo badane pektyny hamowały i ograniczały adhezję inwazyjnego szczepu *E. coli* (AIEC) LF82 oraz szczepu laboratoryjnego K-12C600 do komórek raka jelita grubego.

Stwierdzono, że stosunkowo niska masa cząsteczkowa pektyny PCX (pektyna izolowana przez współdziałanie endo-celulazy i endoxylanazy) wraz ze stosunkowo wysokim udziałem regionów RG I nadaje tej pektynie lepsze właściwości przeciwnowotworowe.

Podsumowując, nowe enzymatycznie otrzymane pektyny jabłkowe zostały zidentyfikowane jako pektyny o potencjale adiuwantów terapii irynotekanem oraz mogące dodatkowo złagodzić jego efekty uboczne poprzez hamowanie bakteryjnej GUS i w ten sposób zwiększyć skuteczność terapeutyczną tego leku.

SUMMARY

The effective treatment of cancer is an important clinical problem of great societal importance. Colorectal cancer (CRC) is an increasingly common malignancy of the gastrointestinal tract. Among diagnosed malignancies, it ranks second in terms of mortality after breast cancer in women and lung cancer in men. It affects not only the elderly. It is also not uncommon to diagnose CRC in young people with a long history of pre-malignant conditions. About 10 per cent of CRC cases develop due to hereditary syndromes such as familial adenomatous polyposis and Lynch syndrome. Patients with inflammatory bowel disease, such as ulcerative colitis and Crohn's disease, are also at higher risk of developing CRC. In addition, CRC progression may be influenced by the gut microbiota, which is significantly altered in cancer patients compared to healthy individuals. Changes in the microbiota are thought to play an important role not only in maintaining intestinal homeostasis and integrity, but also in modulating inflammatory responses. Cytotoxic *E. coli* strains are overrepresented in CRC patients. The composition and enzymatic activity of the gut microbiota can significantly influence the efficacy of chemotherapy. On the other hand, chemotherapeutic drugs can affect the gut microbiota. Irinotecan is a widely used chemotherapeutic agent for the treatment of colorectal cancer. However, its degradation in the gut back to the active form of the drug by bacterial β-glucuronidase (GUS) is the main reason for the observed intestinal toxicity of irinotecan. Supportive treatment to alleviate adverse effects is therefore as important as chemotherapy itself. The aim of this project was to evaluate the interaction of genotoxic *E. coli* strains with the colonic epithelium and the effect of pectins on the antitumor activity of irinotecan.

Pectins are a complex, heterogeneous family of polysaccharides, constituting about 35 % of the dry weight of the cell walls of higher dicotyledonous and monocotyledonous plants. They are the main component of dietary fiber. When consumed with the diet, they exhibit a wide variety of biological activities. They have the ability to lower serum lipid, cholesterol and glucose levels. Pectins also act as immunomodulating agents. The beneficial effect of the presence of pectins in the diet on the microflora of the gastrointestinal tract, as well as their proapoptotic and anticancerogenic properties, has also been demonstrated. They are characterized by high adhesion to the intestinal mucosa, thus forming a protective barrier against colonisation by opportunistic microflora. Almost 90% of orally administered pectin reaches the distal portion of the colon. Pectins create a favourable environment for a healthy microbiome and provide a source of nutrients for the bacteria inhabiting the colon.

Industrially, pectins are extracted from the plant matrix by treatment in a high temperature and acidic environment. Such a procedure causes many of the branched structures of rhamnogalacturonan I or rhamnogalacturonan II formed by the sugars to then be destroyed. It is believed that it is these regions that are crucial for the prebiotic, immunomodulatory and anticancer properties of pectins.

The pectins studied in this project were enzymatically extracted from apple pomace using endocellulase and endoxylanase from the filamentous fungus Trichoderma viride. Both enzymes cleave β-1,4 glycosidic bonds in the cellulose and xylan chains, respectively. Both enzymes cooperate in the release of pectin from cell walls. Three unique preparations of apple pectin were obtained (PC, PX, PCX). The anticancer properties of apple pectins were tested in a panel of colon cancer cell lines (HCT116, Caco-2 and HT-29). Colorectal cancers are molecularly heterogeneous and can be divided into clinically relevant subtypes related to patient prognosis and response to treatment. HT-29, HCT116 and Caco-2 are model colon cancer cell lines commonly used in research. They differ in gender origin and mutation status. The toxicity of the tested pectins was also evaluated against non-cancerous cells, FHC cells (human fetal colonic epithelium).

It was demonstrated that the new enzymatically extracted apple pectins could be promising candidates as an adjunct to irinotecan therapy. The obtained pectins themselves reduced the viability of colon cancer cells, induced apoptosis and increased intracellular production of reactive oxygen species. They enhanced the cytotoxic and proapoptotic effects of irinotecan. The results concluded that the molecular mechanism of the anti-inflammatory and anticancer effects of the isolated apple pectins involves both: Gal-3 and TLR4.

Furthermore, the pectin responsible for changes in β-glucuronidase activity was identified, as well as changes in β-glucuronidase activity produced by strains of *E. coli*, the commensal intestinal microflora. In addition, the pectins tested inhibited the adhesion of the invasive *E. coli* (AIEC) strain LF82 and the laboratory strain K-12C600 to colon cancer cells.
It was found that the relatively low molecular weight of PCX pectin (pectin isolated by both endo-cellulase and endoxylanase), together with the relatively high proportion of RG I regions, gave this pectin superior anticancer properties.

In conclusion, novel enzymatically obtained apple pectins have been identified as pectins with potential as adjuvants to irinotecan therapy and with the potential to further alleviate its side effects by inhibiting bacterial β-glucuronidase and thus enhance the therapeutic efficacy of this drug.

WSTĘP

Rak jelita grubego (colorectal cancer — CRC) zajmuje trzecie miejsce wśród najczęściej występujących nowotworów na świecie. Jak podaje International Agency for Cancer Research of the World Health Organization (WHO) w 2020 roku rozpoznano ponad 1,9 mln nowych przypadków CRC i odnotowano ponad 900 000 zgonów [1]. W zdecydowanej większości CRC występuje jako choroba sporadyczna, rozpoznawana głównie u pacjentów po 50. roku życia i zwykle związana z czynnikami środowiskowymi, takimi jak dieta typu zachodniego bogata w czerwone mięso, tłuszcze i cukry. Około 10% przypadków CRC rozwija się z powodu zespołów dziedzicznych, takich jak rodzinna polipowatość gruczolakowata i zespół Lyncha [2]. Większe ryzyko zachorowania na CRC obserwuje się również u pacjentów z nieswoistymi zapaleniami jelit, takimi jak wrzodziejące zapalenie jelita grubego i choroba Leśniowskiego-Crohna. Miejscowy stan zapalny może modulować patogenezę CRC, głównie poprzez złożoną sieć mediatorów stanu zapalnego ekspresjonowanych zarówno przez same komórki nowotworowe, jak i przez różne typy komórek tworzących mikrośrodowisko guza [3,4].

Dodatkowo na progresję CRC może wpływać mikrobiota jelita grubego, która jest znacznie zmieniona u chorych na raka w porównaniu z osobami zdrowymi. Uważa się, że zmiany w mikrobiocie odgrywają ważną rolę nie tylko w utrzymaniu homeostazy i integralności jelit, ale także w modulacji reakcji zapalnych poprzez interakcję m. in. z receptorami TLR (ang. toll-like receptors) i receptorami domenowymi oligomeryzacji nukleotydów. Cytotoksyczne szczepy *E. coli* są nadreprezentowane u pacjentów cierpiących na CRC [5,6,7]. Wykazano również, że mikrobiota moduluje odpowiedź komórek nowotworowych na chemioterapię. Ponadto bierze udział w metabolizmie cytostatyków, czego przykład stanowi przemiana irynotekanu [8].

Irynotekan (CPT–11) jest szeroko stosowanym chemioterapeutykiem w leczeniu raka jelita grubego. Należy do trzech pierwszych rodzajów chemioterapii stosowanych w leczeniu przerzutowego raka jelita grubego [9,10]. Lek ten stanowi pochodną alkaloidu-kamptotecyny [11]. W wyniku hydrolizy irynotekanu przez karboksyloesterazy pacjenta powstaje jego aktywna forma-SN-38, inhibitor topoizomerazy. SN-38 jest metabolizowany poprzez glukuronidację w wątrobie do glukuronidu irynotekanu (SN-38G), który dostaje się do jelita cienkiego wraz z żółcią. Niestety, w jelicie grubym na skutek aktywności bakteryjnej β-glukuronidazy (GUS), SN-38G jest z powrotem rozkładany do aktywnej postaci (SN-38), która działa toksycznie na nabłonek jelitowy, powodując skutki niepożądane chemioterapii [12,13]. Najczęstszymi dla irynotekanu są biegunka, nudności i wymioty.

Biorąc pod uwagę kluczową rolę mikroflory jelitowej w biegunce istotne staje się zahamowanie jelitowej aktywności bakteryjnej GUS bez pogorszenia skuteczności przeciwnowotworowej irynotekanu. Farmakologiczne hamowanie bakteryjnej

β-glukuronidazy pozwalające na ograniczenie objawów biegunkowych wywołanych irynotekanem, zaobserwowano jak dotąd jedynie na modelu mysim po zastosowaniu pochodnej pirazolo [4,3-c] chinoliny (TCH-3562) [14]. Jak pokazują dane literaturowe zidentyfikowanych zostało jeszcze kilka inhibitorów cechujących się znaczną supresją biegunki i uszkodzeń jelita przez CPT-11, niestety jednocześnie zmieniających farmakokinetykę SN-38 w osoczu, która jak wiadomo jest czynnikiem determinującym skuteczność przeciwnowotworową irynotekanu.

Wysoce prawdopodobne wydaje się, że wykorzystanie wyselekcjonowanych pektyn, jako dodatkowego źródła węglowodanów podczas podawania irynotekanu, może ograniczać hydrolizę SN-38 przez bakteryjną β-glukuronidazę.

Pektyny jako element diety wykazują w organizmie działanie plejotropowe. Jako podstawowy składnik ścian komórkowych roślin stanowią istotną frakcję błonnika pokarmowego [15]. Charakteryzują się dość różnorodną strukturą, która zależy od gatunku rośliny, typu tkanki i jej wieku. Podstawowym elementem strukturalnym pektyny jest liniowy polimer kwasu galakturonowego (GalUA). W natywnej pektynie obecne są także odrębne regiony strukturalne tj. ramnogalakturonian I (RG I) składający się z powtarzających się dimerów GalUA i ramnozy, do której przyłączone są różne boczne łańcuchy cukrowe głównie arabiniany i galaktany oraz ramnogalakturonian II (RG II), będący poligalakturonianem z czterema typami łańcuchów bocznych. Łańcuchy boczne ramnogalakturonanu II (RG II) zawierają nietypowe, rzadkie monosacharydy, w tym L-fukozę, apiozę i kwasy: acerowy, glukuronowy, 2-okso-3-deoksy-D-manno-oktulozorowy (KDO) i 2-okso-3-deoksy-heptulozarowy (DHA). Dostępne w handlu pektyny są zwykle pozyskiwane ze skórek cytrusów lub wytłoków jabłkowych poprzez ekstrakcję gorącym kwasem [16]. Pektyny wyekstrahowane tą metodą są, w porównaniu z cząsteczkami natywnymi, zubożone w RG I i RG II [17]. Frakcje pektyn bogate w RG-I mogą blokować galektynę 3 (Gal-3) wykazując silne działanie antynowotworowe i immunomodulujące [18,19].

In vitro wykazano możliwość synergistycznego działania preparatu modyfikowanej pektyny cytrusowej (Pect-MCP) i paklitaxelu (PTX) wobec komórek raka jajnika. Pect-MCP uwrażliwia komórki raka jajnika na PTX właśnie poprzez hamowanie ekspresji Gal-3. Na wielokierunkowy mechanizm działania przeciwnowotworowego pektyn wskazują również wyniki badań przeprowadzonych m.in. na ludzkich komórkach czerniaka linii B16-F1 [20] i komórkach raka stercza MAT-LyLu [21] hodowanych w obecności niskocząsteczkowej pektyny cytrusowej. Proapoptotyczne właściwości pektyn odnotowano w komórkach wielu linii nowotworowych. W zależności od typu komórek pektyny mogą indukować różne szlaki molekularne inicjujące apoptozę w tym wykonawczą kaspazę 3 w komórkach szpiczaka i prostaty [22].

Znana jest także zdolność pektyn do obniżenia ekspresji lub aktywności niektórych markerów stanu zapalnego w różnych typach komórek stymulowanych LPS. Przykładowo modyfikowana pektyna cytrusowa zmniejsza ekspresję COX-2 i indukowalnej syntazy tlenku azotu (iNOS) [23], a pektyna malinowa hamuje wytwarzanie NO i IL-6 [24] w stymulowanych makrofagach. Obniżony poziom ekspresji IL-6 w obecności bogatej w RG I pektyny z zarodków lotosu, zaobserwowano także w komórkach mysiego mikrogleju stymulowanego LPS [25]. Podobny efekt był widoczny w komórkach raka okrężnicy (HT-29 i SW-620) hodowanych w obecności oligogalaktanu jabłkowego [26] lub modyfikowanych polisacharydów jabłkowych [27]. Wówczas, ekspresja COX-2, jak również innych markerów zapalenia, była znacząco obniżona.

Wyjątkowo dużą zdolność adhezji do warstwy mucynowej na powierzchni jelit wykazują natomiast pektyny o dużym udziale homogalakturonianu (HG). Tworzą w ten sposób barierę chroniącą epitelium przed mikroflorą oportunistyczną. Warto podkreślić, że do okrężnicy dociera prawie 90% pektyn podawanych doustnie, co stanowi korzystne siedlisko dla prawidłowego mikrobiomu, a dodatkowo stanowi istotne źródło substancji odżywczych dla zasiedlających układ pokarmowy bakterii [28,29].

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CELE PROJEKTU BADAWCZEGO

1. Omówienie najnowszych doniesień z zakresu patofizjologii i leczenia biegunki indukowanej chemioterapią, które zostały przedstawione w publikacji pt.: **„Chemotherapy-induced diarrhea - pathophysiology, current and future treatment trends."**

2. Ocena wpływu enzymatycznie otrzymywanej pektyny jabłkowej PC (izolacja endo-celulazą) na komórki raka jelita grubego i porównanie jej właściwości z komercyjnie dostępną pektyną cytrusową SolC o potwierdzonej aktywności przeciwnowotworowej.

Zadania badawcze obejmowały:

I. Ocenę aktywności cytotoksycznej pektyny jabłkowej PC,
II. Określenie mechanizmu działania badanych związków poprzez zbadanie ich wpływu na:

- proliferację komórek nowotworowych,
- cykl komórkowy,
- proces apoptozy,
- zdolność do generowania reaktywnych form tlenu,
- modulację odpowiedzi zapalnej,

3. Potwierdzenie możliwości uzyskania korzystnych interakcji (synergizm) w układzie irynotekan:pektyna jabłkowa PC.
4. Ocena hamowania aktywności bakteryjnej β-glukuronidazy (GUS) oraz adhezji komórek bakteryjnych *E. coli* na powierzchni komórek nowotworowych jelita grubego przez pektynę jabłkową PC.
5. Ocena i porównanie aktywności przeciwnowotworowej dwóch enzymatycznie otrzymanych pektyn jabłkowych PX (izolacja endo-ksylanazą) i PCX (izolacja endo-celulazą i endo-ksylanazą)
6. Ocena efektu synergistycznego przy jednoczesnym zastosowaniu irynotekanu wraz z pektynami jabłkowymi PX oraz PCX.
7. Ocena skuteczności działania PX i PCX jako substancji blokujących adhezję *E. coli* do powierzchni komórek jelita grubego

Wyniki badań zostały przedstawione w publikacjach pt.:

„**Newly-obtained apple pectin as an adjunct to irinotecan therapy, presumptively reducing its side effects via influence on colonic *E. coli* β-glucuronidase activity**”

oraz

„The Use of Endo-Cellulase and Endo-Xylanase for Extraction of Apple Pectins as the Factor Modifying Their Anticancer Prop-erties and Affecting Their Synergy with Active Form of Iri-notecan”

MATERIAŁY I METODY

Badania przedstawione w niniejszym opracowaniu przeprowadzono w Katedrze i Zakładzie Biofizyki i Neurobiologii Uniwersytetu Medycznego we Wrocławiu.

W ramach publikacji **"Newly-obtained apple pectin as an adjunct to irinotecan therapy, presumptively reducing its side effects via influence on colonic *E. coli* β-glucuronidase activity"** oraz **"The Use of Endo-Cellulase and Endo-Xylanase for Extraction of Apple Pectins as the Factor Modifying Their Anticancer Prop-erties and Affecting Their Synergy with Active Form of Iri-notecan"** zastosowano następujące materiały, odczynniki i metody:

1. Chemikalia/Odczynniki

SN-38 (7-etylo-10-hydroksykamptotecyna), aktywny metabolit irynotekanu, pochodził z firmy Sigma-Aldrich. PectaSol-C (SolC) niskocząsteczkowa modyfikowana pektyna cytrusowa o udokumentowanej aktywności przeciwnowotworowej została zakupiona w firmie Econugenics. Pektyny jabłkowe izolowane enzymatycznie uzyskano w ramach współpracy z dr hab. Agnieszką Wikierą z Katedry Biotechnologii i Ogólnej Technologii Żywności Uniwersytetu Rolniczego im. Hugona Kołłątaja w Krakowie. Roztwory pektyn przygotowywano w wodzie podwójnie destylowanej. Roztwór podstawowy SN-38 przygotowano w dimetylosulfotlenku (DMSO).

2. Izolacja pektyn

Makrocząsteczkowe pektyny jabłkowe ekstrahowano z wysuszonych wytłoków jabłkowych przy użyciu celulazy (endo-β-1,4-glukanazy, EC 3.2.1.4) i ksylanazy (endo-β-1,4-ksylanazy, EC 3.2.1.8, Sigma-Aldrich, Poznań, Polska) wytwarzanych przez grzyba strzępkowego *Trichoderma viride.*

3. Hodowla komórkowa

Badania przeprowadzono na ludzkich liniach komórkowych raka jelita grubego: HT-29, HCT116, Caco-2 i FHC, uzyskanych z kolekcji ATCC. Komórki hodowano w temperaturze 37°C w atmosferze nasyconej 5% CO_2.

4. Testy żywotności komórek

Stosowano dwa testy: Sulforhodamine B colorimetric assay (SRB assay) oraz (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay (MTT assay)

5. Analiza izobolograficzna

Wykorzystując oprogramowanie CompuSyn (ComboSyn Inc., Paramus, NJ, USA), zgodnie z klasycznym równaniem mediany efektu, opisanym przez Chou i Martina, obliczono wartości wskaźnika kombinacji (CI).

$$CI = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2}$$

Gdzie: (Dx)1 to dawka samego leku 1, która hamuje układ o x%, (Dx) 2 to dawka samego leku 2, która hamuje układ o x%, a (D) 1+ (D)2 to dawki leku 1 i 2 w połączeniu, które również hamują układ o x%. Wartości CI poniżej 1 oznaczają synergizm, wartości CI równe 1 oznaczają efekt addytywny (tj. brak interakcji), a wartości powyżej 1 wskazują na antagonizm.

6. Analiza cytometrii przepływowej

W celu zbadania cyklu komórkowego, apoptozy oraz wewnątrzkomórkowego poziomu reaktywnych form tlenu (ROS) zastosowano metodę cytometrii przepływowej. Apoptozę badano przy użyciu testu apoptozowego Annexin-V (BioLegend) i roztworu jodku propidium (PI) (100 µg/ml; Sigma Aldrich). Poziom ROS mierzono przy użyciu przepuszczalnej dla komórek sondy niefluorescencyjnej - dwuoctanu 2′,7′-dichlorofluorescyny (DCFH-DA, Sigma-Aldrich, Poznań, Polska) Analizy cytometryczne wykonywano natychmiast przy użyciu cytometru przepływowego Aria III (Becton Dickinson) w konfiguracji FITC (wzbudzenie 488 nm; emisja: lustro LP 503, filtr BP 530/30) lub PE (wzbudzenie 547 nm; emisja: 585 nm) i zliczano co najmniej 10 000 komórek.

7. Test aktywności kaspazy-3

Aktywność kaspazy-3, enzymu należącego do rodziny endoproteaz, oceniano przy użyciu komercyjnie dostępnego zestawu (GenScript) zgodnie z protokołem dostarczonym przez producenta.

8. Ocena poziomu dialdehydu malonowego (MDA)

Poziom dialdehydu malonowego (MDA), produktu peroksydacji lipidów, który jest biologicznym markerem stresu oksydacyjnego, analizowano przy użyciu zestawu Lipid Peroxidation (MDA) Assay Kit (Abcam/Symbios, Straszyn, Polska), zgodnie z instrukcją producenta z niewielkimi modyfikacjami.

9. Test immunoenzymatyczny ELISA

W celu ilościowego oznaczania cyklooksygenazy-2 (COX-2), interleukiny-6 (IL-6), receptora toll-podobnego 4 (TLR4) i Gal-3 zastosowano metodę immunoenzymatyczną (ELISA). Testy wykonywano zgodnie z instrukcjami producenta.

10. Szczepy *E. coli*

Testy przeprowadzono wykorzystując dwa szczepy referencyjne.: prototypowy adherentno-inwazyjny szczep *E. coli* (AIEC) LF82 (O83: H1), otrzymany dzięki uprzejmości dr Arlette Darfeuille-Michaud, Université d'Auvergne (Francja) oraz laboratoryjny szczep *E. coli* K-12. C600.

11. Ocena adhezji komórek bakteryjnych do komórek raka jelita grubego

Obrazowanie procesu adhezji komórek bakteryjnych do komórek raka jelita grubego w obecności pektyn: PC, PX lub PCX wykonano pod mikroskopem świetlnym po zabarwieniu metodą Wrighta-Giemsy.

12. Ocena proliferacji komórek *E. coli*

Wpływ pektyn jabłkowych na proliferację komórek *E. coli* oceniano na podstawie pomiarów spektrofotometrycznych przy długości fali 600 nm i porównywano ze wzrostem komórek *E. coli* w czasie zerowym.

13. Oznaczanie aktywności β-glukuronidazy (GUS)

Aktywność β-glukuronidazy obliczano wg: stężenie 4-nitrofenolu/100 mg/mL białka x 60 min.

14. Analiza statystyczna

Wszystkie eksperymenty zostały powtórzone trzykrotnie. Dane przedstawiają średnie ± odchylenie standardowe (SD) z co najmniej trzech powtórzeń. Zastosowano test t-Studenta, a wartości p mniejsze niż 0,05 uznano za istotne statystycznie.

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The Use of Endo-Cellulase and Endo-Xylanase for the Extraction of Apple Pectins as Factors Modifying Their Anticancer Properties and Affecting Their Synergy with the Active Form of Irinotecan

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Abstract: Pectin constitutes an essential component of dietary fiber. Modified pectins from various sources possess potent anticancer and immunomodulatory activities. In this study, two pectins isolated from apple pomace by *Trichoderma* enzyme treatment, PX (with endo-xylanase) and PCX (with both endo-cellulase and endo-xylanase), were studied in colon cancer cell lines (HCT 116, Caco-2, and HT-29). Both pectins reduced colon cancer cell viability, induced apoptosis, and increased intracellular amounts of reactive oxygen species. Additionally, synergy between pectin and an active form of irinotecan, SN-38, in all aspects mentioned above, was discovered. This drug is a common component of cytotoxic combinations recommended as treatment for colon cancer patients. PX and PCX demonstrated significant anti-inflammatory activity in lipopolysaccharide-stimulated cells. Interaction of apple pectins with galectin-3 and Toll-like Receptor 4 (TLR4) was suggested to be responsible for their anticancer and anti-inflammatory effect. Since PCX was more active than PX in almost all experiments, the role of the enzyme used to obtain the pectin for its biological activity was discussed. It was concluded that co-operation between both enzymes was needed to obtain the molecule of the most beneficial properties. The low molecular mass of PCX together with a high proportion of rhamnogalacturonan I (RG I) regions seemed to be crucial for its superior activity.

Keywords: colon cancer; pectin; synergy; SN-38 (irinotecan); apoptosis; inflammation; enzymatic extraction

1. Introduction

Pectins are acidic heteropolysaccharides that may constitute up to 40% of the dry mass of plant cell walls [1] and they are therefore an essential component of dietary fiber consumed by humans. Their structure depends on the plant of origin and the type of plant tissue used as a source, etc. Pectins are complex polymers with highly branched chains. However, some structural regions can always be identified [2]. First, a linear polymer of

galacturonic acid that may be methylated and acetylated constitutes the homogalacturonan (HG) region. Next is the rhamnogalacturonan I (RG I) region, whose backbone consists of repeating dimers of galacturonic acid and rhamnose that are further substituted with galactan and arabinan side chains. Finally, there is a highly conserved rhamnogalacturonan II (RG II) region rich in rare saccharides, e.g., L-fucose, apiose, aceric acid, and 2-keto-3-deoxy-D-manno-octulosonic acid. The importance of RG I and RG II presence in pectin molecules has been emphasized for pectin's biological activity [3,4].

Extracted pectin is mainly used in the food industry as gelling, thickening, emulsifying, and stabilizing agents in a variety of foods [5]. Recently, pectin was also used in the production of edible film coatings [6] as well as drug nanocarriers [7,8]. The recognized biological activities of pectin are very diverse, including the ability to lower serum lipids, cholesterol, and glucose levels, as well as prebiotic activity (reviewed in [9]). Modified pectins were also described as immunomodulating [10], anticancer [11], and proapoptotic agents [12]. These effects are postulated to be mediated via the interaction of pectin with Toll-like Receptor 4 (TLR4) [13] and galectin 3 (Gal-3) [11].

The main commercial sources of pectins are citrus peels and apple pomace [14]. Industrially, pectin is extracted from the plant matrix by temperature treatment (80–100 °C) in acidic conditions (pH 1–3) [15]. Such a procedure, however, results in a reduced amount of RG I and RG II in the product as compared to native pectin. The preparations are enriched in galacturonic acid, while the degree of methylation and the content of neutral sugars is lowered [16]. Therefore, methods in which the enzymatic decomposition of plant material is employed have recently gained more attention [17]. Wikiera et al. [18] have proposed the novel, effective method of enzymatic isolation of apple pectins with the use of fungal endo-cellulase and endo-xylanase. Both enzymes split β–1,4 glycoside bonds within the chains of cellulose and xylan, respectively. Both enzymes co-operate in releasing pectin from cellular walls. The resultant preparation is free from non-pectin sugars. The detailed structure of the obtained pectins was previously described [18], and their properties and biological activity were partially characterized [19]. Shortly, the enzymatically prepared apple pectins had very high molecular masses and contained a significant amount of branched RG I and RG II regions and were highly methylated and rich in neutral sugars (such as arabinose, rhamnose, and galactose), characteristic of pectins in muro.

In our previous study [20], the promising anticancer activity of apple pectin PC, obtained with the use of endo-cellulase, was demonstrated. Its anticancer properties in colon cancer cell line HCT 116 were shown to be superior to commercially available modified citrus pectin, PectaSol. Additionally, the existence of synergy between PC and the active form of irinotecan (SN-38) was proven. In the present work, two other enzymatically extracted apple pectins were studied. PX was obtained with the use of endo-xylanase, and PCX by the treatment of apple pomace by both endo-cellulase and endo-xylanase. Anticancer properties of the apple pectins were studied in the panel of colon cancer cell lines (HCT 116, Caco-2, and HT-29). Colon cancer cell lines have been chosen as a research model since colon cells are likely to come in direct contact with pectin as plant-origin or pectin-containing foods are consumed. Both pectins were demonstrated to reduce colon cancer cell viability, induce apoptosis, and increase the amount of reactive oxygen species (ROS) in cancer cells. Additionally, the synergy between pectin and SN-38 in all aspects mentioned above was discovered. Moreover, apple pectins had an anti-inflammatory effect in colon cancer cells, possibly via the interaction with TLR4. Since PCX turned out to be more active than PX in almost all experiments, the role of the type of enzyme used to obtain the pectin for its biological activity was discussed.

2. Results

2.1. Cytotoxicity Assay

2.1.1. Cytotoxicity of Pectins in Colon Cancer Cells

The anticancer activity of apple pectins was studied in three colon cancer cell lines: HCT 116, Caco-2, and HT-29. Colorectal cancers are molecularly heterogeneous and can be

2.1.2. Cytotoxic Effect of Pectins Combined with SN-38

Figure 1 (right column) also presents the effect of anticancer drug SN-38 (an active form of irinotecan) on the cytotoxic potential of PX and PCX. It was visible that the combination of each apple pectin (at 0.2 mg/mL) with the non-toxic concentration of SN-38 (5 nM) resulted in the enhancement of the cytotoxic effect in all colon cancer cell lines. Both pectins exhibited significant cytotoxicity when applied alone, but the co-treatment of cancer cells with low concentration of SN-38 increased cytotoxicity even more.

This prompted us to perform isobolographic analysis to detect the existence of putative synergy between the apple pectins and the anticancer drug. For pure compounds and their combinations, dose and effect data were obtained from the MTT assay. The analysis via CompuSyn software based on the model of Chou and Martin [23] yielded combination index values (CI) for PX:SN-38 and PCX:SN-38 mixtures (Table 2). For almost all the combinations, CI values were clearly below 1, suggesting synergy between the apple pectins and SN-38. As judged by CI values, in HCT 116 and Caco-2 cells the synergy between PCX and SN-38 was more potent than in case of PX. Surprisingly, this effect was reverted in HT-29 cells. The obtained isobolograms are presented in Figure 2. Additional analysis performed using Combenefit software [24] (Figure 3) corroborated the synergy between the studied substances. Matrix format plots present synergy scores calculated according to the Highest Single Agent (HSA) model [25], and the level of antagonism or synergism is represented by a color scale bar. As can be noticed in both HCT 116 and Caco-2 cells, but not in HT-29 cells, PCX exhibited stronger synergy with SN-38 than PX. Additionally, it can be noticed that the synergistic effect predominantly occurred in low concentrations of anticancer drug, i.e., below its IC_{50} value (that ranged between 7.5 and 8 nM depending on colon cancer cell line).

Table 2. Combination of apple pectins with SN-38 against colon cancer cell growth.

Cell Line	Concentration (mg/mL)			Ratio	Combination Index (CI)
	SN-38	PX	PCX		
HCT 116	0.78×10^{-6}	0.1	-	128,200:1	0.629
	1.96×10^{-6}	0.2	-	102,000:1	0.863
	3.14×10^{-6}	0.5	-	157,730:1	1.010
	0.78×10^{-6}	-	0.1	128,200:1	0.543
	1.96×10^{-6}	-	0.2	102,000:1	0.561
	3.14×10^{-6}	-	0.5	157,730:1	0.905
Caco-2	0.78×10^{-6}	0.1	-	128,200:1	0.895
	1.96×10^{-6}	0.2	-	102,000:1	0.791
	3.14×10^{-6}	0.5	-	157,730:1	0.821
	0.78×10^{-6}	-	0.1	128,200:1	0.599
	1.96×10^{-6}	-	0.2	102,000:1	0.424
	3.14×10^{-6}	-	0.5	157,730:1	0.489
HT-29	0.78×10^{-6}	0.1	-	128,200:1	0.725
	1.96×10^{-6}	0.2	-	102,000:1	0.726
	3.14×10^{-6}	0.5	-	157,730:1	0.735
	0.78×10^{-6}	-	0.1	128,200:1	0.822
	1.96×10^{-6}	-	0.2	102,000:1	0.941
	3.14×10^{-6}	-	0.5	157,730:1	0.956

Dose and effect data were obtained from the MTT assay (mean values of three experiments) and analyzed by CompuSyn software. CI values were calculated by CompuSyn software. CI = 1 indicates additive effect, CI < 1—synergism, and CI > 1—antagonism.

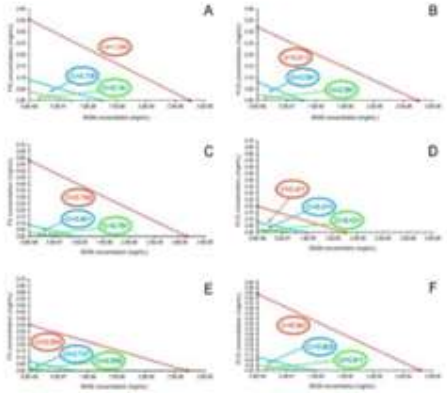

Figure 2. Isobolograms for the interaction of PX (**A**,**C**,**E**) and PCX (**B**,**D**,**F**) with SN-38 in HCT-116 (**A**,**B**), Caco-2 (**C**,**D**), and HT-29 cells (**E**,**F**). The isobolograms were constructed by connecting the IC_{30} (triangles), IC_{50} (circles), and IC_{70} (squares) values of pectins with the appropriate IC values of SN-38. Lines indicate the theoretical lines of additivity.

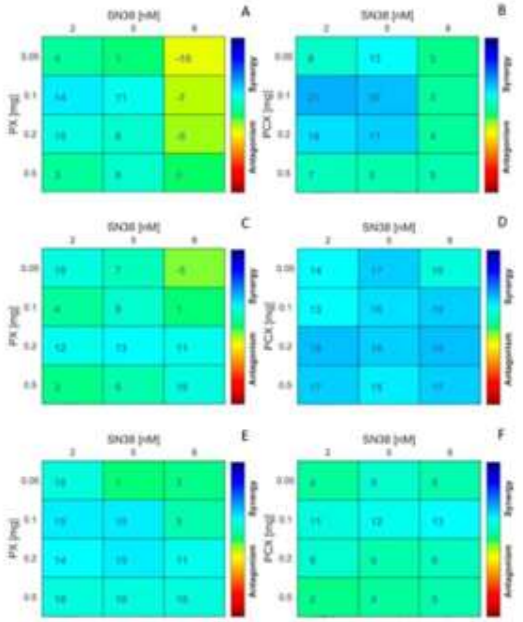

Figure 3. Synergism of apple pectins PX (**A**,**C**,**E**) and PCX (**B**,**D**,**F**) with anticancer drug SN-38 in colon cancer cells HCT 116 (**A**,**B**), Caco-2 (**C**,**D**), and HT-29 (**E**,**F**). The matrices show Combenefit analysis of the combinations. MTT cell viability data after 48 h of incubation (arithmetically averaged data of three independent experiments) were used for the analysis. Synergy score: less than −5—the interaction between two drugs is likely to be antagonistic; from −5 to 5—the interaction is likely to be additive; larger than 5—the interaction is likely to be synergistic.

2.2. Cell Cycle Analysis

Flow cytometric analysis of cellular DNA content was employed to investigate the influence of pectins on the cell cycle of colon cancer cells. Figure 4 presents the proportion of HT-29 cells in different cycle phases and Figure 5 presents the exemplary histograms. It can be noticed that the treatment of cells with 0.2 mg/mL of both PX and PCX caused the significant (* $p < 0.05$ as compared to the untreated control) increase of the sub-G_1 fraction of HT-29 cells with a concomitant decrease in other fractions, especially G_0/G_1. Cells in the sub-G_1 phase are predominantly dead cells, i.e., necrotic or late apoptotic, while G_0/G_1 phase represents living mononuclear cells. The addition of 5 nM of SN-38 also increased the share of sub-G_1 cells, however, this was to a lesser extent than pectins themselves. Most interestingly, the combination of pectin and SN-38 resulted in a robust increase in the number of HT-29 cells in the sub-G_1 phase. This pointed to the mutual enhancement of activity between the anticancer drug and apple pectins. Similar results were obtained when the influence of the studied pectins was investigated in HCT 116 cells and Caco-2 cells (Figure S3).

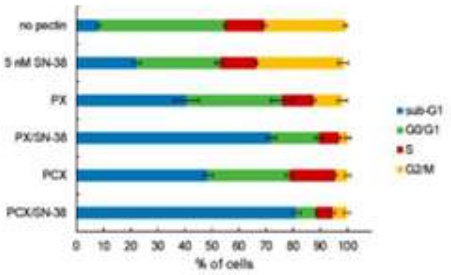

Figure 4. Cell cycle-dependent DNA content in HT-29 cells treated with 0.2 mg/mL of pectins and/or 5 nM SN-38 for 48 h. Sub-G_1 population—dead cells, G_0/G_1—mononuclear cells, S—DNA replication, G_2/M—mitosis. The means of three experiments ± SD are presented.

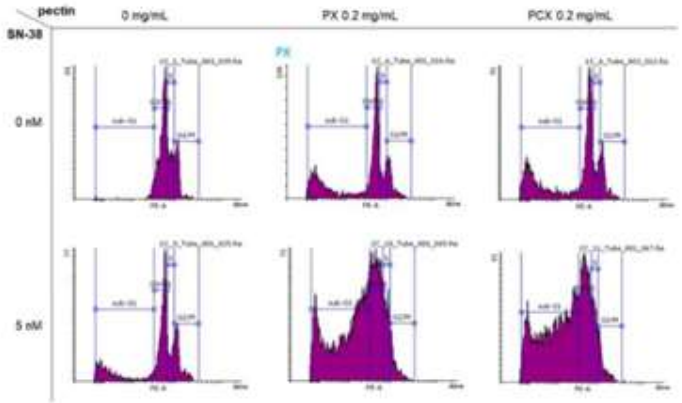

Figure 5. Typical histograms of DNA content (stained with PI) in HT-29 cells treated with 0.2 mg/mL of pectins and/or 5 nM SN-38 for 48 h. Sub-G_1 population—dead cells, G_0/G_1—mononuclear cells, S—DNA replication, G_2/M—mitosis.

2.3. *Apoptosis Detection*

The finding that the treatment of colon cancer cells with pectins affected their cell cycle, causing the increase of dead cells share, prompted us to perform a more detailed analysis of the putative mechanism of cellular death caused by the studied compounds. The results of cytometric experiments on the detection of apoptotic cells are presented in Figure 6. It is clearly visible that the treatment of HT-29 cells by both pectins resulted in an increase in the number of apoptotic cells as compared to the cells not treated with the studied pectins (* $p < 0.05$). SN-38 applied at 5 nM concentration also slightly increased the population of apoptotic cells. However, the most pronounced effect was observed when the pectins were used in combination with the anticancer drug. The proportion of apoptotic cells raised dramatically. It is worth noticing that in all cases, the factor responsible for reducing the normal cell population was the significant increase of the number of apoptotic cells, while the population of necrotic cells increased only slightly. Similar results were also obtained for HCT 116 and Caco-2 cells (Figure S4).

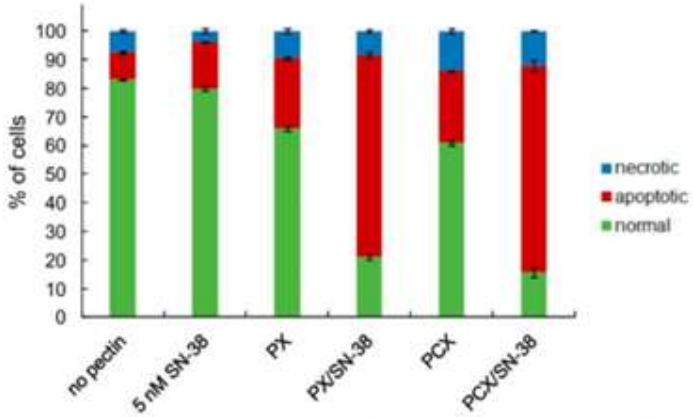

Figure 6. The proportion of normal, apoptotic, and necrotic cell populations as recorded by Annexin-V apoptosis assay in HT-29 cells treated with pectins (0.2 mg/mL) and/or SN-38 for 48 h. The means of three experiments ± SD are presented. Cells were recognized as viable (Annexin-V and PI negative), apoptotic (Annexin-V positive and PI negative), and necrotic (Annexin-V and PI positive) based on the measurement of cell-associated fluorescence of FITC-Annexin-V conjugate and PI.

Additionally, the ability of apple pectins to affect the activity of caspase-3 was studied. This proteolytic enzyme is associated with the execution phase of the apoptotic cascade. As shown in Table 3, both apple pectins caused the activation of caspase-3 in all studied cell lines. At the same concentration (0.2 mg/mL), PCX turned out to be a more potent caspase-3 activator than PX. Anticancer drug SN-38, at 5 nM, virtually did not affect the enzyme's activity. However, the combination of the drug with pectins produced the significant activation of apoptosis-associated caspase. The results of the described above experiments pointed out that apple pectins, especially in combination with SN-38, manifested potent proapoptotic activity in colon cancer cells.

Table 3. Relative caspase-3 activity in colon cancer cells treated with pectins (at 0.2 mg/mL) and pectins in combination with SN-38 (at 5 nM) for 48 h.

Cell Line	Pectin (mg/mL)	Relative Caspase-3 Activity	
			+5 nM SN-38
HCT 116	0	1.00 ± 0.00	1.06 ± 0.06
	0.2 PX	1.40 ± 0.06 *	2.36 ± 0.04 * $^{\$}$
	0.2 PCX	1.47 ± 0.03 *	2.43 ± 0.02 * $^{\$}$
Caco-2	0	1.00 ± 0.00	1.08 ± 0.01
	0.2 PX	1.59 ± 0.05 *	2.40 ± 0.00 * $^{\$}$
	0.2 PCX	1.69 ± 0.02 *	2.63 ± 0.02 * $^{\$}$
HT-29	0	1.00 ± 0.00	1.18 ± 0.19
	0.2 PX	1.59 ± 0.01 *	2.55 ± 0.08 * $^{\$}$
	0.2 PCX	1.67 ± 0.03 *	2.76 ± 0.25 *

The means of three experiments ± SD are presented (* $p < 0.05$). Statistical significance was checked between the studied probes and controls (no pectin), and between the probes containing only pectin and pectin together with SN-38 ($^{\$}$ $p < 0.05$).

2.4. Oxidative Stress Detection

To better understand the mechanism by which apple pectins induced apoptosis in colon cancer cells, the effect of pectins on the degree of oxidative stress in cells was studied. The lipid peroxidation status of cells was determined by measuring the amount of peroxidation product MDA (malondialdehyde) in cells treated by pectins. As shown in Figure 7A, both PX and PCX, when used at 0.2 mg/mL increased the level of lipid peroxidation in HT-29 cells. The active form of irinotecan, SN-38, when applied at low concentration (5 nM), had virtually no effect on the studied parameter. On the other hand, when HT-29 cells were treated with SN-38 in combination with pectins, a further increase of lipid peroxidation was recorded, more pronounced in the case of PCX than PX. Nearly the same results were also obtained in HCT 116 and Caco-2 colon cancer cells (Figure S5).

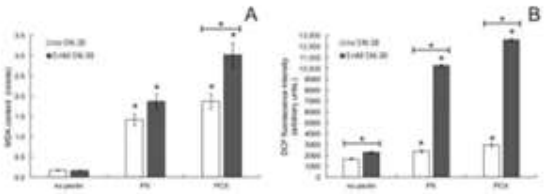

Figure 7. Lipid peroxidation (**A**) and ROS level (**B**) in HT-29 cells treated with 0.2 mg/mL pectins and/or 5 nM SN-38 for 48 h. The means of three experiments ± SD are presented (* $p < 0.05$). Statistical significance was checked between the studied probes and controls (no pectin) as well as between probes containing only pectin and pectin combined with SN-38.

Next, the generation of ROS in colon cancer cells in the presence of apple pectins was quantified. The results of the assay in which intracellular ROS were detected with the use of fluorescent probe DCF are presented in Figure 7B. Both studied pectins (at 0.2 mg/mL) significantly increased ROS levels in HT-29 cells, and the degree of this increase was comparable to the effect caused by SN-38 at 5 nM. The combination of pectins with the low concentration of anticancer drug resulted in a dramatic rise in the amount of ROS generated in colon cancer cells. This effect was slightly stronger for the PCX:SN-38 combination than for PX:SN-38. Similar observations were also made in two other colon cancer cell lines studied (Figure S5); however, in HCT 116 cells, PX was more effective in augmenting the ROS-generating potency of SN-38 than PCX.

2.5. Modulation of Inflammation by Pectins

Colon cancer cells were pretreated by lipopolysaccharide (at 0.5 µM) for 24 h to induce inflammation. The ability of apple pectins to modulate the cellular inflammation process was investigated in a model system prepared in such a way. As shown in Figure 8, the

treatment of HT-29 cells with LPS resulted in a significant increase in both cyclooxygenase 2 (COX-2) and interleukin 6 (IL-6) amounts. Pectins (applied at 0.2 mg/mL) did not affect the level of both inflammation markers in the cells not stimulated by LPS. On the other hand, apple pectins were able to strongly reduce the level of COX-2 (Figure 8A) and IL-6 (Figure 8B) secreted by LPS-pretreated colon cancer cells, which pointed to their anti-inflammatory potency. Similar results were obtained in two other colon cancer cell lines: HCT 116 and Caco-2 (Figure S6).

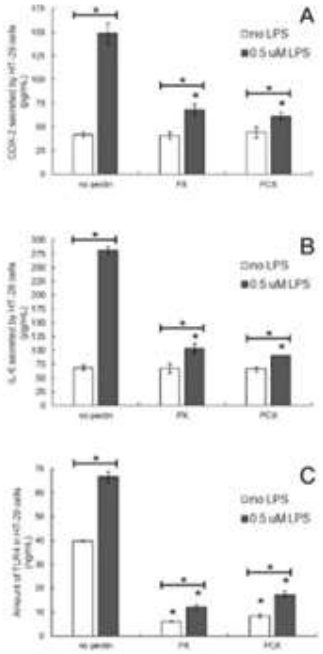

Figure 8. Amount of COX-2 (**A**), IL-6 (**B**) and TLR4 (**C**) in HT-29 cells treated with 0.2 mg/mL pectins and/or 0.5 μM LPS. Cells were pretreated with LPS for 24 h and then incubated with pectins for 48 h. The means of three experiments ± SD are presented (* $p < 0.05$). Statistical significance was checked between the studied probes and controls (no pectin) as well as between probes containing only pectin and pectin combined with LPS.

TLR4 plays the role of main cellular receptor for LPS, and its activation leads to the activation of cytokine production. As shown in Figure 8C, the pretreatment of HT-29 cells by LPS resulted in a significant increase in the amount of TLR4. The studied apple pectins, PX and PCX, caused a significant decrease in the amount of this protein in colon cancer cells that had been both stimulated and not stimulated by LPS.

2.6. Galectin-3 Detection

Since both studied pectins induced apoptosis in colon cancer cells, it was also checked whether they could affect the cellular amount of galectin-3 (Gal-3). Gal-3 possesses many functions. Among others, it is engaged in apoptosis regulation. Investigation of Gal-3 by means of ELISA assay showed that the treatment of HT-29 cells by PX or PCX (at 0.2 mg/mL) resulted in a pronounced drop in the amount of Gal-3 detected in cell lysates

(Figure 9). Additionally, PCX was demonstrated to affect the Gal-3 amount more strongly than PX. Similar observations were made in HCT 116 and Caco-2 cells (Figure S7).

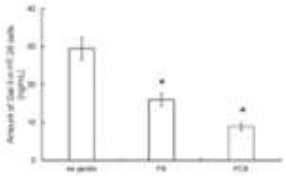

Figure 9. Amount of Gal-3 in HT-29 cells treated with 0.2 mg/mL pectins for 48 h. The means of three experiments ± SD are presented (* $p < 0.05$). Statistical significance was checked between the studied probes and controls (no pectin).

2.7. *E. coli* Adherence to Cancer Cells

Since the interaction of all compounds with colonic cells might be modulated by the local microbiota, the ability of the pectins to interfere with the adherence of *Escherichia coli* was tested in HCT 116 cells (Figure 10). The adherent-invasive strain of bacteria (LF82) isolated from a patient with Crohn's disease was used in experiments. PX inhibited the adherence of LF82 to epithelial cells by ca. 30% (* $p < 0.05$). PCX was less active in this respect. It exerted a slight inhibition on *E. coli* adherence at the highest concentration used, however, statistical significance was not reached.

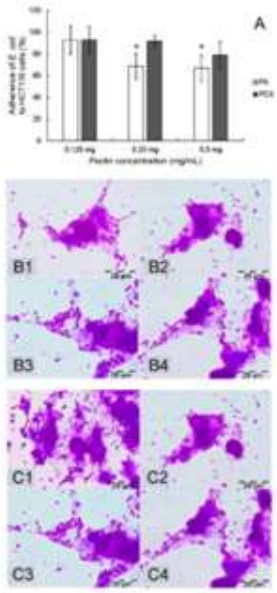

Figure 10. Adherence of *E. coli* LF82 strain to HCT 116 cells (**A**) in the presence of pectins during 2 h of incubation. The means of three experiments ± SD are presented. Statistical significance (* $p < 0.05$) was checked between the studied probes and controls (no pectin) assumed to be 100%. Representative pictures of bacteria adhering to untreated HCT 116 cells (**B1**,**C1**) and treated with PX (**B**) or PCX (**C**) at concentrations of 0.125 mg/mL (**B2**,**C2**), 0.25 mg/mL (**B3**,**C3**), and 0.5 mg/mL (**B4**,**C4**). Wright-Giemsa stain, 100× magnification.

3. Discussion

The anticancer activity of enzymatically extracted apple pectins, PX, and PCX, was demonstrated in a panel of colon cancer cell lines. Both studied pectins were cytotoxic to HCT 116, Caco-2, and HT-29 cells. IC_{50} values recorded for PCX in HT-29 and Caco-2 cells were almost twice lower than those for PX. In HCT 116 cells, IC_{50} values for both pectins were comparable but PCX was still more cytotoxic than PX. Anticancer properties of PX and PCX have been previously studied in adenocarcinoma and melanoma cells [19]. It was found that colon cancer cells were more sensitive to pectins than melanoma cells. In contrast, the viability of normal mouse fibroblasts was not affected by pectins in the studied concentrations. Recently, cellulase-extracted apple pectin, PC, was demonstrated to reduce the viability of colon cancer cells [20]. Pectins isolated from other plant sources, such as potato [26] and ginseng [27], were previously shown to also be anticancer agents in human colorectal adenocarcinoma HT-29 cells. It is worth noticing that the superiority of the anticancer activity of pectin preparations rich in RG I regions over the ones depleted in these regions was reported [12,26]. On the other hand, pectin preparations from jaboticaba fruit [28] and papaya [29], which are rich in galacturonic acid (that suggested high content of HG regions), were demonstrated to be selectively toxic to HCT 116 and HT-29 cells as compared to the preparations with higher contents of neutral sugars.

The cytotoxic properties of the apple pectins towards colon cancer cells were also studied in the presence of a low concentration of the active form of irinotecan (SN-38). The drug was used at the concentration of 5 nM, which was practically non-toxic to none of the studied cell lines. When SN-38 and apple pectins were applied together, the studied compounds exerted a much more pronounced cytotoxic effect on all types of the colon cancer cells. Isobolographic analysis clearly demonstrated the existence of synergy between the apple pectins and SN-38. For PX, the severity of the observed synergistic effect was similar in all three cell lines studied. On the other hand, PCX exhibited more potent synergy with SN-38 in HCT 116 and Caco-2 cells than in HT-29 cells. Therefore, it was concluded that the processes that resulted in synergy were dependent on the type of cancer cells. Together with our previous observation [20], this is the first demonstration of the synergistic effect between apple pectin and the anticancer drug. Previously, only modified citrus pectin was shown to exhibit synergy with paclitaxel in ovarian cancer cells [30], and doxorubicin in prostate cancer cells [31].

The observation of the cytotoxic effect of enzymatically extracted apple pectins on colon cancer cells was supplemented by the study of their effect on cell cycle and apoptosis induction. In all three cell lines, the presence of pectins resulted in an increase in the number of cells in the sub-G_1 phase of the cell cycle (necrotic and late apoptotic cells) as well as the induction of apoptosis (documented as the elevated number of cells that had externalized phosphatidylserine and also as the activation of caspase-3). When the binary combination of the apple pectin and SN-38 was applied to colon cancer cells, the enhancement of the effect of pectins both on the cell cycle and apoptosis induction was recorded. Proapoptotic properties of various pectin preparations in colon cancer cells have been previously reported. Pectins obtained from sugar beetroot and sweet potato were found to induce apoptosis in HT-29 cells [12,32]. The HG-rich ginseng pectin caused HT-29 cell cycle arrest in the G_2/M phase and induced apoptosis accompanied by the activation of caspase-3 [27]. Enzymatically isolated apple pectin, PC, induced apoptosis in human colorectal carcinoma cells HCT 116 [20], and enzymatically prepared low molecular mass citrus pectin reduced viability, induced apoptosis, and caused cell cycle arrest in the S phase in liver cancer HepG2 cells [33]. Finally, it was shown that fish oil- and citrus pectin-enriched diet protected rats from chemically or radiation-induced colon cancer by upregulating apoptosis in colonic mucosa [34].

Next, the ability of the apple pectins to affect the level of lipid peroxidation and intracellular ROS was investigated. It was found that the studied pectins significantly increased the levels of both markers of oxidative stress in colon cancer cells. Additionally, in the presence of SN-38, the effect of pectins was highly augmented. This suggested that

pectins displayed pro-oxidative properties in the experimental settings. Such properties might complement the cytotoxic potential of the apple pectins towards colon cancer cells since the enhancement of ROS production might lead to cellular death via triggering apoptosis. This observation is in apparent contrast with the numerous reports on the antioxidative activity of various pectin preparations (reviewed in [35]), including apple pectin [19]. However, it should be kept in mind that the results obtained in simple laboratory models are not necessarily likely to translate into cellular conditions. The ROS-increasing potency of various pectins has been previously observed. Prostate cancer cells exhibited increased radiosensitivity in the presence of modified citrus pectin, which was associated with the elevated ROS production [36]. Salehi et al. [37] described that citrus and apple pectins induced apoptosis in breast cancer cells through the dysregulation of permeability and subsequent destruction of the mitochondrial membrane that allowed for the excessive ROS release. The increase in ROS production was also recorded in human glioblastoma cells treated with pectins from *Campomanesia xanthocarpa* [38]. Finally, enzymatically extracted apple pectin, PC, has been observed to increase ROS production in colon cancer HCT 116 cells [20]. Pectins are believed to interact with Gal-3, which was shown to influence ROS production, NADPH oxidase enzyme expression, and redox signalling [39]. It can therefore be supposed that the increase of cellular amount of ROS observed in the presence of various pectins might be mediated via their interaction with Gal-3.

The chronic inflammatory process is nowadays believed to lie at the root of the development of many progressive diseases, including cancer. Since the anti-inflammatory activity of pectins has been widely observed (for a review see [40]), it was decided to study apple pectins in this respect. Bacterial LPS was used to induce inflammation. Its application resulted in the significant increase in COX-2 and IL-6 levels in all colon cancer cell lines tested. PX and PCX strongly decreased the amounts of both inflammation markers while not affecting their levels in non-stimulated cells. The ability of pectins to mitigate the expression or activity of various inflammation markers in variable LPS-stimulated cell types has been widely recognized. For example, citrus pectin reduced the expression of COX-2 and inducible nitric oxide synthase (iNOS) [41], and raspberry pectin diminished NO and IL-6 production [42] in stimulated macrophages. Expression of IL-6 was also diminished in LPS-treated murine microglia in the presence of RG I-rich pectin from lotus germs [43]. When LPS-stimulated colon cancer cells (HT-29 and SW-620) were treated by apple oligogalactan [44] or modified apple polysaccharides [45], the expression of COX-2, as well as other inflammation markers, was significantly lowered. Apple pectin, PC, also decreased the amounts of COX-2 and IL-6 in colon cancer cells HCT 116 pretreated with LPS [20]. Ginseng polysaccharides were demonstrated to alleviate colitis symptoms in rats, accompanied by the downregulation of inflammatory cytokines (IL-1β, IL-2, IL-6, and IL-17) [46]. Additionally, antibiotic-associated diarrhea in mice was relieved by the polysaccharide from the rhizome of *Dioscorea opposite*, which also attenuated the expression of IL-1β and IL-6 in colon tissues [47].

Toll-like receptors (TLRs) play a key role in pro-inflammatory signaling networks due to their ability to recognize a variety of pathogen-associated products (e.g., lipids, proteins, lipoproteins, and nucleic acids) and subsequent triggering of the production of inflammatory cytokines, co-stimulatory molecules, interferons, and chemokines [48]. TLR4 is known to be the cellular receptor for LPS produced by Gram-negative bacteria. Since the direct interaction of pectins with TLR4 has been proposed to constitute the mechanism of the immunomodulatory activity of pectins [10], the ability of enzymatically extracted apple pectins to bind to TLR4 was studied by means of an ELISA assay. A measurable amount of TLR4 was detected only in HT-29 cells but not in HCT 116 and Caco-2 cells. The stimulation of colon cancer cells did not change the situation. This is in agreement with the analysis of Suzuki et al. [49], who detected the presence of TLR4 protein in the cytoplasmic fraction of HT-29 and Colo205 cells but not in HCT 116 and Caco-2. This suggested that some other receptors had to be associated with the LPS-responsiveness observed in the two latter cell lines. For instance, TLR2 has also been postulated to serve as the receptor for LPS [50].

As shown by the results of the experiments, both PX and PCX significantly decreased the amount of TLR4 accessible to antibodies in HT-29 cells that were both stimulated and non-stimulated by LPS. Therefore, it was concluded that both studied pectins bound to the TLR4 receptor. The effect of pectins on TLR4 function and expression has already been observed in several laboratory settings, including the experimental models in which the conditions of inflammation-associated diseases were mimicked in animals. Modified citrus pectin downregulated the expression of TLR4 in rats with myocardial fibrosis [51], and an oligogalactan from apple pectin effectively reduced the elevated levels of TLR4 in a mouse model of colitis-associated colon cancer [52]. Moreover, modified apple polysaccharide was shown to suppress TLR4 expression and thus the TLR4-signalling pathway in colon cancer cells [45]. The authors pointed to the competition between LPS and apple polysaccharide for binding to TLR4. Park et al. [53] demonstrated that the anti-TLR4 antibody blocked the immunomodulatory effect of RG I-type polysaccharide from citrus in macrophages. Additionally, the colocalization of RG II with TLR4 in bone marrow dendritic cells was shown [54].

Another essential regulatory protein with which pectins are known to interact is galectin-3. Gal-3 belongs to the lectin family and specifically recognizes β-galactosides (see [55] for a review). Due to the specific binding to various protein targets, Gal-3 mediates numerous biological processes associated with cellular growth, cancer transformation, invasion, and metastasis. The changes in its expression have been reported to occur in many cancer and pre-cancerous conditions. Intracellular Gal-3 is believed to act as an anti-apoptotic factor, so its overexpression is likely to make cancer cells partially resistant to apoptosis. In the present study, an ELISA assay was employed to investigate the interaction of the apple pectins with Gal-3. In all colon cancer cell lines, the presence of pectins caused a decrease in the amount of Gal-3 detected by the antibodies. Additionally, PCX seemed to affect Gal-3 to a larger extent than PX. Therefore, it was concluded that the studied pectins directly interacted with Gal-3, which might be responsible for their observed antiproliferative and proapoptotic activity. Since the first observation of Nangia-Makker et al. [56]—that modified citrus pectin can bind to Gal-3 and act as its competitive inhibitor, many studies have reported the direct interaction between these molecules. Binding between various types of pectins or polysaccharides with Gal-3 has been detected via nuclear magnetic resonance [57,58], surface plasmon resonance [59,60], and atomic force microscopy [61], as well as fluorescence microscopy and flow cytometry [60]. The inhibition of the Gal-3-mediated hemagglutination of erythrocytes by pectins has also been observed [29,62,63].

Interaction of all dietary compounds with colonic cells might be modulated by the local microbiota. Therefore, it was checked whether enzymatically extracted apple pectins could affect the adherence of *Escherichia coli* to colon cancer cells. As both colon cancer and Crohn's disease have been associated with the local inflammation of the colon, an adherent-invasive strain of bacteria (LF82) isolated from a Crohn's disease patient was used in the experiments. Reduced adhesion of LF82 to cancerous epithelial cells in the presence of PX demonstrated the interference of pectin with *E. coli* adherence. LF82 adhesion is determined by mannose-sensitive type 1 fimbriae that bind to mannose receptors on the surface of intestinal epithelial cells. Pectins containing, among others, mannose groups, can interfere with the adhesion of LF82 to the intestinal epithelium that was also shown in our previous study on endo-cellulase-extracted pectin [20].

In summary, we have clearly shown that the apple pectins PX and PCX exhibited antiproliferative, proapoptotic, and anti-inflammatory activities in colon cancer cells. Moreover, both pectins exhibited synergy with SN-38 in reducing the viability of cancer cells. As with the molecular mechanism of anticancer and anti-inflammatory properties of the pectins, the putative interaction with Gal-3 and TLR4 was suggested, respectively. Interestingly, PCX turned out to be a more active antiproliferative agent than PX, while their anti-inflammatory activity was comparable. Moreover, PCX also exhibited a stronger synergy with SN-38. This prompted us to attempt to analyze the features of three different

enzymatically extracted apple pectins that might be important for their anticancer activity. PX was obtained by apple pomace treatment with endo-xylanase, PC with endo-cellulase, and PCX with both enzymes [18]. The anticancer properties of PC have been already studied in HCT 116 cells [20], and it turned out to be more potent than commercially available modified citrus pectin, PectaSol, but less active than PCX. The composition of all three apple pectins has been already characterized [18]. Compared with the standard acid-extracted pectin, all had high molecular masses, were highly methylated, and contained a significant amount of branched RG I and RG II regions. PX was characterized by the highest mass (ca. 900 kDa), the lowest content of galacturonic acid, and the highest content of neutral sugars. PC was intermediate in all discussed aspects, while PCX had the lowest mass (ca. 420 kDa) and the smallest amount of neutral sugars. However, it must be stressed that PCX still contained more branched regions than commercially produced apple pectin. When one looks at literature reports discussing the parameters crucial for the antiproliferative activity of pectins and/or their ability to interact with Gal-3 (which is believed to be the primary mechanism responsible for pectin's anticancer potency), a complicated picture emerges. First, relatively low molecular mass was associated with the beneficial activity, as various types of modified pectins were more potent anticancer agents than naturally large pectins [29,64,65]. The presence of RG I regions has been stressed to be necessary for antiproliferative effect of pectins by many authors [12,26,65]. Also, the content of various types of arabinogalactan side chains was pointed out as an important factor [12,66,67]. In particular, galactans rich in terminal β-galactosides are frequently considered to be "pharmacophores" for Gal-3 binding [67,68]. On the other hand, some reports suggested that the presence of non-esterified HG regions (that translated to high content of glacturonic acid) was necessary for the anticancer activity of pectins [28,29,69]. This apparent discrepancy was explained by realizing that both the RG I/HG backbone and the galactan/arabinan side chains were engaged in the anticancer activity of pectins [12]. The requirement for the correct RG I/HG ratio and the co-operation of both regions was also noticed [61]. Zhang et al. [69] proposed that the interaction of HG with RG I forced the correct spatial arrangement of the pectin molecule in which binding epitopes for Gal-3 (containing galactose and arabinose residues) were exposed. The right proportion between HG and RG I regions and their three-dimensional arrangement seemed also to be the factor explaining the observed differences in activity between the three apple pectins. Although PX contained the highest proportion of branched regions, it was not the most active anticancer compound. The obstacle might be its high molecular mass; such a large molecule was likely to adopt a conformation in which arabinogalactan side chains would be covered and protected from the binding with Gal-3. PCX, which was characterized by the lowest molecular mass, exhibited the superior antiproliferative activity that led us to the conclusion that the co-operation between both endo-xylanase and endo-cellulase was needed to obtain the molecule of the most beneficial properties. An interesting observation was recently made in the study on pectins isolated from papaya fruit at different stages of ripening process [65]. It was shown that the activity of natural plant enzymes, such as polygalacturonases and β-galactosidases, produced during ripening and causing the solubilization of cell walls and fruit softening, resulted in the pectin preparation with potent antiproliferative activity towards colon cancer cells HCT 116 and HT-29 and high potency for Gal-3 binding. This suggests that using natural, plant, fungal or microbial enzymes may constitute a promising direction in the development of pectin extraction methods.

4. Materials and Methods

4.1. Chemicals

SN-38 (7-ethyl-10-hydroxy-camptothecin), an active metabolite of irinotecan, was a product of Sigma-Aldrich (Poznan, Poland). Stock solution of SN-38 was prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C. Stock solutions of pectins were prepared at a concentration of 10 mg/mL in double-distilled water. Stock solutions were diluted in a culture medium just before the experiments.

4.2. Pectin Isolation

Macromolecular apple pectin was extracted from dried apple pomace using endo-cellulase (endo-β-1,4-glucanase, EC 3.2.1.4, Sigma-Aldrich, Poznan, Poland) and endo-xylanase (endo-β-1,4-xylanase, EC 3.2.1.8, Sigma-Aldrich, Poznan, Poland) produced by filamentous fungus *Trichoderma viride*. Isolation was performed with 50 U of enzyme per 1 g of pomace, at pH 5.0, in 40 °C, for 10 h, according to the method described by Wikiera et al. [18]. Briefly, dried and ground apple was treated by the appropriate enzyme for 10 h in conditions of pH 5.0 at 40 °C with constant shaking. After the extraction, the samples were cooled down to 20 °C and centrifuged. Next, cold 96% ethanol (4 °C) was added to the supernatants to the final concentration of 70%. The precipitated pectin was collected by centrifugation, washed with 70% ethanol, centrifuged again and the pellets were dried at 60 °C for 24 h until the constant weight was achieved. The resulting pectins were ground to a particle passing through a 60-mesh screen (0.251 mm). The subsequent characterization of the obtained pectins yielded the following parameters [18]. Molecular mass of PX was 899 kDa, galacturonic acid content was 61.1%, degree of methylation was 73.4%, and neutral sugars content was 29.8%. PCX molecules were smaller (molecular mass of 419 kDa), less methylated (56.1%) contained more galacturonic acid (74.7%), and less neutral sugars (17.9%).

4.3. Cell Culture

The studies were performed on human colon cancer cell lines: HT-29, HCT 116, Caco-2 and FHC, obtained from ATCC collection. The cells were cultured in medium DMEM-F12 (PAA) supplemented with 1% antibiotics (10,000 µg/mL streptomycin and 10,000 units/mL penicillin; Sigma-Aldrich (Poznan, Poland) and fetal bovine serum (FBS; Eurx). The concentration of FBS in medium was 10% for HCT 116. HT-29, and FHC cells, and 20% for Caco-2 cells. Moreover, DMEM-F12 for Caco-2 cells contained non-essential amino acid solution (1×) (Sigma-Aldrich, Poznan, Poland). The medium for FHC cell line was additionally supplemented with 0.005 mg/mL transferrin, 100 ng/mL hydrocortisone, 20 ng/mL human recombinant EGF. The cells were cultivated at 37 °C in a humidified atmosphere with 5% CO_2, under standard conditions. The medium was changed twice a week. Confluent cultures were passaged using 0.25% trypsin. All experimental procedures were carried out in the log-phase of cell growth.

4.4. Cell Viability Assay

Cells were seeded on 96-well plates (150,000 cells/mL) 24 h before the experiment. Then, the medium was removed, and a fresh medium containing studied pectins (PX or PCX) in the concentration of 0.05; 0.1; 0.2; or 0.5 mg/mL was added. In the experiments in which the combination of pectin and SN-38 was used, the concentration of PX or PCX was 0.2 mg/mL, whereas the concentration of the drug was 5 nM. The appropriate control containing DMSO in fresh medium was also prepared. The plates were incubated for 48 h at 37 °C in a humidified atmosphere with 5% CO_2. Cell viability was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method, as described previously [20]. In general, the assay is based on the reduction of a yellow tetrazolium salt (MTT) to purple formazan crystals by metabolically active cells. The absorbance of the final product was read at 570 nm using a microplate reader. Survival rate was expressed as the percentage of cell survival and calculated from the ratio (A_{570} of treated cells/A_{570} of control cells) × 100%. The experiments were repeated three times.

The sulforhodamine B (SRB) assay [70] was modified and used for estimation of cytotoxic properties of the studied compounds. Cells were seeded (60,000/well) onto 96-well plates with the compounds: pectins or SN-38, used alone or in combination in the appropriate concentrations. DMSO concentration in the samples did not exceed 0.5%. The plates were incubated for 48 h at 37 °C. Subsequently, 50% cold trichloroacetic acid was added. After incubation for 60 min at 4 °C, cells were washed with tap water and stained with 0.4% sulforhodamine B (Sigma-Aldrich, Poznan, Poland) for 30 min at room temperature. The dye was removed by washing under 1% acetic acid. Then, the plates were

dried, and 10 mmol/L Tris (pH = 10.5) was added to each well. Protein concentration was estimated from the measurements of absorbance at 491 nm. Survival rate was expressed as the percentage of cell survival and calculated from the ratio (A_{491} of treated cells/A_{491} of control cells) × 100%. The experiments were repeated three times.

4.5. Isobolographic Analysis

The combination index (CI) was calculated using CompuSyn software (ComboSyn Inc., Paramus, NJ, USA) according to the classic median-effect Equation (1), as was previously described by [23].

$$CI = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2} \quad (1)$$

In the Equation (1), $(Dx)_1$ is the dose of drug 1 alone that inhibits a system by x%, $(Dx)_2$ is the dose of drug 2 alone that inhibits a system by x%, and $(D)_1 + (D)_2$ are the doses of drug 1 and 2 in combination that also inhibit a system by x%. CI values below 1 represent synergism, CI value equal to 1 indicates additive effect (i.e., no interaction), and CI values above 1 point to antagonism.

Combenefit is a software tool that enables the visualization, analysis, and quantification of substance combination effects [24]. Data taken from MTT assays of pectin:drug combinations were processed using classical Highest Single Agent (HSA) synergy model [25].

4.6. Flow Cytometry

In order to study cell cycle, an apoptosis and intracellular level of reactive oxygen species (ROS) flow cytometry method was implemented. The cells (150,000 cells/mL) were seeded onto 12-well plates 24 h before the experiment. Then, the medium was removed, and a fresh medium containing pectin (PX or PCX) at the concentration of 0.2 mg/mL with or without SN-38 at the concentration of 5 nM was added. The appropriate control containing DMSO in fresh medium was also prepared. The cells were incubated for the next 48 h at 37 °C. Further procedures was carried out as described previously [20]. Briefly, the cells were harvested and centrifuged (235× *g*, 3 min, room temperature). The apoptosis was investigated using an Annexin-V apoptosis assay (BioLegend, San Diego, CA, USA). To study cell cycle, the cells were fixed with hypotonic buffer containing propidium iodide (PI) at 100 µg/mL in PBS; 5 mg/L of citric acid; 1:9 Triton-X solution; RNase 100 µg/mL in PBS (Sigma-Aldrich, Poznan, Poland). The level of ROS was measured using a cell-permeable non-fluorescent probe, 2',7'-dichlorofluorescin diacetate (DCFH-DA, Sigma-Aldrich, Poznan, Poland), which undergoes hydrolysis by cellular esterases into its polar form DCFH. Due to the intracellular ROS and other peroxides oxidation process takes place and DCFH turns into highly fluorescent compound 2',7'-dichlorofluorescein (DCF). Cytometric analyses were performed using an Aria III flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with FITC configuration (488 nm excitation; emission: LP mirror 503, BP filter 530/30) or with PE configuration (547 nm excitation; emission: 585 nm), and at least 10,000 cells were counted. All experiments were performed in triplicate.

4.7. Caspase-3 Activity

The activity of caspase-3 was analyzed using a commercially available kit (GenScript Biotech, Leiden, the Netherlands), according to the manufacturer's protocol. Cells were seeded (800,000 cells/well) onto a 6-well plate in 2 mL of medium and incubated for 24 h at 37 °C. Then, the medium was removed, and a fresh medium containing pectin (PX or PCX) at concentration of 0.2 mg/mL with or without SN-38 at 5 nM was added. Control wells contained the cancer cells in fresh medium with DMSO. The cells were incubated for the next 48 h (at 37 °C), then scraped and centrifuged (2000× *g*, 5 min, 25 °C). A further procedure was carried out as described previously [20]. In general, the assay based on the spectrophotometric detection of chromophore p-nitroaniline (pNA) (at 405 nm) released by caspase-3 from the labeled substrate DEVD-pNA. The relative increase of caspase-3 activity was determined by calculating the ratio of the absorbance of pNA in the studied

sample (treated with the compound) to the control (with no compound). Experiments were performed in triplicate.

4.8. MDA Content

The level of malondialdehyde (MDA), a product of lipid peroxidation which is a biological marker of oxidative stress, was analyzed using Lipid Peroxidation (MDA) Assay Kit (Abcam/Symbios, Straszyn, Poland), according to the manufacturer's instructions with minor modifications. Cells were seeded (800,000 cells/well) onto a 6-well plate in 2 mL of medium and incubated for 24 h at 37 °C. Then, the medium was removed, and a fresh medium containing pectin (PX or PCX) at concentration of 0.2 mg/mL with or without SN-38 at 5 nM was added. The cells were incubated for next 48 h (37 °C). After incubation, the cells were harvested and washed with PBS. The pellet of cells was suspended in Lysis Solution containing MDA Lysis Buffer and butylated hydroxytoluene (BHT). The cells were homogenized on ice and then centrifuged (13,000× *g*, 10 min) to remove insoluble material. In order to generate MDA-TBA adducts, supernatant was mixed with tiobarbituric acid (TBA) and incubated for 60 min at 95 °C. Then, the samples were cooled to room temperature and added into a 96-well microplate for analysis. A microplate reader was used to measure the absorbance at 532 nm. In order to determine MDA concentration in the samples, a standard curve was used.

4.9. Enzyme-Linked Immunosorbent Assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) method was used to detect and quantify cyclooxygenase-2 (COX-2), interleukin-6 (IL-6), toll-like receptor 4 (TLR4), and Gal-3. First, the cells (HT-29, HCT 116 or Caco-2) were seeded in 96-well plates (150,000 cells/mL). In order to stimulate immune response lipopolysaccharide (LPS; trinitrophenol-lipopolysaccharide from *E. coli* O111:B4, Sigma-Aldrich, Poznan, Poland) in the concentration of 5 µM was added to each well 24 h before the experiment. This step was omitted in case of Gal-3 assay. Next, the cells were treated by the studied pectins at the concentration of 0.2 mg/mL for 48 h. IL-6, COX-2, TLR4, and Gal-3 were detected in cancer cells culture lysates using IL-6 Human ELISA Kit (ThermoFisher Scientific, Waltham, MA, USA), Human COX-2 ELISA Kit (Sigma-Aldrich, Poznan, Poland), Human TLR-4 ELISA Kit (Sigma-Aldrich, Poznan, Poland), and Human Galectin-3 ELISA Kit (Sigma-Aldrich, Poznan, Poland), respectively. The studies were performed according to the manufacturer's instructions. All experiments on the detection and quantification of selected proteins were repeated three times.

4.10. Adherence Assay

The adherence of bacteria cells to colon cancer cells in the presence of pectins, PX or PCX, was investigated as previously described [20]. An adherent-invasive strain of *E. coli* (LF82) isolated from a patient with Crohn's disease was kindly provided by Dr. Arlette Darfeuille-Michaud (Université d'Auvergne, France). HCT 116 cells were infected with *E. coli* and incubated with PX or PCX in concentrations of 0.125, 0.25, and 0.5 mg/mL. Cells untreated with pectins and infected with bacteria constituted a positive control. After 2 h of incubation (37 °C, 5% CO_2), the cells were washed with PBS and lysed with 0.1% Triton X-100. Serial dilutions of bacterial lysates were plated onto nutrient agar and incubated overnight at 37 °C to count bacterial colonies (CFU). The experiment was repeated three times in duplicate. The results are presented as the percentage of *E. coli* adhering to untreated cells, with negative control established to be 100%.

Adhesion images were taken under a light microscope after Wright–Giemsa staining. HCT 116 cells were cultured on slides in the wells of a 24-well plate. After 2 h of adherence experimentation, HCT 116 cells were washed and fixed with formaldehyde in the concentration of 4% (10 min, room temperature). Then, formaldehyde was washed off and the cells were stained with Wright–Giemsa for 30 min. The dye was rinsed off, and the slides were dried and inspected under a microscope.

4.11. Statistical Analysis

The values of measured parameters were presented as the means ± standard deviation (SD) from three independent experiments. The statistical significance was determined by Student *t*-test (0.05 as threshold value) with the use of Statistica 10 software.

5. Conclusions

Summing up, the antiproliferative, proapoptotic, and anti-inflammatory properties of enzymatically extracted apple pectins were demonstrated in colon cancer cells. Additionally, the synergy between pectin and SN-38 was discovered. The interaction of the apple pectins with Gal-3 and TLR4 was the molecular mechanism for their anticancer and anti-inflammatory effect. It was concluded that the relatively low molecular mass of PCX together with the relatively high proportion of RG I regions gave this pectin superior anticancer activity.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ph15060732/s1. Figure S1: SRB cytotoxicity assay of pectins in HCT 116 (**A**), Caco-2 (**C**), HT-29 cells (**E**), and 0.2 mg/mL pectins in combination with SN-38 ((**B**,**D**,**F**) for HCT 116, Caco-2, and HT-29 cells, respectively), incubation time 48 h. Figure S2: MTT cytotoxicity assay of pectins at 0.2 mg/mL in FHC cells. Incubation time was 48 h. Figure S3: Cell cycle-dependent DNA content in HCT 116 (**A**) and Caco-2 (**B**), cells treated with 0.2 mg/mL of pectins and/or SN-38 for 48 h. Figure S4: The proportion of normal, apoptotic, and necrotic cell populations as recorded by annexin V apoptosis assay in HCT 116 (**A**) and Caco-2 (**B**) cells treated with pectins (0.2 mg/mL) and/or SN-38 for 48 h. Figure S5: Lipid peroxidation (**A**,**C**) and ROS level (**B**,**D**) in HCT 116 (**A**,**B**) and Caco-2 (**C**,**D**) cells treated with 0.2 mg/mL pectins and/or 5 nM SN-38 for 48 h. Figure S6: Amount of COX-2 (**A**,**C**) and IL-6 (**B**,**D**) in HCT 116 (**A**,**B**) and Caco-2 (**C**,**D**) cells treated with 0.2 mg/mL pectins and/or 0.5 µM LPS (cells were pretreated with LPS for 24 h and then incubated with pectins for 48 h). Figure S7: Amount of Gal-3 in HCT 116 (**A**) and Caco-2 (**B**) cells treated with 0.2 mg/mL pectins for 48 h.

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Newly Obtained Apple Pectin as an Adjunct to Irinotecan Therapy of Colorectal Cancer Reducing *E. coli* Adherence and β-Glucuronidase Activity

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Simple Summary: Colorectal cancer (CRC) is the second cause of cancer death worldwide. Irinotecan is a drug widely used in CRC treatment. Unfortunately, colonic bacteria decompose the metabolite of irinotecan back to the active form of the drug resulting in severe side-effects of the treatment, such as diarrhea. The present work demonstrated that new apple pectin (PC) enhanced cytostatic action of irinotecan and, at the same time, reduced the activity of bacterial enzymes responsible for the appearance of side-effects in patients. Thus, novel pectin PC constitutes a promising candidate for an adjunct to irinotecan therapy that might alleviate its side-effects, increasing its therapeutic efficacy.

Abstract: Colorectal cancer (CRC) is the second cause of cancer death worldwide. The composition and enzymatic activity of colonic microbiota can significantly affect the effectiveness of CRC chemotherapy. Irinotecan is a drug widely used to treat colon cancer. However, the transformation of a drug-glucuronide (SN-38G) back to its active form (SN-38) by bacterial β-glucuronidase (GUS) constitutes the primary reason for the observed intestinal toxicity of irinotecan. It was demonstrated that novel enzymatically extracted apple pectin (PC) might be a promising candidate for an adjunct to irinotecan therapy. PC itself reduced the viability of HCT 116 and Caco-2 colorectal cancer cells, induced apoptosis, and increased intracellular reactive oxygen species production. Moreover, PC enhanced the cytotoxic and proapoptotic effect of irinotecan (at concentrations below its IC_{50}), i.e., synergistic effect was recorded. Additionally, PC exhibited potent anti-inflammatory properties and prevented adhesion of prototype adherent-invasive *E. coli* (AIEC) LF82 strain and laboratory K-12_{C600} strain to colon cancer cells. PC was also identified to be an effective inhibitor of bacterial GUS activity. Altogether, novel apple pectin was identified as a promising candidate for a supplement to irinotecan therapy that might alleviate its side-effects via inhibition of bacterial GUS and thus increasing its therapeutic efficacy.

Keywords: colon cancer; pectin; irinotecan; apoptosis; inflammation; *E. coli*; bacterial β-glucuronidase; side-effects alleviation

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1. Introduction

Colorectal cancer (CRC) takes the third position among the most frequently occurring cancers worldwide [1]. As reported by International Agency for Cancer Research of the

World Health Organization (WHO) in 2020 more than 1.9 million new CRC cases were diagnosed and over 900,000 deaths were recorded [1]. In the great majority, CRC appears as a sporadic disease, typically diagnosed in patients over 50 and usually linked to environmental factors, such as western-type diet rich in red meat, fat, and sugar [2]. About 10% of CRC cases develop due to inherited syndromes, such as familial adenomatous polyposis and Lynch syndrome [2]. Moreover, the higher risk of CRC has been observed in patients with inflammatory bowel disease, such as ulcerative colitis and Crohn's disease [3]. Local inflammation may modulate the pathogenesis of CRC, mostly via the complex network of inflammation mediators, such as cytokines, produced both by cancer cells themselves and by various types of cells constituting tumor microenvironment [4]. Additionally, the progression of CRC can be influenced by colonic microbiota [5,6] that is altered in cancer patients as compared to the healthy subjects [7,8]. The microbiota was also shown to modulate the response of tumor cells to chemotherapy [9,10].

The treatment options of CRC depend on the stage of disease. For locally advanced colon or rectal tumors concomitant combination of surgery and chemotherapy is recommended, while in metastatic CRC, chemotherapy appears to be preferable [11]. Irinotecan is a derivative of an alkaloid, camptothecin. Irinotecan hydrolysis by patients' carboxylesterases yields its active form, SN-38, an inhibitor of topoisomerase I [12]. This drug is a common component of cytotoxic combinations recommended as a treatment for CRC patients, such as FOLFIRI (fluorouracil, leucovorin, and irinotecan) or FOLFOXIRI (fluorouracil, leucovorin, oxaliplatin, and irinotecan) [13,14]. Common side-effects of irinotecan include diarrhea, nausea, and vomiting [15,16]. SN-38 is metabolized via glucuronidation in the liver to irinotecan glucuronide (SN-38G) that enters the small intestine via biliary excretion [17]. Unfortunately, bacteria in the colon produce β-glucuronidase (GUS) that decomposes SN-38G back to its active form (SN-38), which is toxic for intestinal epithelium [18], causing the adverse effects of chemotherapy.

Pectin constitutes a significant component of dietary fiber. This structural acidic heteropolysaccharide is a component of plant cell walls [19]. The basic structural skeleton of pectin is a linear polymer of galacturonic acid (GalUA). In native pectin molecule, some distinct structural regions are present. In rhamnogalacturonan I (RG I), the backbone consists of repeating dimers of GalUA and rhamnose, and is substituted with side chains composed of galactose and arabinose. Sidechains of rhamnogalacturonan II (RG II) contain atypical, rare monosaccharides, including L-fucose, apiose, and acids: aceric, glucuronic, ketodeoxyoctonic (KDO), and 3-deoxy-lyxo-2-heptulopyranosylaric (DHA). Structural differences may depend on the plant source of the polysaccharide. Commercially available pectins are usually prepared from citrus peels or apple pomace by hot acid extraction [20]. Pectins extracted using this method are, in comparison with the native molecule, depleted in RG I and RG II that are considered to be crucial for prebiotic, immunomodulatory, and anticancer potency of pectins [21,22]. Recently, Wikiera et al. [23] developed an effective method for enzymatic isolation of apple pectins using limited doses of endo-cellulase and endo-xylanase. This procedure protects native molecules, especially its neutral sugars that are not damaged during isolation [23]. Pectin studied in the present work (PC) was enzymatically extracted from apple pomace using endo-cellulase from filamentous fungus *Trichoderma viride*. Its detailed structure was previously described [23], and its biological properties were partially characterized [24].

In the present work, we studied the influence of enzymatically obtained pectin, PC, on colon cancer cells and compared its properties to commercially available modified citrus pectin, SolC. It was assumed that PC is going to exhibit intrinsic anticancer activity. Next, due to presumed anticancer properties of PC, the enhancement of the anticancer activity of irinotecan (i.e., synergistic effect) was expected when the drug would be applied together with PC. Additionally, considering the crucial role of colonic microbiota in causing irinotecan-induced diarrhea, we supposed that enzymatically obtained pectin might alleviate side-effects of irinotecan both via inhibition of bacterial GUS in the colon or

providing an additional substrate for the enzyme. The results presented below confirmed all of the above hypotheses.

2. Materials and Methods

2.1. Chemicals

SN-38 (7-ethyl-10-hydroxy-camptothecin), an active metabolite of irinotecan, was from Sigma-Aldrich (Poznan, Poland). PectaSol-C (SolC) low molecular weight modified citrus pectin with a documented antitumor activity [25] was purchased from ecoNugenics (Santa Rosa, C, USA). Stock solutions of pectins were prepared at a concentration of 10 mg/mL in double-distilled water. About 50 mM stock solution of SN-38 was prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C. Stock solutions were diluted in a culture medium just before the experiments.

2.2. Pectin Isolation

Macromolecular apple pectin was extracted from dried apple pomace using cellulase (endo-β-1,4-glucanase, EC 3.2.1.4) produced by filamentous fungus Trichoderma viride (Sigma-Aldrich, Poznan, Poland, Cat. No. C9422). Isolation was performed with 50 U enzyme per 1 g of pomace, at pH 5.0, in 40 °C, for 10 h, according to a method described by Wikiera et al. 2016 [23]. The detailed characteristics of the obtained pectin are presented in the previous works [23,24].

2.3. Cell Culture

The experiments were performed on the human colon cancer cell lines HCT-116 and Caco-2 (obtained from ATCC collection). The cells were cultivated in medium DMEM-F12 (PAA) supplemented with fetal bovine serum (10% FBS for HCT 116 and 20% for Caco-2; Eurx) and 1% antibiotics (10,000 µg/mL streptomycin and 10,000 units/mL penicillin; Sigma-Aldrich (Poznan, Poland), at 37 °C in a humidified atmosphere with 5% CO_2, under standard conditions. In addition, the medium for Caco-2 cells contained non-essential amino acid solution (1×) (Sigma-Aldrich, Poznan, Poland). The medium was changed twice a week. Confluent cultures were passaged using 0.25% trypsin. All experimental procedures were carried out in the log-phase of cell growth.

2.4. Cell Viability Assay

SRB assay. The sulforhodamine B (SRB, Sigma-Aldrich, Poznan, Poland) assay was performed, as previously described [26], with modifications. Briefly, 15,000 cells were seeded in 96-well plates and allowed for a 24-h attachment period (at 37 °C). Then, the cells were treated with the studied pectins (0.05–0.5 mg/mL with or without 5 nM SN-38) for 48 h. Control wells only contained medium. The further procedure was carried out as previously described [27]. Cytotoxicity of DMSO to the cells was found to be negligible. All experiments were repeated three times.

MTT assay. Cells were seeded on 96-well plates in the concentration of 150,000 cells/mL. About 24 h later, the medium was removed, and a fresh medium containing tested pectins (0.05–0.5 mg/mL with or without 5 nM SN-38) was added. The control wells contained DMSO diluted in a medium. The plates were incubated for 48 h at 37 °C in a humidified atmosphere with 5% CO_2. Then, the medium was removed, and cells were washed with physiological saline (1 × PBS; Sigma-Aldrich, Poznan, Poland). In the next step of the experiment MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in the concentration of 0.5 mg/mL (diluted in RPMI 1640 without phenol red; Sigma-Aldrich, Poznan, Poland) was added to each well. The plates were incubated for at least 2 h yielding purple MTT formazan crystals in the wells. The incubation with MTT was followed by the dissolution of crystals in the mixture of acidic isopropanol (isopropanol:HCl, v/v 1:0.04). The absorbance of the obtained product was read at 570 nm using a microplate reader. Survival rate was expressed as the percentage of cell survival and calculated from the

ratio (A_{570} of treated cells/A_{570} of control cells) × 100%. The experiments were repeated three times.

2.5. Isobolographic Analysis

By the CompuSyn software (ComboSyn Inc., Paramus, NJ, USA) according to the classic median-effect equation, as described by Chou and Martin, the combination index (CI) values were calculated [28].

$$CI = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2} \quad (1)$$

where: $(Dx)_1$ is the dose of drug 1 alone that inhibits a system by x%, $(Dx)_2$ is the dose of drug 2 alone that inhibits a system by x%, and $(D)_1 + (D)_2$ are the doses of drug 1 and 2 in combination that also inhibit a system by x%. CI values below 1 represent synergism, CI values equal to 1 indicate additive effect (i.e., no interaction), and CI values above 1 point to antagonism.

2.6. Flow Cytometry Analysis

In order to study the cell cycle, apoptosis, and intracellular level of reactive oxygen species (ROS), flow cytometry was applied. In these studies, the cells in concentration 150,000 cells/mL were seeded onto 12-well plates 24 h before the experiment. Then, the medium was removed, and a fresh medium containing the compounds (0.2 mg/mL pectins with or without 5 nM SN-38) was added. The plates were incubated for 48 h at 37 °C in a humidified atmosphere with 5% CO_2.

The apoptosis was investigated using an Annexin-V apoptosis assay (BioLegend, San Diego, CA, USA) and propidium iodide (PI) solution (100 µg/mL; Sigma-Aldrich, Poznan, Poland). The cells were collected and centrifuged for 3 min at 235× *g*. After supernatant removal, the cells were washed with PBS and centrifuged once again. In the next step, the pellet of cells was suspended in cold Annexin-V binding buffer and stained with FITC-labeled Annexin-V and PI in the dark for 20 min at 37 °C. After this time, the final volume of Annexin-V binding buffer was added to the samples.

In order to analyze the cell cycle, collected cells were centrifuged for 3 min at 235× *g* (room temperature). After supernatant removal the cells were fixed with hypotonic buffer containing PI 100 µg/mL in PBS; 5 mg/l of citric acid; 1:9 Triton-X solution; RNase 100 µg/mL in PBS. The samples were incubated for 20 min (room temperature) in the dark and then stored on ice till they were measured.

The level of ROS in the cells treated with studied compounds was quantified using 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich, Poznan, Poland), a cell-permeable fluorogenic probe. The experiments were performed after 72 h of incubation with given compounds. The cells were harvested and centrifuged, washed with PBS, and centrifuged again. The pellet was resuspended in a culture medium and a cell-permeable non-fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich, Poznan, Poland) in a final concentration of 30 µM was added. The samples were incubated in darkness for 30 min at 37 °C and then stored on ice till they were measured. DCFH-DA is hydrolyzed by cellular esterases into its polar form DCFH. Due to the intracellular ROS and other peroxides DCFH undergoes oxidation and turns into highly a fluorescent form 2′,7′-dichlorofluorescein (DCF).

Cytometric analyses were performed immediately using an Aria III flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with the FITC configuration (488 nm excitation; emission: LP mirror 503, BP filter 530/30) or PE configuration (547 nm excitation; emission: 585 nm) and at least 10,000 cells were counted.

2.7. Caspase-3 Activation Assay

The activity of caspase-3 was analyzed using a commercially available kit (GenScript Biotech, Leiden, the Netherlands). Cells were seeded (800,000 cells/well) onto a 6-well plate

in 2 mL of medium and incubated for 24 h at 37 °C. Then, the cells were treated with PC or SolC alone (at 0.2 mg/mL), and in combination with SN-38 (5 nM). After incubation with studied compounds for 48 h cells were scraped and centrifuged (2000× *g*, 5 min, 25 °C). After cell lysis, spectrophotometric detection (A_{405}) of the chromophore *p*-nitroanilide (pNA) was used to measure caspase-3 activity. The relative increase of caspase-3 activity was determined by calculating the ratio of the absorbance of pNA in the studied sample (treated with the compound) to the control (with no compound). Experiments were performed in triplicate.

2.8. Enzyme-Linked Immunosorbent Assay (ELISA)

HCT 116 cells were seeded in 96-well plates in the concentration of 150,000 cells/mL. The colorectal cancer cells were treated with 5 µM of lipopolysaccharide (LPS; trinitrophenol-lipopolysaccharide from *E. coli* O111:B4, Sigma-Aldrich, Poznan, Poland) added at the time of seeding. A fresh medium containing the compounds (0.2 mg/mL) was added after 24 h. After incubation with the studied compounds for 48 h interleukin 6 (IL-6) and cyclooxygenase 2 (COX-2) in the cancer cell culture lysates were quantified using ELISA (Human COX-2 ELISA Kit; Sigma-Aldrich, Poznan, Poland; IL-6 Human ELISA Kit-ThermoFisher Scientific, Waltham, MA, USA). ELISA was performed according to the manufacturer's instructions. Each plate test was repeated three times.

2.9. E. coli Strains

Tests were performed on two reference strains. A prototype adherent-invasive *E. coli* (AIEC) LF82 (O83: H1) strain, kindly provided by Dr. Arlette Darfeuille-Michaud, Université d'Auvergne, France, and laboratory *E. coli* K-12_{C600} strain. *E. coli* strains were routinely cultured overnight in Luria broth (LB) with shaking at 37 °C.

2.10. Adherence Assay

Overnight *E. coli* cultures in LB medium were harvested and suspended in PBS to the optical density 6×10^8 CFU/mL established spectrophotometrically at 600 nm. HCT 116 or Caco-2 cells were washed three times with pre-warmed PBS, and 200 µL of pectin solutions in PBS at concentrations 0.125, 0.25, and 0.5 mg/mL were added to the cells following 200 µL of *E. coli* suspensions. Cells untreated with pectins and infected with *E. coli* served as a positive control. Then the cells were incubated for two hours at 37 °C in a humid atmosphere with 5% CO_2. At 2 h post-infection, cells were washed three times with PBS and lysed with 0.1% Triton X-100. Serial dilutions of bacterial lysates were plated onto nutrient agar and incubated overnight at 37 °C to count bacterial colonies (CFU). The assay was repeated three times in duplicate. The results are presented as the percentage of *E. coli* adhering to cells concerning negative control established to be 100%.

Wright-Giemsa staining. Intestinal epithelial cells were cultured on slides in the wells of a 24-well plate. After 2 h of *E. coli* adherence with the epithelial cells, the cells were washed three times and fixed with 4% formaldehyde for 10 min at room temperature. After washing off the formaldehyde, cells were stained with Wright-Giemsa for 30 min. The dye was rinsed off, and the slides were dried. Adhesion images were taken under a light microscope at 100× magnification.

2.11. E. coli Proliferation

The impact of apple pectins on *E. coli* proliferation was assessed by mixing 75 µL of *E. coli* suspensions in PBS at the optical density 6×10^8 CFU/mL obtained from overnight cultures in LB with the equal volumes of pectins solutions in PBS at concentrations 0.125, 0.25, and 0.5 mg/mL. According to the adherence assay, the plate was incubated for two hours at 37 °C in an atmosphere with 5% CO_2. The proliferation of *E. coli* was measured spectrophotometrically at 600 nm and compared to the growth of *E. coli* at time zero established to be 100%.

2.12. β-Glucuronidase (GUS) Activity Assay

Overnight *E. coli* LF82 and K-12$_{C600}$ cultures in 3 mL of tryptic soy broth (TSB) were supplemented with 0.3 µM 4-nitrophenyl β-D-glucuronide (PNPG; Sigma-Aldrich, Poznan, Poland) one hour before the end of the incubation to induce GUS synthesis. Then, the cultures were centrifuged (16,000× *g*; 10 min; 4 °C). Bacterial cell pellets were washed twice in cold 100 mM sodium phosphate buffer (pH 6.8), followed by suspension in 1 mL of cold phosphate buffer, and mixing with 0.1 mm silica glass beads. The samples were then beaten in BeadBugTM microtube homogenizer five times for 30 sec each, with 30-sec incubations on ice in between. The tubes were centrifuged for 10 min, 16,000× *g* at 4 °C, and bacterial extracts were collected and stored on ice. Bacterial extracts were prewarmed at 37 °C, and samples of 75 µL were mixed in microtitrate plate with prewarmed equal volumes of the 10 mM PNPG in phosphate buffer. The yellow 4-nitrophenol product was recorded in a spectrophotometer after 60 min of incubation at 37 °C at 405 nm. The 4-nitrophenol concentration was determined from a standard curve according to Aich et al. [29]. Protein concentrations in bacterial extracts were determined with a bicinchoninic acid kit (Pierce, Waltham, MA, USA). GUS activity was calculated as follows: 4-nitrophenol concentration/100 mg/mL protein × 60 min. The effect of pectins SolC and PC on *E. coli* GUS activity was assayed as described above. SolC and PC solutions in PBS at final concentrations of 0.25, 0.5, and 0.7 mg/mL were mixed with an appropriate amount of *E. coli* lysates and 10 mM PNPG in the wells of a 96-well plate and incubated at 37 °C for 18 h.

2.13. Statistical Analysis

All of the experiments were repeated three times. Data represent the mean ± standard deviation (SD) of at least three replications. The Student's *t*-test was applied and *p*-values less than 0.05 were considered to achieve statistical significance.

3. Results

3.1. Anticancer Activity of Pectins

3.1.1. Cytotoxicity of Pectins

The influence of two pectins on colon cancer cell viability was assessed. Commercially available pectin PectaSol-C (SolC) was compared to PC that had been enzymatically extracted from apple pomace using endo-cellulase. SolC is derived from water-insoluble citrus pectin modified by pH/temperature treatment to increase its water solubility and anticancer properties [25].

Both pectins were revealed to possess anticancer activity against human colorectal carcinoma HCT 116 cells (Figure 1A,B), as shown by the results of SRB as well as MTT assays. In both types of assays, PC exhibited significantly greater cytotoxic activity as compared to SolC ($p < 0.05$ for all concentrations tested). IC_{50} values obtained for PC were 0.098 ± 0.011 mg/mL and 0.103 ± 0.014 mg/mL for SRB and MTT assay, respectively. In the case of SolC, the SRB assay yielded an IC_{50} value of 0.590 ± 0.019 mg/mL, whereas the MTT assay gave IC_{50} equal to 0.640 ± 0.018 mg/mL. The appearance of HCT 116 cells cultures treated with 0.2 mg/mL of pectins is presented in Figure 1E.

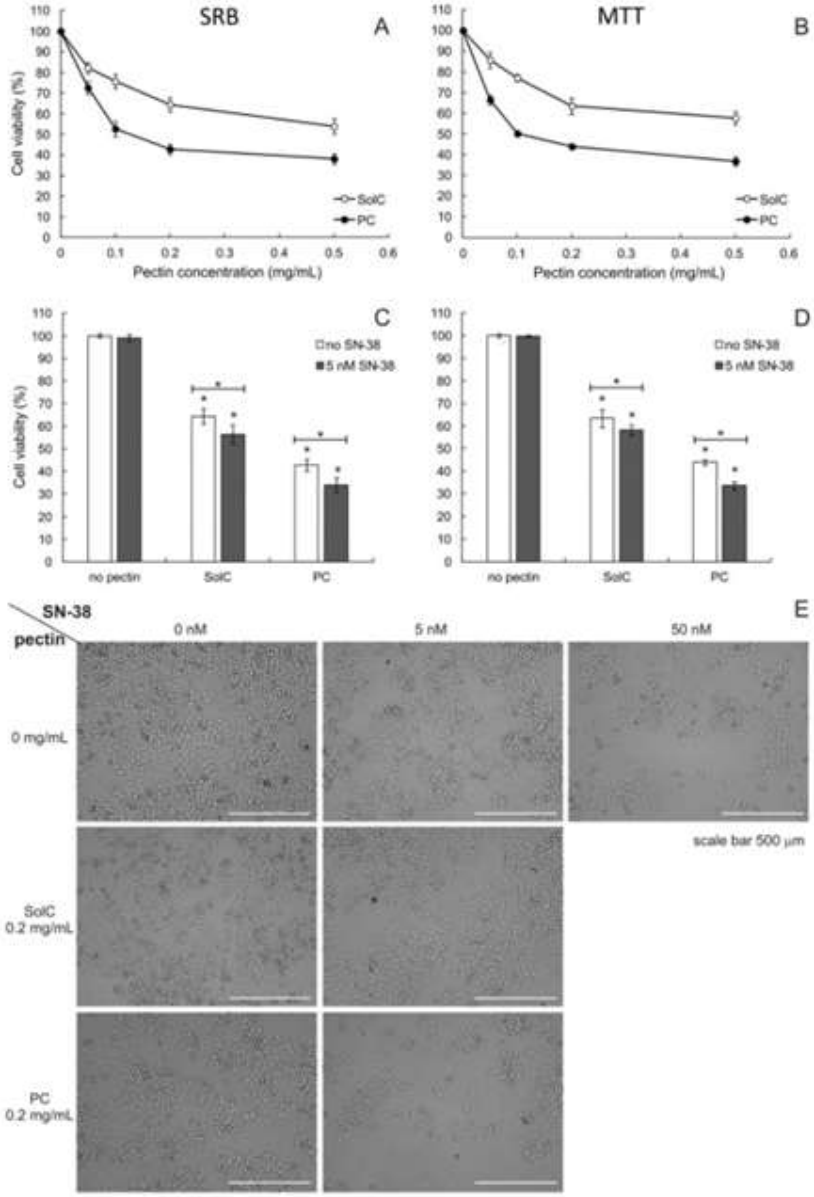

Figure 1. SRB (**A**) and MTT (**B**) cytotoxicity assay of pectins and 0.2 mg/mL pectins in combination with SN-38 (**C**,**D**) for SRB and MTT, respectively) in HCT 116 cells (incubation time 48 h). Light microscopy images (magnification 10×) of HCT

116 cells (**E**) treated with pectins (0.2 mg/mL) and/or 5 nM SN-38. The means of three experiments ± SD are presented (* $p < 0.05$). Statistical significance was checked between the studied probes and controls (no pectin) and between probes containing only pectin and pectin combined with SN-38. In panels A and B all measurements were significantly different from the control (not marked on the graph for the sake of clarity).

Cytotoxic activity of the studied pectins was also studied in human colorectal adenocarcinoma cells Caco-2 (Figure S1). The results of SRB and MTT cell viability assays revealed that PC was more toxic to Caco-2 cells than SolC, similarly as in the case of HCT 116 cells. IC_{50} values calculated for SolC were 0.440 ± 0.016 mg/mL (SRB) and 0.490 ± 0.015 mg/mL (MTT) that were slightly lower than that obtained in HCT 116 cells. On the other hand, IC_{50} values obtained for PC in Caco-2 cells, 0.210 ± 0.009 mg/mL for SRB and 0.270 ± 0.013 mg/mL for MTT, were almost twice higher than in HCT 116 cells. Therefore, it was concluded that the difference in cytotoxicity between SolC and PC was more pronounced in the case of HCT 116 cell line than in Caco-2.

3.1.2. Cytotoxicity of Pectins Combined with SN-38

Additionally, the anticancer activity of pectins was studied in the presence of SN-38, an active form of irinotecan—the drug commonly used to treat colon cancer. Its IC_{50} value in HCT 116 cell line was found to be 10.2 ± 0.8 nM (3.5×10^{-6} mg/mL). Figure 1E presents the appearance of cell cultures treated with 5 and 50 nM of SN-38 as well as with the mixtures of 0.2 mg/mL of pectin and 5 nM of SN-38. It can be noticed that in the case of PC:SN-38 combination the number of HCT 116 cells is reduced to a greater degree than in case of the treatment with SN-38 (at 5 nM) alone. Figure 1C,D present the results of the SRB and MTT assay, respectively. Cell viability was significantly reduced in the presence of both 0.2 mg/mL of SolC and PC. In addition, the combination of pectins with a low concentration of SN-38 reduced cell viability even more. The isobolographic analysis was employed to evaluate the possible synergy between the anticancer drug and pectins. Dose and effect data obtained from SRB assay for pure compounds and for two-component combinations (pectin:SN-38) were subjected to CompuSyn analysis (Table 1). The cytotoxic effects of PC and SN-38 were synergistic as demonstrated by the combination index (CI) values well below 1. On the other hand, no synergy was detected between SolC and SN-38, their effect was purely additive.

Table 1. Combination of pectins with SN-38 against HCT 116 cell growth.

Concentration (mg/mL)		Ratio	Combination Index (CI)
SN-38	**SolC**		
1.96×10^{-6}	0.2	102,000:1	1000
SN-38	**PC**		
0.96×10^{-6}	0.1	104,400:1	0.822
1.96×10^{-6}	0.2	102,000:1	0.779
3.92×10^{-6}	0.5	127,550:1	0.792

Dose and effect data were obtained from the sulforhodamine B (SRB) assay (mean values of three experiments) and analyzed by CompuSyn software (ComboSyn Inc., Paramus, NJ, USA). CI values were calculated by CompuSyn software. CI = 1 indicates additive effect, CI < 1—synergism, and CI > 1—antagonism.

3.2. *Proapoptotic Activity of Pectins*

3.2.1. Annexin V/PI Double Staining Assay

After confirming the anticancer properties of SolC and PC, it was checked whether the treatment of colon cancer cells with the studied pectins led to cellular death by apoptosis. Therefore, Annexin V/PI double staining assay was performed in HCT 116 cells. Typical dot plots obtained during the assay are presented in Figure S2. As shown in Figure 2, PC at 0.2 mg/mL slightly increased the number of apoptotic cells as compared to the control not treated by the pectin. Such an effect was not observed for SolC tested at the same concentration. The application of SN-38 also resulted in a significant increase of apoptotic

cell population, both at 5 and 50 nM concentration. The drug when applied in the highest concentration also caused necrosis of HCT 116 cells. The apoptosis-inducing activity of pectins was also tested in combination with SN-38 at the concentration of 5 nM. It was observed (Figure 2B) that in the case of PC the number of cells undergoing apoptosis was much bigger as compared to the probe treated with SN-38 only ($p < 0.05$). On the other hand, the combined SolC:SN-38 treatment resulted in lower number of apoptotic cells as compared to the probe treated with SN-38 alone ($p < 0.05$). It was concluded that PC augmented the proapoptotic activity of SN-38.

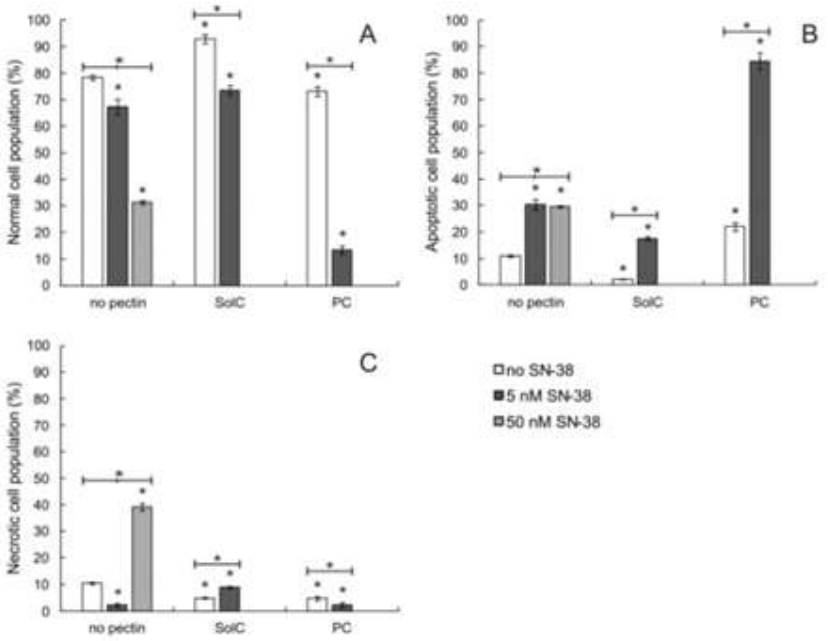

Figure 2. The proportion of normal (**A**), apoptotic (**B**), and necrotic (**C**) cell populations as recorded by Annexin-V apoptosis assay in HCT 116 cells treated with pectins (0.2 mg/mL) and/or SN-38 for 48 h. The means of three experiments ± SD are presented (* $p < 0.05$). Statistical significance was checked between the studied probes and controls (no pectin), and between probes containing only pectin and pectin combined with SN-38. Cells were recognized as viable (Annexin-V and PI negative), apoptotic (Annexin-V positive and PI negative), and necrotic (Annexin-V and PI positive) based on the measurement of cell-associated fluorescence of FITC-Annexin-V conjugate and PI.

3.2.2. Caspase-3 Activation

Additionally, the ability of pectins to activate the apoptosis-involved protease, caspase-3, was investigated (Figure 3). Caspase-3 is an enzyme associated with the initiation of the apoptotic cascade. Its activation ultimately leads to the execution of the program of cell death. Both studied pectins activated this enzyme in concentrations higher than 0.1 mg/mL. The addition of 5 nM of SN-38 to the cells also treated with 0.2 mg/mL of pectin significantly enhanced the activity of caspase-3. SN-38 used alone at 5 nM did not, however, cause any activation of caspase-3. This experiment additionally confirmed the proapoptotic properties of the studied pectins and demonstrated that PC enhanced the ability of SN-38 (applied in concentration below IC_{50}) to induce cell death.

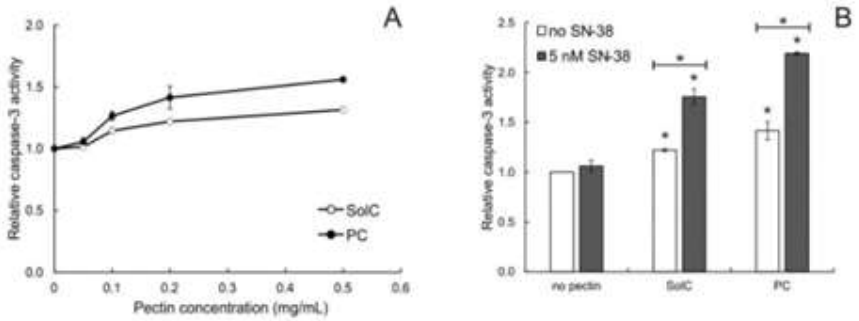

Figure 3. Relative caspase-3 activity in HCT 116 cells treated with pectins (**A**) and 0.2 mg/mL pectins in combination with SN-38. (**B**) for 48 h. The means of three experiments ± SD are presented (* $p < 0.05$). Statistical significance was checked between the studied probes and controls (no pectin), and between probes containing only pectin and pectin combined with SN-38. In panel A only the measurements for 0.05 mg/mL of SolC and PC were not significantly different from the control (not marked on the graph for the sake of clarity).

3.3. The Influence of Pectins on Cell Cycle

The influence of pectins on the cell cycle of HCT 116 cells was also tested (see Figure 4 and Figure S3 for the histograms). The cytometric evaluation indicated that both pectins at the concentration of 0.2 mg/mL elevated sub-G_1 cellular fraction (dead cells), with PC being significantly more active than SolC. The number of dead cells was also elevated when HCT 116 cells were treated with SN-38, the higher concentration of the drug inevitably causing the more pronounced effect. The most interesting observation was made for the PC:SN-38 mixture. When the cells were treated by the two compounds in combination, the very strong increase of sub-G_1 cellular fraction occurred that was accompanied by the reduction of the abundance of all other fractions. On this basis, it was concluded that PC enhanced the cytotoxic potential of SN-38.

3.4. The Effect of Pectins on Reactive Oxygen Species (ROS) Production

The assay based on the detection of intracellular ROS by the fluorescent probe DCF was applied to quantify the generation of ROS in the presence of the studied pectins. As shown in Figure 5. the treatment of HCT 116 cells with SolC at 0.2 mg/mL concentration resulted in a slight decrease in ROS level, whereas PC applied at the same concentration elevated the production of ROS by cancer cells. SN-38 (at 5 nM) gently increased ROS level. When the anticancer drug was combined with pectins, the increase in ROS generation was observed, by more than 60% in the case of SolC and almost 4.5-fold in the case of PC. The conclusion was drawn that SolC itself possessed some antioxidative properties, in contrast to PC that was prooxidative. On the other hand, both pectins strengthened ROS production induced by SN-38.

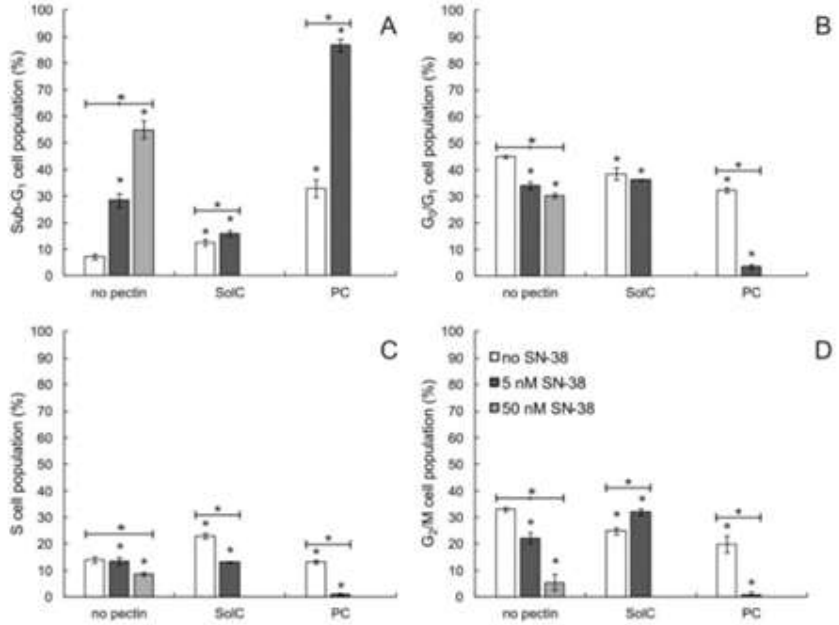

Figure 4. Cell cycle-dependent DNA content in HCT 116 cells treated with 0.2 mg/mL of pectins and /or SN-38 for 48 h. Sub-G1 population (**A**)—dead cells, G0/G1 (**B**)—mononuclear cells, S (**C**)—DNA replication, G2/M (**D**)—mitosis. Statistical significance was checked between the studied probes and controls (no pectin) as well as between probes containing only pectin and pectin combined with SN-38. * $p < 0.05$.

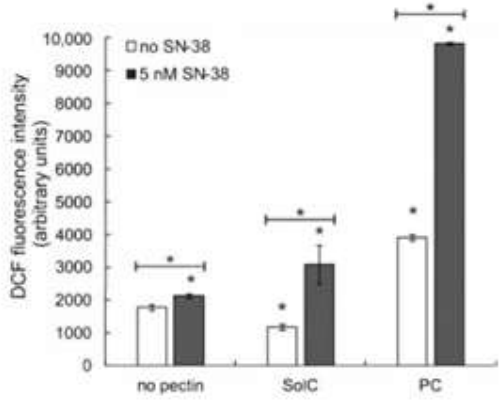

Figure 5. ROS level in HCT 116 cells treated with 0.2 mg/mL pectins and/or 5 nM SN-38 for 48 h. The means of three experiments ± SD are presented (* $p < 0.05$). Statistical significance was checked between the studied probes and controls (no pectin) as well as between probes containing only pectin and pectin combined with SN-38.

3.5. Anti-Inflammatory Activity of Pectins

To check whether the studied pectins could modify the cellular inflammatory response HCT 116 cells were pretreated by 5 μM of bacterial lipopolysaccharide (LPS, endotoxin) for 24 h before the experiment to induce inflammation. After the treatment with pectins the amounts of cyclooxygenase 2 (COX-2) and interleukin 6 (IL-6) were quantified in cell culture lysates. As presented in Figure 6 in not-stimulated cells pectins tested at 0.2 mg/mL had no influence on either COX-2 or IL-6 levels. As expected, the treatment of colon cancer cells by LPS induced inflammatory response resulting in the substantial increase of COX-2 and IL-6 produced by the cells (Figures 6A and 6B, respectively). The addition of any of the studied pectins resulted in the reduction of LPS-induced levels of both inflammation markers. The effect of PC was more pronounced than that of SolC (statistical significance ($p < 0.05$) was obtained both in the case of COX-2 and IL-6). It was demonstrated that both studied pectins were able to modulate the inflammatory response of colon cancer cells.

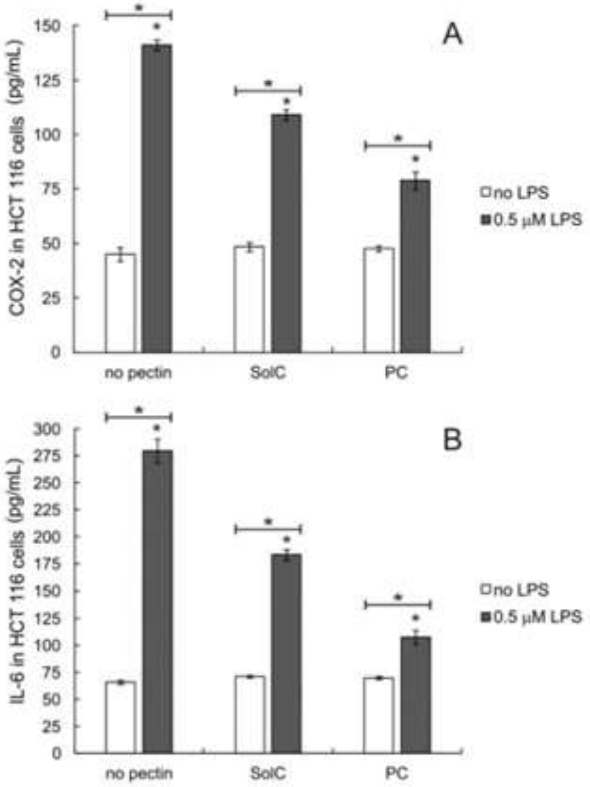

Figure 6. Amount of COX-2 (**A**) and IL-6 (**B**) in HCT 116 cells treated with 0.2 mg/mL pectins and/or 0.5 μM LPS. Cells were pretreated with LPS for 24 h and then incubated with pectins for 48 h. The means of three experiments ± SD are presented (* $p < 0.05$). Statistical significance was checked between the studied probes and controls (no pectin) as well as between probes containing only pectin and pectin combined with LPS.

3.6. Adherence of E. coli to Colon Cancer Cells

Since colonic microbiota is known to affect colorectal cancer initiation and progression, adherence of bacteria *E. coli* to cancer cells in the presence of the studied pectins was monitored. Two bacterial strains were employed, a laboratory *E. coli* K-12$_{C600}$ strain, and an adherent-invasive *E. coli* (AIEC) LF82 strain. The latter was isolated from a patient with Crohn's disease. SolC did not influence the ability of either LF82 (Figure 7A) or K-12$_{C600}$ (Figure 7B) strains to adhere to HCT 116 cells. On the other hand, PC in concentrations 0.25 mg/mL or higher significantly reduced the adherence of both *E. coli* strains to cancer cells. The observed effect seemed slightly stronger in AIEC *E. coli* than in the control laboratory strain. It was also confirmed that none of the studied pectins influenced cancer cell viability (Figure S5) and *E. coli* proliferation (Figure S6) under experimental conditions. The same experiment was performed in human colorectal adenocarcinoma cells Caco-2 (Figure S4), and it showed virtually the same results. Representative pictures of *E. coli* LF82 strain adhering to untreated Caco-2 cells and in the presence of PC at different concentrations are shown in Figure 8. It was therefore found that PC was able to reduce adherence of *E. coli* to colon cancer cells.

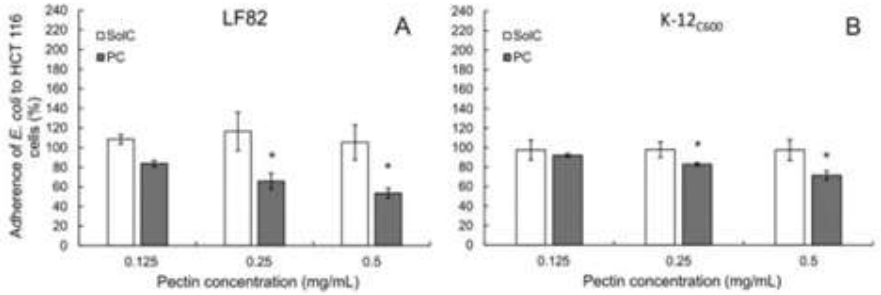

Figure 7. *E. coli* LH82 (**A**) and K-12$_{C600}$ strains (**B**) adherence to HCT 116 cells in the presence of pectins during 2 h of incubation. The means of three experiments ± SD are presented. Statistical significance (* $p < 0.05$) was checked between the studied probes and controls (no pectin) assumed to be 100%.

3.7. The Effect of Pectins on β-Glucuronidase (GUS) Activity

Colonic bacteria may influence intestinal toxicity of irinotecan by enzymatic decomposition of SN-38G back to highly cytotoxic SN-38. The influence of pectins on the activity of β-glucuronidase produced by *E. coli* strains was tested. As shown in Figure 9, both studied pectins reduced GUS activity in a concentration-dependent manner. PC exhibited much stronger enzyme-inhibiting potency as compared to SolC. It was concluded that pectins might potentially alleviate irinotecan-induced diarrhea by reducing enzymatic transformation of the drug in the colon.

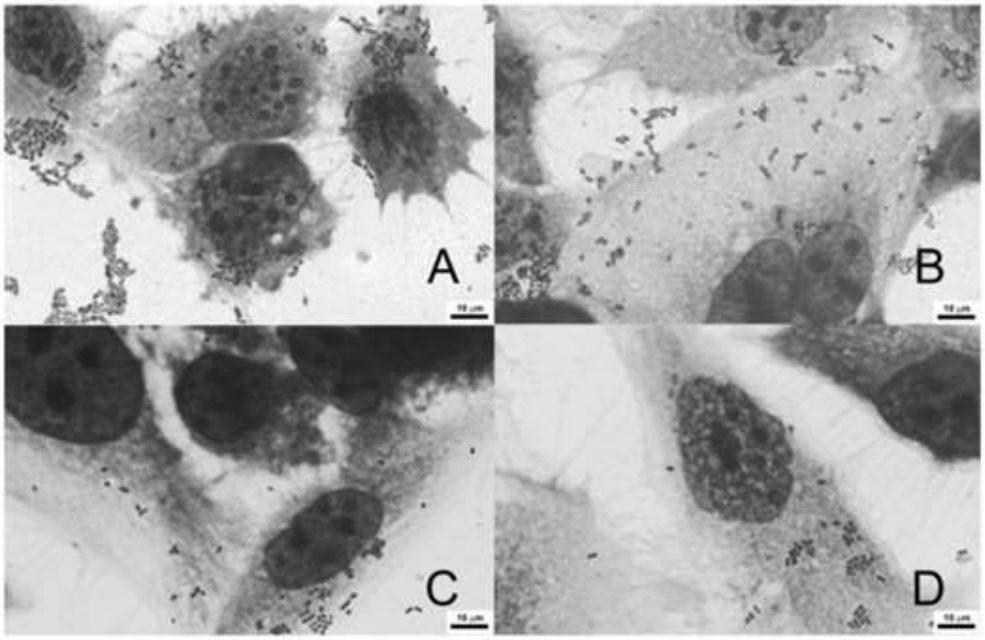

Figure 8. Adherence of *E. coli* (LF82 strain) to Caco-2 cells. Representative pictures of bacteria adhering to untreated Caco-2 cells (**A**), and treated with PC at concentration of 0.125 mg/mL (**B**), 0.25 mg/mL (**C**), and 0.5 mg/mL, (**D**). Wright-Giemsa stain, 100× magnification.

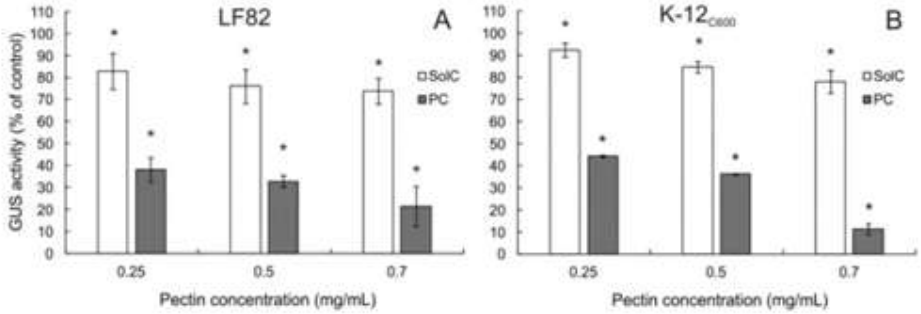

Figure 9. The influence of pectins on GUS activity in *E. coli* LH82 (**A**) and K-12$_{C600}$ lysates (**B**). Pectin solution were mixed with 0.5 U of *E. coli* GUS and incubated for 18 h. The means of three experiments ± SD are presented (* $p < 0.05$). Statistical significance was checked between the studied probes and controls (no pectin) assumed to be 100%.

4. Discussion

In the present work, it was demonstrated that both studied pectins, PC and SolC, significantly reduced the viability of two colon cancer cell lines, HCT 116 and Caco-2. In both types of cells, enzymatically isolated apple pectin (PC) exhibited greater cytotoxicity in comparison to commercially available modified citrus pectin (SolC). Previous studies on PC revealed its ability to reduce proliferation, cell adhesion, and invasion of human adenocarcinoma HT-29 and melanoma B16F10 cells [24]. Pectin treatment was much more destructive to colon cancer cells than to melanoma cells. At the same time, it was shown that PC (tested in concentrations up to 1 mg/mL) exerted virtually no effect on proliferation and adhesion of normal mouse fibroblasts L929 [24]. Anticancer activity of various pectins has been already recognized for several years (see [21,30] for a review). Pectins isolated from ginseng [31] and potato [32] were demonstrated to exert an antiproliferative effect on colon cancer cells (HT-29). Interestingly, it seems that anticancer activity of commercial pectin preparations was limited while enzymatically modified pectins, especially the ones rich in RG I regions, significantly reduced the proliferation of various cancer cell lines [33,34].

Moreover, PC but not SolC was demonstrated to act synergistically with SN-38 in reducing colon cancer cells viability. Pectins have not been reported to potentiate the activity of this drug before. In contrast, the augmentation of the antiproliferative effect of irinotecan toward HCT 116 cells was observed in case of flavopiridol [35]. On the other hand, the synergistic anticancer effect of modified citrus pectin preparation and paclitaxel [36], as well as doxorubicin [37] was recorded in ovarian and prostate cancer cell lines, respectively.

To understand better the mechanism of anticancer properties of pectins, their ability to induce apoptosis in colon cancer cells was studied. It was shown that PC but not SolC significantly increased the size of the apoptotic cell population. Both pectins activated caspase-3, an effector caspase in the apoptotic pathway. Cell cycle analysis demonstrated both pectins elevated sub-G_1 cellular fraction that is composed of cellular debris as well as late apoptotic and necrotic cells. SN-38, tested at low concentrations, also induced apoptosis in HCT 116 cells and raised the abundance of sub-G_1 cellular fraction but it did not activate caspase-3. The most interesting findings came from the analysis of HCT 116 cells treated with PC and SN-38 simultaneously. Strong increases of apoptotic cell population, as well as sub-G_1 cellular fraction, suggested that PC augmented proapoptotic and cytostatic properties of this anticancer drug. Proapoptotic activity of pectins have been already demonstrated for many types of pectin preparations in a variety of experimental settings, e.g., in ovarian cancer (SolC [36]), prostate cancer (fractionated pectin powder [38], SolC [39]) as well as in colon cancer (pectic oligosaccharides [40], sweet potato [41], ginger [31], and beetroot pectin [33]). Modified citrus pectin was recorded to increase caspase-3 activity and to modify the cell cycle of ovarian cancer cells in a similar way as observed for apple pectin PC in the present work [36]. In the same study, the synergy of pectin and paclitaxel in apoptosis induction was observed. However, the same pectin preparation induced G_2/M phase arrest, activation of caspase-3, and the cleavage of poly (ADP-ribose) polymerase (PARP) in urinary bladder cancer cells [42]. Since dietary pectins are not digested before they reach the distal portion of the gastrointestinal tract, they cannot reach circulation. Pectins are known to bind strongly to the mucous layer of the intestine, and in this way, they can modulate the function of epithelial cells. Avivi-Green et al. [43] fed rats in which carcinogenesis had been chemically induced a high-pectin diet and observed an increased apoptotic index of their colon crypt cells that was likely to result in reduced tumor volumes in comparison with rats fed with a standard diet. It has been presumed that proapoptotic, and more generally, anticancer activity of pectins was related to their interaction with galectin-3 (Gal-3). Gal-3 is a lectin that specifically binds β-galactosides. It is present in a variety of cells and often over-expressed in cancer (see [44,45] for a review). Gal-3 specifically binds many intra- and extracellular proteins mediating numerous biological processes including the ones necessary for cancer growth, transformation, invasion, and metastasis. The studies trying to identify the structural features of pectin needed for proapoptotic activity pointed to the

importance of an ester-based cross-link within the pectin molecule [38] as well as to the high content RG-I regions (rich in neutral sugars) within the pectin preparation [33].

The proapoptotic properties of chemical compounds are often associated with their ability to increase the level of reactive oxygen species (ROS) within cells. This possibility was tested in the case of the studied pectins. It was found that PC but not SolC (both at 0.2 mg/mL) increased ROS content in colon cancer cells. SN-38, when applied alone at the concentration of 5 nM increased ROS level only slightly. The SN-38-induced increase in ROS content was pretty elevated when the drug was combined with SolC and considerably raised in the case of the PC:SN-38 combination. Enzymatically isolated pectin, PC, has been previously demonstrated to effectively scavenge free radicals, and its antioxidative potency was more significant as compared to commercial apple pectin [24]. Antioxidative activity of various types of pectins has been widely reported (see [46] for a review). However, it should be taken into consideration that the laboratory-detected ability of a compound to scavenge free radicals does not have to translate into its ROS-mitigating behavior in the cellular environment. Modified citrus pectin has been recently demonstrated to increase ROS production in prostate cancer cells that contributed to their increased radiosensitivity in the presence of pectin [47]. Both citrus and apple pectins were observed to increase ROS release in breast cancer cells and to induce apoptosis via the mitochondrial pathway [48].

Local inflammation in the colon has been associated with a higher risk of CRC development [5]. Therefore, it was decided to investigate the ability of the studied pectins to modulate the inflammatory response of colon cancer cells. Since HCT 116 cells express Toll-Like Receptor 4 (TLR4) [49,50], LPS produced by *E. coli* was used to induce inflammatory processes in these cells. Interaction of TLR4 with LPS switches on the signaling pathway in which many components (including nuclear factor NFκB) are engaged and that triggers the expression of pro-inflammatory cytokines (e.g., IL-6) and enzymes, like COX-2 [51,52]. The results showed that LPS induced a significant increase in both IL-6 and COX-2 levels produced by HCT 116 cells. In the presence of pectins the levels of both proteins significantly decreased. The immunomodulatory effect was, however, much more pronounced in the case of PC than SolC. The interplay of pectins with various components of LPS/TLR4-induced pathways has already been observed. Citrus pectin caused the decrease of expression of inducible nitric oxide synthase (iNOS) and COX-2 in LPS-activated macrophages [53]. An apple oligogalactan suppressed both the expression and activity of COX-2 in LPS-stimulated colon carcinoma cells [54]. In the same experimental setting modified apple polysaccharides significantly decreased LPS-induced expression of TLR4, COX-2, matrix metallopeptidases (MMP) 2 and 9, iNOS, as well as prostaglandin E2 [55]. Oral application of pectins was also demonstrated to alleviate LPS-induced shock in mice [56] and to decrease the expression of pro-inflammatory markers in model mouse colitis [57]. The direct interaction of pectins with TLR4, thus competing with LPS binding, was proposed to constitute the molecular basis for the modulation of the inflammatory process by pectins [55,58]. Interestingly, the sugar profile and structure of RG II region present in PC molecule (but not in SolC) highly resemble the structure of O-antigen of LPS [59,60].

Pectins are characterized by high adhesion to the intestinal mucous layer. They can both affect the function of epithelial cells and form the protective barrier against colonization by opportunistic microflora [61]. Additionally, they form a favorable environment for the normal microbiome and constitute the source of nutrients for bacteria populating the colon [62]. The disruption of gut microbiome equilibrium, called dysbiosis, often precedes the development of certain pathological conditions, from mild as irritable bowel syndrome to severe such as CRC. Colonic microbiota of CRC patients has been shown to differ from healthy individuals colonic flora [8,9]. Therefore, the impact of PC and SolC on the adherence of *E. coli* to colorectal cancer cells was studied. Two bacterial strains have been applied, laboratory K-12_{C600} strain, and adherent-invasive *E. coli* (AIEC) LF82 strain, isolated from a Crohn's disease patient. SolC did not change the adherence of *E. coli* to HCT 116 and Caco-2 cells, whereas PC significantly diminished the adherence of bacteria to colon cancer

cells. Moreover, the inhibitory action of PC seemed to be stronger in AIEC *E. coli* than in laboratory strain. The inhibitory effect of pectic oligosaccharides (POS) on the adhesion of pathogenic bacterial strains has been demonstrated in numerous studies. In human colon adenocarcinoma epithelial cells HT-29 POS from citrus significantly reduced the adherence of *Vibrio cholerae* [63], several enteropathogenic as well as verotoxigenic *E. coli* strains [64], and Shiga-toxin-producing *E. coli* O157:H7 [65]. Although orange peel POS did not affect *Campylobacter jejuni* adhesion, they reduced its invasion into intestinal epithelial cells Caco-2 [66]. In the study on POS produced from apple pomace, Wilkowska et al. [67] presented evidence for their inhibitory effect on adhesion of pathogenic strains of *E. coli*, *Listeria monocytogenes*, and *Salmonella typhimurium* to Caco- 2 cells. In contrast, the adhesion of probiotic bacterial strains to the intestine epithelium was increased in the presence of pectins. Similarly, pectin-like oligosaccharides from *Panax ginseng* roots was shown to reduce adhesion of *Helicobacter pylori Actinobacillus actinomycetemcomitans*, *Propionibacterium acnes*, and *Staphylococcus aureus* to human gastric adenocarcinoma cells with no inhibitory effects against non-pathogenic *Lactobacillus acidophilus*, *E. coli*, and *Staphylococcus epidermidis* [68]. These studies pointed to the beneficial effects of pectins in maintaining healthy microbiota.

The composition and enzymatic activity of colonic microbiota can significantly affect the effectiveness of chemotherapy [10,11]. On the other hand, chemotherapeutic drugs can influence intestinal microbiota [69]. Decomposition of a drug-glucuronide back to an active form of the drug by bacterial β-glucuronidase constitutes the major reason for the observed intestinal toxicity of irinotecan [18] but also other xenobiotics [70]. The application of GUS inhibitors as an adjuvant to irinotecan therapy has been proposed and demonstrated to be effective in alleviating the drug-induced gut damage and diarrhea [71,72]. For this reason, it was decided to investigate the effect of the studied pectins on the activity of GUS produced by two *E. coli* strains, laboratory strain and the one isolated of Crohn's disease patient. Both SolC and PC were demonstrated to inhibit GUS activity. However, the reduction caused by PC was much stronger as compared to SolC. The analysis of previous studies dealing with the influence of pectins on bacterial GUS revealed lots of inconsistencies. Depending on the type of pectin and experimental setting pectins were reported to increase [73,74], to have no effect [75–77], or to reduce GUS activity [78–81]. Based on the results obtained in the present study, it was concluded that PC strongly inhibited the enzymatic activity of *E. coli* GUS and might therefore reduce the backward transformation of SN-38G to SN-38.

5. Conclusions

The novel pectin, PC, obtained by enzymatic extraction of apple pomace, demonstrated to possess superior anticancer activity as compared to the modified citrus pectin, SolC. The key findings of the present study are summarized in Figure 10. PC reduced colon cancer cell viability, induced apoptosis, and increased intracellular ROS production. Moreover, the enhancement of the cytotoxic and proapoptotic effects of low concentrations of irinotecan was observed in the presence of PC. Additionally, novel apple pectin exhibited potent anti-inflammatory properties, prevented adhesion of *E. coli* to colon cancer cells, as well as inhibited the activity of bacterial GUS. With the reservation that the conclusions are based on the in vitro study only, it was demonstrated that novel pectin might be a promising candidate for an adjunct to irinotecan therapy that might alleviate its side effects and thus increase its therapeutic efficacy.

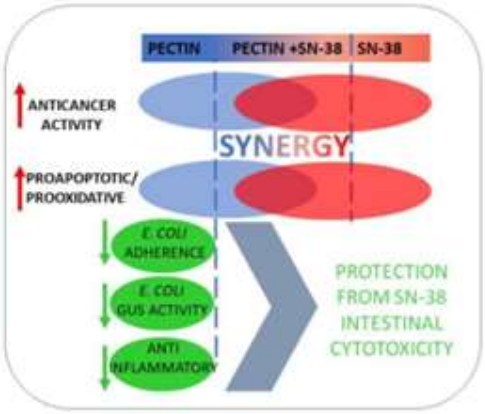

Figure 10. Schematic representation of activities of novel apple pectin in colon cancer cells.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cancers13122952/s1. Figure S1: SRB (A) and MTT (B) cytotoxicity assay of pectins in Caco-2 cells (incubation time 48 h). Figure S2: Typical dot plots of Annexin V/PI double staining apoptosis assay in HCT 116 cells. Figure S3: Typical histograms of DNA content (stained with PI) in HCT 116 cells treated with 0.2 mg/mL of pectins and/or SN-38 for 48 h. Sub-G_1 population—dead cells, G_0/G_1—mononuclear cells, S—DNA replication, G_2/M—mitosis. Figure S4: *E. coli* LH82 (A) and K-12_{C600} strains (B) adherence to Caco-2 cells in the presence of pectins during 2 h of incubation. Figure S5: MTT (B) cytotoxicity assay of pectins in HCT-116 (A) and Caco-2 cells (B) during 2 h of incubation. Figure S6: *E. coli* LH82 (A) and K-12_{C600} strains (B) proliferation during 2 h of incubation in the presence of pectins.

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9. Biegunka indukowana chemioterapią - patofizjologia, obecne i przyszłe kierunki leczenia

Chemotherapy-induced diarrhea - pathophysiology, current and future treatment trends

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Streszczenie

Chemioterapia stanowi jedną z podstawowych metod leczenia chorób nowotworowych. Jej zastosowanie znacznie poprawiło rokowanie pacjentów. Pomimo jej niezaprzeczalnych zalet niestety istotny problem kliniczny stanowi znaczna toksyczność leczenia chemicznego. Wiąże się to z objawami niepożądanymi, które w istotny sposób obniżają jakość życia chorych. Jednym z nich jest biegunka indukowaną chemioterapią CID (ang.chemotherapy-induced diarrhea). Chociaż częstość występowania CID jest trudna do oszacowania, uważa się, że dotyczy ona około 80% pacjentów poddanych działaniu chemioterapii. Mechanizmy leżące u podstaw CID, pozostają niejasne, aczkolwiek uważa się, że wynikają one z synergii nakładających się procesów patofizjologicznych, w tym zapalenia, dysfunkcji wydzielniczej, zaburzeń motoryki jak również zmian w unerwieniu przewodu pokarmowego. Obecne postępowanie medyczne CID zakłada głównie leczenie objawowe i nierzadko prowadzi do pogorszenia i tak już istniejących skutków chemioterapii wywołując szereg skutków ubocznych. Pojawiające się nowe schematy postępowania, stanowią obiecującą alternatywę leczenia CID.

1. Wstęp

Nowotwory stanowią główną przyczyną zgonów na świecie. Tylko w 2018 r. odnotowano około 18,1 mln nowych zachorowań oraz 6,2 mln zgonów na raka (Bray i in. 2018). Chociaż postęp w medycynie przyczynił się do udoskonalenia technik diagnozowania i leczenia nowotworów, oczekuje się, że w nadchodzących dziesięcioleciach obciążenie związane z chorobami nowotworowymi na świecie znacznie wzrośnie. Starzenie się populacji oraz styl życia w tym palenie tytoniu, brak aktywności fizycznej i nieodpowiednia dieta, zostały uznane za podstawowe czynniki przyczyniające się do wzrostu zachorowalności na całym świecie (Jemal i in. 2011).

Chemioterapię stosuje się na różnych etapach leczenia przeciwnowotworowego i chociaż znacznie poprawiła ogólną przeżywalność w wielu rodzajach nowotworów to cytotoksyczne efekty uboczne stanowią przeszkodę znacznie utrudniającą jej kliniczne zastosowanie (Kannarkatt i in. 2017). Objawy uboczne ze strony układu pokarmowego, takie jak nudności, wymioty, bóle brzucha, wzdęcia, owrzodzenia, zaparcia a w szczególności biegunka należą do najczęstszych przeszkód powodującymi opóźnienia a nawet przerwanie leczenia. Jednocześnie w znacznym stopniu obniżają jakość życia wielu pacjentów. Objawy uboczne stwierdzono u 40 % pacjentów otrzymujących chemioterapię w standardowych dawkach oraz u 100% otrzymujących chemioterapię wysokodawkową (McQuade i in.2014).

2. Opis zagadnienia, przegląd literatury

Biegunka wywołana chemioterapią jest istotnym problemem klinicznym, który w istotny sposób wpływa na śmiertelność chorych na raka na całym świecie. Częstość występowania i nasilenie CID są bardzo zróżnicowane w zależności od chemioterapeutyku, sposobu podawania i dawkowania. Niektóre protokoły leczenia, szczególnie te zawierające 5-fluorouracyl i irynotekan, wiążą się ze

wysokim odsetkiem występowania biegunki, aż do 80%. U jednej trzeciej pacjentów występuje ciężka biegunka (stopień 3 lub 4) (Maroun i in. 2007). Trwała i ciężka biegunka może prowadzić do znacznego niedożywienia, odwodnienia - kacheksji i w konsekwencji do niewydolności nerek. Odwodnienie z powodu CID jest związane z przedwczesną śmiercią u około 5% pacjentów poddawanych leczeniu przeciwnowotworowemu (Rothenberg i in. 2001). Co więcej, chemioterapeutyki mogą również wywołać ciężkie zapalenia jelit prowadzące do zaburzeń klinicznych o potencjalnie zagrażających życiu konsekwencjach (McQuade i in. 2016).

U ponad 30% chorych na CID występuje znaczne obniżenie jakości życia. Uporczywa CID wiąże się z lękiem, depresją, izolacją społeczną oraz niską samooceną (Viele 2003), co podkreśla znaczenie zarówno wyjaśnienia podstawowych mechanizmów choroby, jak i poprawy skuteczności leczenia.

2.1 Patofizjologia

Biegunka stanowi częsty efekt uboczny działania irynotekanu, stosowanego w leczeniu raka okrężnicy. 50–80% pacjentów poddanych działaniu irynotekanu cierpi na ciężką biegunkę. Zmiany histologiczne, występujące w całym przewodzie pokarmowym w odpowiedzi na podanie irinotekanu, potwierdziły wyniki badań *in vivo* na zwierzętach. Odnotowano wyraźną ablację krypt, skrócenie kosmków oraz zanik nabłonka w jelicie cienkim i grubym (Gibson i in. 2003). Chociaż pacjenci nie są poddawani rutynowym badaniom endoskopowym uważa się, że CID jest w dużej mierze formą lub skutkiem ubocznym zapalenia błony śluzowej przewodu pokarmowego. Inicjacja zapalenia błony śluzowej jest wynikiem bezpośredniego i pośredniego oddziaływania chemioterapeutyków na szybko dzielące się komórki nabłonka przewodu pokarmowego, prowadząc do ich apoptozy. Dochodzi do aktywacji jądrowego czynnika transkrypcyjnego kappa B (NFκB), następnie do wyrzutu prozapalnych cytokin, w tym m. in. interleukiny 1. Przyczyniają się one do powstania owrzodzeń i zapalenia nabłonka błony śluzowej przewodu pokarmowego (Gibson i in. 2003; Stringer 2009). Potwierdza to fakt, że w jelicie grubym po podaniu irinotekanu obserwuje się wzrost ekspresji cyklooksygenazy COX-2 (ang. cyclooxygenase 2), związany ze zwiększonym uwalnianiem prostaglandyny E2 (ang. PGE2-Prostaglandin E2) (Yang i in. 2005).

Kluczowy element patofizjologii wszystkich rodzajów biegunek stanowi zakłócenie równowagi wodno-elektrolitowej w przewodzie pokarmowym. Biegunka może być wynikiem działania siły osmotycznej, która działając w świetle przewodu pokarmowego, "wciąga" wodę do jelita, lub wyniku aktywnego stanu wydzielniczego w enterocytach. W pierwszym przypadku biegunka ma charakter osmolarny, co obserwuje się po spożyciu niewchłanialnych cukrów, np. takich jak laktuloza. Dla CID charakterystyczny jest drugi mechanizm. Poprzez uszkodzenie śluzówki dochodzi do wydzielania szeregu cytokin które wywołują hiperperystaltykę jelit oraz indukują nadmierne wydzielanie elektrolitów, w szczególności chlorków(Yang i in. 2005).

2.2 Mikrobiota jelitowa

Integralną rolę w homeostazie jelitowej odgrywają drobnoustroje jelitowe. Uważa się, że pełnią także istotną funkcję w rozwoju zapalenia błony śluzowej przewodu pokarmowego. Zachwianie naturalnej równowagi flory bakteryjnej chorego, poprzez zastosowanie chemioterapii, może mieć także istotny wpływ na skuteczność leczenia przeciwnowotworowego(Stringer 2009). Ponadto, mikrobiota bierze udział w metabolizmie cytostatyków, czego przykład stanowi przemiana irynotekanu. Irynotekan jest stosowany jako prolek (CPT-11), którego aktywacja zachodzi przez hydrolizę wiązania estrowego w aktywną formę (SN-38). Następnie SN-38 jest metabolizowany w wątrobie do nieaktywnej postaci glukuronidu SN-38 (SN-38G), a następnie eksportowany do okrężnicy. Tutaj SN-38G ulega hydrolizie w obecności bakteryjnej β-glukuronidazę (βG). Proces ten prowadzi do reaktywacji aktywnej postaci irynotekanu odpowiedzialnej za toksyczność jelitową. Biorąc pod uwagę kluczową rolę mikrobioty okrężnicy w wywoływaniu biegunki wywołanej irynotekanem, hamowanie bakteryjnej βG w okrężnicy lub zapewnienie dodatkowego substratu dla tego enzymu stanowi wyzwanie współczesnej onkologii i mikrobiologii.

Jak zauważono w obecności irynotekanu liczba bakterii *Bacteroides spp*. maleje przy jednoczesnym wzroście liczby bakterii z rodzaju S*taphylococcus spp., Clostridium spp. i E. coli*. (Stringer i in. 2009).Uważa się, że zmiany w mikrobiocie odgrywają ważną rolę nie tylko

w utrzymaniu homeostazy i integralności jelit, ale także w modulacji reakcji zapalnych poprzez interakcję z receptorami TLR (ang. toll-like receptors) i receptorami domenowymi oligomeryzacji nukleotydów, które aktywują transkrypcyjny czynnik NFκB. Jak wiadomo, prawidłowa proliferacja i różnicowanie nabłonka przewodu pokarmowego powracają po około 2 tygodniach po zakończeniu terapii. Natomiast zmiany funkcjonalne utrzymują się dłużej. Patofizjologia leżąca u podstaw tych trwałych zmian w funkcji przewodu pokarmowego obejmuje nie tylko mechanizm osmotyczny i zapalny ale także neurogenny. (McQuade i in. 2014). Chemioterapia indukując uszkodzenia jelitowego układu nerwowego ENS (ang.enteric nervous system) przyczynia się do zaburzeń motoryki przewodu pokarmowego i wystąpienia ciężkiej postaci biegunki. Do niedawna pomijano wpływ chemioterapeutyków na neurony jelitowe i dysfunkcję przewodu pokarmowego. Jednak dowiedziono, że przewlekłe leczenie cisplatyną powoduje utratę neuronów jelitowych, wzrost amplitudy skurczów fałdów błony śluzowej dna żołądka, a także biegunki (McQuade i in. 2014).

2.3 Aktualne metody leczenia CID

Zgodnie z "National Cancer Institute's Common Terminology Criteria for Adverse Effects grading system" CID może być sklasyfikowana jako:
1. Biegunka niepowikłana (stopień 1-2, bez powikłań) lub
2.Biegunka powikłana (stopień 3-4 z jednym lub więcej powikłaniami), w tym:
(A) Biegunka o wczesnym początku (<24 h po podaniu dawki leczenia chemoterapeutycznego)
(B) Biegunka o późnym początku (>24 h po podaniu dawki leczenia chemoterapeutycznego),
(C) Biegunka przewlekła (obecna przez >4 tygodnie) lub
(D) nieprzewlekła (obecna przez <4 tygodnie).

Niepowikłana postać CID może być leczona poprzez modyfikację diety oraz stosowanie standardowych leków przeciwbiegunkowych. Postać powikłana wymaga aplikacji wysokich dawek leków oraz hospitalizacji (McQuade i in. 2014). Zalecenia dotyczące postępowania w CID zostały opublikowane w 1998 r. zaktualizowane w 2004 r. (Benson i in. 2004) i zawierają wytyczne i oceny postępowania. Obecnie jedynymi lekami zalecanymi w zaktualizowanych wytycznych dotyczących leczenia są pochodne opioidów, takie jak: loperamid, oktreotyd oraz laudanum/nalewka z opium.

2.4 Loperamid

Loperamid to syntetyczny agonistą receptorów opioidowych μ w ścianie jelit. Wywiera hamujący wpływ na uwalnianie prostaglandyn i acetylocholiny, a także zwiększa resorpcję wody i elektrolitów. Wysokie dawki loperamidu znajdują zastosowanie w łagodzeniu CID. Jego stosowanie prowadzi jednak do szeregu działań niepożądanych, takich jak silne zaparcia, bóle brzucha, zawroty głowy, wysypki, a także do pogorszenia już istniejących wzdęć, mdłości i wymiotów. Wysokie dawki loperamidu zwiększają częstość występowania porażennej niedrożności jelit. Pomimo tych poważnych działań niepożądanych, loperamid stanowi lek pierwszego rzutu w CID (McQuade i in. 2014).

2.5 Oktreotyd

Oktreotyd jest syntetycznym analogiem somatostatyny i hamuje wydzielanie hormonu wzrostu GH (ang. growth hormon) serotoniny i peptydów uwalnianych przez wewnątrzwydzielniczy układ żołądkowo-jelitowo-trzustkowy. Oktreotyd jest stosowany w leczeniu powikłanej CID oraz jako lek drugiego rzutu u pacjentów, którzy nie reagują na działanie loperamidu po 48 h, pomimo zwiększania dawki. Chociaż oktreotyd skutecznie zmniejsza objawy CID, u ponad 10% pacjentów występują skutki uboczne klasyfikowane jako ciężkie, w tym: bradykardia, ciężkie zaparcia, silne bóle brzucha, zaburzenia czynności tarczycy, kamica żółciowa, bóle i zawroty głowy (Stein i in. 2010).

2.6 Laudanum/ nalewka z opium

Nalewka z opium jest stosowana w biegunkach opornych na leczenie pierwszego rzutu. Podobnie jak loperamid jest agonistą receptorów opioidowych μ w obrębie przewodu pokarmowego. Hamując perystaltykę jelit, wydłuża czas pasażu oraz sprzyjaja reabsorpcji płynów. Skuteczność Laudanum w leczeniu CID dotychczas nie jest poparta badaniami, jednak lek ten jest powszechnie

stosowany jako lek drugiego rzutu w przewlekłych i niepowikłanych biegunkach (Stein i in. 2010). Nalewka z opium wywołuje wiele działań niepożądanych, w tym euforię, nudności, wymioty, bolesne/trudne oddawanie moczu, napady drgawek i reakcje alergiczne. Ponadto, podawanie Laudanum może wiązać się z uzależnieniem psychologicznym i fizycznym, depresją oddechową oraz ciężkimi zaparciami.

2.7 Potencjalnie nowe terapie

Ponieważ obecnie stosowane terapie w leczeniu CID mają ograniczoną skuteczność i wiele działań niepożądanych, poszukiwanie i stosowanie nowych leków przeciwbiegunkowych jest niezbędne do poprawy jakości życia pacjentów cierpiących na nowotwory. Stosunkowo nowe i już stosowanych terapie wykorzystywane w leczeniu schorzeń takich jak IBS z dominującą biegunką (IBS-D) i przewlekła idiopatyczna biegunka, mogłyby znaleźć zastosowanie w leczeniu CID.

2.8 Inhibicja kanału chlorkowego

Chlor jest kluczowym jonem biorącym udział w wydzielaniu i wchłanianiu jelitowym. Nadmierna sekrecja jest napędzana przez aktywne wydzielanie chlorków, a następnie wtórny ruch wody i sodu do światła jelita. Pomimo braku silnych selektywnych inhibitorów napięciowo zależnych kanałów chlorkowych, hamowanie aktywności kanałów chlorkowych aktywowanych wapniem skutecznie ogranicza wydzielanie chlorku do światła przewodu pokarmowego. Wykorzystanie modelu mysiego z ciężką biegunką wydzielniczą wywołaną rotawirusem, w którym dokonano inhibicji kanałów chlorkowych aktywowanych wapniem stosując ekstrakt z czerwonego wina pozwoliło na zmniejszenie wydzielania płynu jelitowego, zmniejszając przy tym objawy biegunki (Ko i in. 2014).

2.9 Aktywacja receptora kannabinoidalnego

Mechanizm działania kannabinoidów opiera się na wiązaniu z dwoma głównymi receptorami sprzężonymi z białkiem G. Receptory CB1 i CB2 to receptory licznie występujące w układzie pokarmowym. Mimo, że ich aktywność jest zmienna w zależności od odcinka przewodu pokarmowego, stwierdzono, że aktywacja receptorów CB1 hamuje czynność skurczową mięśniówki okrężnicy. Jak dowiedziono dronabinol, jako nieselektywny agonista receptorów kannabinoidowych, spowalnia pasaż zarówno u osób zdrowych, jak i u pacjentów z biegunką związaną z IBS (IBS-D) (Wong i in. 2011). Ponadto jak wykazano w oparciu o badania z wykorzystaniem modelu szczurzego, niska dawka nieselektywnego agonisty receptorów kannabinoidowych WIN55,212-2 zmniejsza nasilenie biegunki wywołanej 5-fluorouracylem. (McQuade i in. 2014).

2.10Probiotyki, antybiotyki i inhibitory β-glukuronidazy

Wraz z uznaniem, że mikrobiom odgrywa kluczową rolę w patofizjologii zapalenia błony śluzowej i rozwoju CID, zarówno antybiotyki, jak i probiotyki stały się obiecującymi opcjami terapeutycznymi. Dowiedziono, że podaż probiotyków obniża częstość występowania CID zarówno przy stosowaniu 5-fluorouracylu, jak irynotekanu (McQuade i in. 2014). Ponadto, dieta wzbogacona w błonnik, suplementacja *Lactobacillus rhamnosus* sprzyja zmniejszeniu objawów związanych z nasileniem biegunki stopnia 3/4 wywołanej toksycznością 5-fluorouracylu aż u 15% pacjentów leczonych z powodu raka jelita grubego. Jednocześnie wykazano, że w agresywnym leczeniu CID zalecana jest aplikacja doustnych antybiotyków, takich jak fluorochinolony (Benson i in. 2004; Maroun i in. 2007).

Analiza wyników badań przeprowadzonych z wykorzystaniem modelu mysiego, potwierdziła że selektywne hamowanie bakteryjnej β-glukuronidazy łagodzi indukowane przez cytostatyki uszkodzenie śluzówki przewodu pokarmowego. Ponadto, dowiedziono, że doustne podawanie silnych inhibitorów β-glukuronidazy bakteryjnej zmniejsza toksyczność irynotekanu (Wallace i in. 2010). Wyniki badań klinicznych udowodniły, że lek o nazwie handlowej Kampo Hangeshashinto (TJ-14), zmniejsza częstość występowania, jak i czas trwania indukowanego chemioterapią zapalenia błony śluzowej jamy ustnej u pacjentów z rakiem jelita grubego Substancją aktywną TJ-14 stanowi bajkalina, będąca inhibitorem β-glukuronidazy (Matsuda i in. 2015).

W porównaniu grupą kontrolną u pacjentów leczonych TJ-14 stwierdzono zmniejszenie częstości występowania biegunki 3 i 4 stopnia (McQuade i in. 2014).

2.11Pektyny

Pektyny stanowią główny składnik błonnika pokarmowego. Charakteryzują się wysoką adhezją do błony śluzowej jelita, tworząc w ten sposób barierę ochronną przed kolonizacją flory oportunistycznej. Prawie 90% pektyn podanych doustnie dociera do dystalnego fragmentu okrężnicy. Pektyny tworzą sprzyjające środowisko dla prawidłowego mikrobiomu i stanowią źródło składników odżywczych dla bakterii zamieszkujących okrężnicę. W wyniku bakteryjnego rozkładu pektyn powstają krótkołańcuchowe kwasy tłuszczowe, co prowadzi do przejściowego obniżenia pH w jelitach. Uważa się, że proces ten jest odpowiedzialny za hamowanie wzrostu szczepów patogennych przy jednoczesnym zachowaniu fizjologicznej flory jelitowej. Ponadto pektyny są w stanie wiązać związki organiczne, takie jak toksyny. działając ochronnie na śluzówkę jelit. Wyniki badań z udziałem badaniu 255 pacjentów pediatrycznych w wieku od 6 miesięcy do 6 lat stosujących doustnie ekstrakt pektyny z jabłka, potwierdziły istotne obniżenie częstości oddawania stolca, szczególnie w przypadkach, gdy biegunka nie ustępowała do trzeciego dnia leczenia (Becker i in. 2011).Wydaje się wysoce prawdopodobne, że zastosowanie pektyn jako dodatkowego źródła węglowodanów dostarczanych podczas leczenia irynotekanem może zahamować proces hydrolizy SN-38G przez bakteryjną βG, a tym samym zwiększyć ograniczoną skuteczność terapii w leczeniu CID.

3. Wnioski

CID stanowi jeden z najczęstszych objawów niepożądanych związanych z chemioterapią. Przyczynia się do opóźnień w leczeniu, redukcji dawki, a przez to do obniżenia skuteczności leczenia, niekiedy do jego zaprzestania. Pomimo licznych badań klinicznych mających na celu ocenę skuteczności substancji terapeutycznych i/lub profilaktycznych w łagodzeniu CID, w aktualnych wytycznych zalecane są tylko trzy leki: loperamid, nalewka z opium i oktreotyd. Ponadto, obecnie wykorzystywanym metodom leczenia CID towarzyszą liczne współistniejące objawy niepożądane. Nowe terapie stanowią obiecującą alternatywę. Rozwój nowych metod leczenia CID jest niezbędny dla poprawy wyników klinicznych i jakości życia osób leczonych onkologicznie. Biorąc pod uwagę kluczową rolę mikrobioty w przebiegu biegunki, hamowanie aktywności bakteryjnej βG w okrężnicy lub zapewnienie dodatkowego substratu dla tego enzymu stanowi wyzwanie współczesnej onkologii i mikrobiologii.

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PODSUMOWANIE I WNIOSKI

Na podstawie przeprowadzonych doświadczeń i wyników zamieszczonych w publikacji **"Newly-obtained apple pectin as an adjunct to irinotecan therapy, presumptively reducing its side effects via influence on colonic *E. coli* β-glucuronidase activity"** można wysunąć następujące wnioski:

1. Pektyny, PC i SolC, znacząco obniżają żywotność komórek dwóch linii komórkowych raka jelita grubego, HCT116 i Caco-2. W obu typach komórek enzymatycznie izolowana pektyna jabłkowa (PC) wykazuje wyższą cytotoksyczność w porównaniu z komercyjnie dostępną modyfikowaną pektyną cytrusową (SolC).

2. Pektyna PC, ale nie SolC, działa synergistycznie z SN-38, co zaobserwowano na podstawie oceny żywotności komórek raka jelita grubego. Co ważne, pektyny nie były wcześniej opisywane jako adiuwanty potęgujące aktywność tego leku.

W celu lepszego zrozumienia mechanizmu przeciwnowotworowego działania pektyn, zbadano ich zdolność do indukowania apoptozy i generowania reaktywnych form tlenu w komórkach raka jelita grubego. Pozwoliło to na wysunięcie następujących wniosków:

3. Pektyna jabłkowa PC, ale nie cytrusowa SolC, istotnie zwiększa wielkość populacji komórek apoptotycznych.

4. PC, ale nie SolC zwiększa zawartość ROS w komórkach raka jelita grubego.

5. SN-38, zastosowany samodzielnie w stężeniu 5 nM, tylko nieznacznie zwiększał poziom ROS podczas gdy PC w kombinacji z SN-38 indukowała znacznie większe poziomy ROS niż w przypadku kombinacji SN-38:SolC.

Miejscowy stan zapalny w jelicie grubym wiąże się z wyższym ryzykiem rozwoju CRC. Dlatego zbadano zdolność badanych pektyn do modulowania odpowiedzi zapalnej komórek raka jelita grubego. Wyniki badań wykazały:

6. LPS indukuje znaczący wzrost poziomu IL-6 i COX-2 produkowanych przez komórki HCT116. W obecności pektyn poziomy obu białek ulegają znacznemu obniżeniu.

7. Efekt immunomodulacyjny jest znacznie wyraźniejszy w przypadku PC niż SolC.

Zaburzenie równowagi mikrobiomu jelitowego, zwane dysbiozą, często poprzedza rozwój niektórych stanów patologicznych, w tym CRC. Wykazano, że mikrobiota jelita grubego pacjentów z CRC różni się od flory jelita grubego osób zdrowych. Zbadano wpływ PC i SolC na adherencję *E. coli* do komórek raka jelita grubego.

8. SolC nie zmienia adherencji *E. coli* do komórek HCT116 i Caco-2, natomiast PC znacząco zmniejsza adherencję bakterii do komórek raka jelita grubego.
9. Hamujące działanie PC jest silniejsze u *E. coli* szczepu AIEC niż u szczepu laboratoryjnego.

Skład i aktywność enzymatyczna mikrobioty jelita grubego może w istotny sposób wpływać na skuteczność chemioterapii. Z drugiej strony, leki chemioterapeutyczne mogą wpływać na mikrobiotę jelitową. Rozkład SN-38G z powrotem do aktywnej formy leku przez bakteryjną β-glukuronidazę stanowi główną przyczynę obserwowanej toksyczności jelitowej irynotekanu, ale także innych ksenobiotyków. Zbadano wpływ badanych pektyn na aktywność GUS wytwarzanej przez dwa szczepy *E. coli*, laboratoryjny i wyizolowany od pacjenta z chorobą Leśniowskiego-Crohna. Wykazano, że:

10. SolC, jak i PC hamują aktywność GUS.
11. Redukcja spowodowana przez PC jest znacznie silniejsza w porównaniu z SolC. PC może silniej ograniczać transformację wsteczną SN-38G do SN-38.

Podsumowując, wykazano, że nowa pektyna, PC, otrzymana w wyniku enzymatycznej ekstrakcji wytłoków jabłkowych, posiada wyższą aktywność przeciwnowotworową w porównaniu z modyfikowaną pektyną cytrusową, SolC. PC zmniejsza żywotność komórek raka jelita grubego, indukuje apoptozę i zwiększa produkcję wewnątrzkomórkowych ROS. Ponadto, w obecności PC obserwuje się wzmocnienie cytotoksycznego i proapoptotycznego działania niskich stężeń irynotekanu. Dodatkowo, nowa pektyna jabłkowa wykazuje silne właściwości przeciwzapalne, zapobiega adhezji *E. coli* do komórek raka jelita grubego, a także hamuje aktywność bakteryjnej GUS. Z zastrzeżeniem, że wnioski oparte są na podstawie wyników badań *in vitro*, wykazano, że nowa pektyna może być obiecującym kandydatem jako dodatek do terapii irynotekanem, który mógłby złagodzić jej efekty uboczne i zwiększyć skuteczność terapeutyczną.

Na podstawie przeprowadzonych doświadczeń i wyników zamieszczonych w publikacji **"The Use of Endo-Cellulase and Endo-Xylanase for Extraction of Apple Pectins as the Factor Modifying Their Anticancer Properties and Affecting Their Synergy with Active Form of Irinotecan"** można wysunąć następujące wnioski:

1. Obie pektyny jabłkowe PX i PCX wykazują aktywność cytotoksyczną w grupie linii komórkowych raka jelita grubego HCT116, Caco-2 i HT-29.
2. Wartości IC_{50} odnotowane dla PCX w komórkach HT-29 i Caco-2 są prawie dwukrotnie niższe niż dla PX. W komórkach HCT116 wartości IC_{50} dla obu pektyn są porównywalne, mimo to PCX jest bardziej cytotoksyczny niż PX.

Badano również właściwości cytotoksyczne pektyn jabłkowych w stosunku do komórek raka jelita grubego w obecności niskiego stężenia aktywnej formy irynotekanu (SN-38). Lek stosowano w stężeniu 5 nM, o znikomej toksyczności względem badanych linii komórkowych. Otrzymane wyniki badań wykazały:

3. Istnienie synergizmu pomiędzy pektynami jabłkowymi PX i PCX i SN-38.
4. W przypadku PX, nasilenie obserwowanego efektu synergicznego jest podobne we wszystkich trzech badanych liniach komórkowych.
5. PCX wykazywał silniejszą synergię z SN-38 w komórkach HCT116 i Caco-2, ale nie w komórkach HT-29. Stwierdzono zatem, że procesy powodujące synergizm są zależne od rodzaju komórek nowotworowych.

Obserwację cytotoksycznego działania enzymatycznie ekstrahowanych pektyn jabłkowych na komórki raka jelita grubego uzupełniono o badanie ich wpływu na cykl komórkowy i indukcję apoptozy.

6. We wszystkich trzech liniach komórkowych obecność pektyn powoduje wzrost liczby komórek w fazie sub-G1 cyklu komórkowego (komórki nekrotyczne i późno apoptotyczne), jak również indukcję apoptozy.
7. W przypadku zastosowania dwuskładnikowego połączenia pektyny jabłkowej i SN-38 na komórki raka jelita grubego odnotowuje się wzmocnienie wpływu pektyn zarówno na cykl komórkowy, jak i indukcję apoptozy.

Ponadto zbadano zdolność pektyn jabłkowych do indukcji peroksydacji lipidów i akumulacji wewnątrzkomórkowych ROS. Stwierdzono, że:.

8. PX i PCX istotnie zwiększają poziom obu markerów stresu oksydacyjnego w komórkach raka jelita grubego.
9. W obecności SN-38, efekt działania pektyn jest silnie wzmocniony.

Sugeruje to, że w warunkach eksperymentalnych pektyny wykazywały właściwości prooksydacyjne. Takie właściwości mogą uzupełniać potencjał cytotoksyczny pektyn jabłkowych w stosunku do komórek raka jelita grubego, ponieważ zwiększenie produkcji ROS może prowadzić do śmierci komórek poprzez wywołanie apoptozy. Obserwacja ta pozostaje w oczywistej sprzeczności z licznymi doniesieniami na temat aktywności antyoksydacyjnej różnych preparatów pektynowych. Należy jednak pamiętać, że wyniki uzyskane w prostych modelach laboratoryjnych nie muszą mieć przełożenia na warunki hodowli komórkowych.
Obecnie uważa się, że przewlekły proces zapalny leży u podstaw rozwoju wielu postępujących chorób, w tym nowotworów. Ponieważ aktywność przeciwzapalna pektyn została szeroko zaobserwowana, zbadano pod tym kątem nowo otrzymane pektyny jabłkowe. Do wywołania stanu zapalnego użyto bakteryjnego LPS. Jego zastosowanie spowodowało znaczący wzrost poziomu COX-2 i IL-6 we wszystkich badanych liniach komórkowych raka jelita grubego.

10. PX i PCX silnie obniżają poziom obu markerów stanu zapalnego, nie wywołując jednocześnie zmiany w komórkach niestymulowanych.

Receptory Toll-podobne (TLR) pełnią główną funkcję w rozpoznaniu zagrożenia i inicjacji odpowiedzi immunologicznej Pobudzenie receptorów TLR przez produkty drobnoustrojów stanowi sygnał aktywujący mechanizmy odporności immunologicznej nieswoistej, co można zaobserwować poprzez między innymi wzmożoną syntezę czynników przeciwbakteryjnych i cytokin prozapalnych.
Wykazano, że:

11. PX i PCX znacząco obniżają poziom TLR4 dostępny dla przeciwciał w komórkach HT-29.

Innym, ważnym białkiem regulatorowym, z którym pektyny wchodzą w interakcje, jest galektyna-3. Ze względu na specyficzne wiązanie się z różnymi białkami docelowymi Gal-3 pośredniczy w wielu procesach biologicznych związanych ze wzrostem komórek, transformacją nowotworową, inwazją i przerzutami. Stwierdzono, że zmiany w jej ekspresji występują w wielu stanach nowotworowych i przednowotworowych. Uważa się, że wewnątrzkomórkowa Gal-3 działa jako czynnik antyapoptotyczny, dlatego jej nadekspresja może powodować częściową oporność komórek nowotworowych na apoptozę. Dowiedziono, że:

12. We wszystkich badanych liniach komórkowych raka jelita grubego obecność pektyn powoduje zmniejszenie ilości Gal-3 wykrywanej przez przeciwciała.
13. PCX wpływa na obniżenie poziomu Gal-3 w większym stopniu niż PX.

Podsumowując, wykazano, że pektyny jabłkowe, PX i PCX, wykazują aktywność antyproliferacyjną, proapoptotyczną i przeciwzapalną w komórkach raka jelita grubego. Co więcej, obie pektyny wykazują synergizm z SN-38 w zmniejszaniu żywotności komórek nowotworowych. Jako molekularny mechanizm przeciwnowotworowych i przeciwzapalnych właściwości pektyn zasugerowano prawdopodobieństwo interakcji, odpowiednio z Gal-3 i TLR4. Co ciekawe, PCX okazuję się być bardziej aktywnym czynnikiem antyproliferacyjnym niż PX, podczas gdy ich aktywność przeciwzapalna była porównywalna. Co więcej, PCX wykazuje również silniejszą synergię z SN-38.

W pracy przeglądowej “Chemotherapy-induced diarrhea-pathophysiology current and future treatment trends”opisano rozbudowaną patofizjologię biegunki indukowanej chemioterapią. Na podstawie aktualnych wytycznych opisano leczenie oraz potencjalnie nowe terapie.

OŚWIADCZENIA WSPÓŁAUTORÓW

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OŚWIADCZENIE

Oświadczam że w pracy *Maksymowicz, Jerzy, Anna Palko-Łabuz, Beata Sobieszczańska, Mateusz Chmielarz, Mirosława Ferens-Sieczkowska, Magdalena Skonieczna, Agnieszka Wikiera, Olga Wesołowska, and Kamila Środa-Pomianek. 2022. "The Use of Endo-Cellulase and Endo-Xylanase for the Extraction of Apple Pectins as Factors Modifying Their Anticancer Properties and Affecting Their Synergy with the Active Form of Irinotecan" Pharmaceuticals 15, no. 6: 732.* mój współudział polegał na; konceptualizacji, porządkowaniu danych, analizie formalnej, pozyskaniu funduszy, prowadzeniu badań, administracji projektu, walidacji, wizualizacji, recenzji i redakcji.

Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej lek. Jerzego Maksymowicza pt."Ocena interakcji genotoksycznych szczepów *E. Coli* z nabłonkiem jelita grubego i wpływu pektyn na aktywność przeciwnowotworową irynotekanu"

Podpis

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Wyrażam zgodę na użycie powyższej publikacji w rozprawie doktorskiej lek. Jerzego Maksymowicza pt.:"Ocena interakcji genotoksycznych szczepów *E. Coli* z nabłonkiem jelita grubego i wpływu pektyn na aktywność przeciwnowotworową irynotekanu"

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Wrocław, 11.09.22

OŚWIADCZENIE

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Podpis

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I NEUROBIOLOGII
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Wrocław 13.06.2022

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Katedra Inżynierii i Biologii Systemów
Politechnika Śląska

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Wrocław, 13.06.2022

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