



Szkoła Doktorska Nauk Ścisłych i Przyrodniczych
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Charakterystyka potencjału przeciwnowotworowego kwasu roburowego

Characterization of anticancer potential of
roburic acid

Praca doktorska wykonana
pod kierunkiem promotora
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Wykaz skrótów:

ADMET – absorpcja, dystrybucja, metabolizm, wydalanie, toksyczność, (ang. *absorption, distribution, metabolism, excretion, toxicity*)

AKT – kinaza białkowa B, (ang. *protein kinase B*)

AO – oranż akrydyny, (ang. *acridine orange*)

c-Myc – czynnik transkrypcyjny Myc, (ang. *MYC proto-oncogene*)

CAII – anhydraza węglanowa II, (ang. *carbonic anhydrase II*)

CDK2 – kinaza zależna od cyklin 2, (ang. *cyclin-dependent kinase 2*)

CDKN1A – gen inhibitora kinaz zależnych od cyklin 1A (ang. *cyclin-dependent kinase inhibitor 1A*)

CES1 – karboksyoesteraza 1, (ang. *carboxylesterase 1*)

DMSO – dimetylosulfotlenek, (ang. *dimethyl sulfoxide*)

EB – bromek etydyny, (ang. *ethidium bromide*)

EGFR – receptor nabłonkowego czynnika wzrostu, (ang. *epidermal growth factor receptor*)

FITC – izotiocyanian fluoresceiny, (ang. *fluorescein isothiocyanate*)

I κ B α –inhibitor κ B α , (ang. *inhibitor kappa B alpha*)

IKK – kinaza inhibitorowa κ B, (ang. *I κ B kinase*)

Ki-67 – antygen Kiel 67, (ang. *antigen Kiel 67*)

MAPK – kinazy aktywowane mitogenami, (ang. *mitogen-activated protein kinase*)

MKI67 – gen kodujący marker proliferacji Ki-67, (ang. *marker of proliferation Ki-67*)

NF- κ B – jądrowy czynnik transkrypcyjny kappa B, (ang. *nuclear factor kappa-light-chain-enhancer of activated B cells*)

p21 – inhibitor kinaz zależnych od cyklin p21, (ang. *cyclin-dependent kinase inhibitor 1*)

p38 – kinaza białkowa aktywowana stresem p38, (ang. *p38 mitogen-activated protein kinase*)

PCNA - jądrowy antygen komórek proliferujących, (ang. *proliferating cell nuclear antigen*)

PI – jodek propidyny, (ang. *propidium iodide*)

PI3K – kinaza 3-fosfatydylinozytolu, (ang. *phosphoinositide 3-kinase*)

PLA2G2A – fosfolipaza A2, (ang. *phospholipase A2*)

RA – kwas roburowy, (ang. *roburic acid*)

SA- β -Gal - SA- β -galaktozydaza (ang. *senescence-associated β -galactosidase*)

SD – odchylenie standardowe, (ang. *standard deviation*)

STAT3 – przekaźnik sygnału i aktywator transkrypcji 3, (ang. *signal transducer and activator of transcription 3*)

TNF- α – czynnik martwicy nowotworów α , (ang. *tumor necrosis factor alpha*)

TP53 - gen supresorowy kodujący białko TP53, (ang. *tumor protein TP53*)

Spis publikacji i manuskryptów wchodzących w skład rozprawy doktorskiej:

- **Praca przeglądowa:**

1. **Gielecińska A.**, Kciuk M., Mujwar S., Celik I., Kołat D., Kałuzińska-Kołat Ż., Kontek R., ***Substances of Natural Origin in Medicine: Plants vs. Cancer. Cells.*** 2023 Mar 23;12(7):986. doi: 10.3390/cells12070986.
IF: 5.1; punkty MEiN: 140 pkt.

- **Prace oryginalne:**

1. **Gielecińska A.**, Kciuk M., Mujwar S., Schmid J.A., Kontek R. ***Anticancer Activity of Roburic Acid: In Vitro and In Silico Investigation.*** **International Journal of Molecular Sciences.** 2025 Jul 3;26(13):6420. doi: 10.3390/ijms26136420.
IF: 4.9; punkty MEiN: 140 pkt.
2. **Gielecińska A.**, Kciuk M., Gruszka R., Wawrocki S., Kontek R. Roburic Acid as a Therapeutic Candidate: ***Antiproliferative Activity and Secondary Cell Death Response in Colorectal Cancer Cells.*** **International Journal of Molecular Sciences.** 2026 Mar 8;27(5):2478. doi: 10.3390/ijms27052478.
IF: 4.9; punkty MNiSzW: 140 pkt.

Sumaryczny IF: 14,9; punkty MEiN/MNiSzW: 420.

- **Manuskrypt:**

1. 2026; **Gielecińska A.**, Wawrocki S., Marciniak B., Kciuk M., Kontek R. ***Revisiting Apoptosis: What Lies Behind the Antiproliferative Activity of Roburic Acid in Colorectal Cancer Cells?*** Manuskrypt wysłany do Phytomedicine. IF: 8.3; punkty MNiSzW: 140 pkt.

Wprowadzenie

Współczesna farmakologia coraz częściej sięga po rozwiązania, które natura testowała przez miliony lat. Wśród bogactwa związków naturalnych szczególne miejsce zajmują związki pochodzenia roślinnego, stanowiące źródło aktywnych biologicznie cząsteczek. Ze względu na brak zdolności roślin do przemieszczania się, wykształciły one złożone mechanizmy percepcji bodźców i adaptacyjnej odpowiedzi na zmieniające się warunki środowiska. Jednym z ich kluczowych elementów są metabolity wtórne, pełniące funkcje regulacyjne i ochronne [1,2]. Wykazując pleiotropowe działanie na poziomie komórkowym, modulują one złożone sieci sygnałowe, co odróżnia je od związków projektowanych w ramach popularnego nurtu farmakologii ukierunkowanej na pojedynczy cel molekularny. W konsekwencji część z nich znajduje zastosowanie jako samodzielne substancje czynne, podczas gdy inne są strukturalnym punktem wyjścia do dalszych modyfikacji.

Wielokierunkowy charakter działania metabolitów wtórnych roślin wyraźnie przejawia się w ich zdolności do modulowania odpowiedzi zapalnej, której przewlekła aktywacja odgrywa istotną rolę w patogenezie wielu chorób cywilizacyjnych [3,4]. Stan zapalny jest podstawowym mechanizmem obronnym organizmów, inicjowanym w odpowiedzi na pojawienie się czynników uszkadzających [5]. Stanowi zintegrowany układ procesów adaptacyjnych i naprawczych, których celem jest eliminacja zagrożenia oraz przywrócenie stanu fizjologicznego. W przebiegu odpowiedzi zapalnej dochodzi do czasowych zmian w mikrośrodkowisku tkanek, obejmujących aktywację komórek układu odpornościowego, przebudowę macierzy zewnątrzkomórkowej oraz nasilenie proliferacji, sprzyjające regeneracji uszkodzonych struktur [6]. Procesy te mają charakter przejściowy i ulegają wyciszeniu po usunięciu stresora. Jednak w sytuacji wzmożonej ekspozycji na bodźce uszkadzające lub zaburzeniu mechanizmów regulacyjnych, stan zapalny może utrzymywać się dłużej, prowadząc do trwałych zmian o charakterze patologicznym. Przewlekłe środowisko zapalne sprzyja wtedy dalszej akumulacji uszkodzeń, zaburza równowagę między proliferacją, a śmiercią komórkową oraz ułatwia przełamywanie mechanizmów kontroli wzrostu komórkowego [7,8]. Współczesna

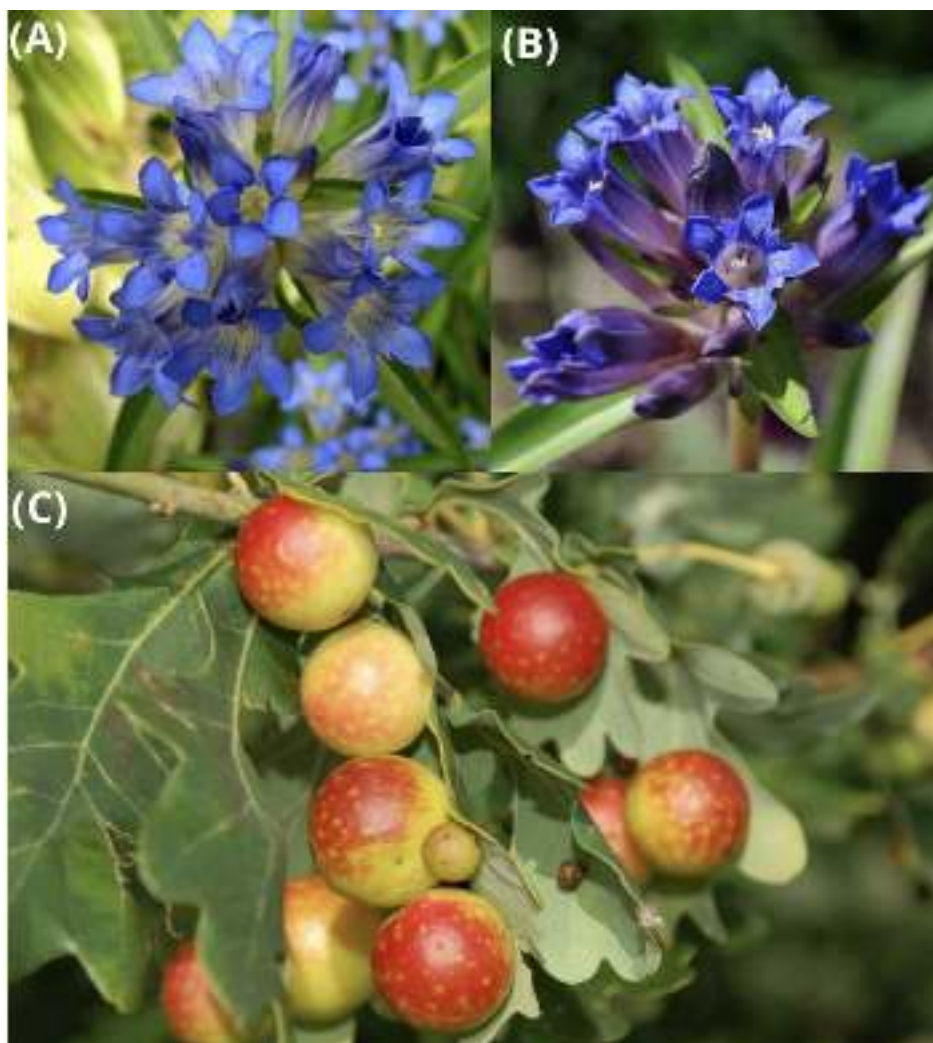
farmacja wykorzystuje roślinne metabolity wtórne zarówno w formie naturalnych ekstraktów, jak i jako prekursory do syntezy bardziej zaawansowanych leków. Ich wielokierunkowe działanie sprawia, że są one skuteczne w terapiach chorób złożonych, takich jak nowotwory czy zaburzenia metaboliczne.

Konsekwencją długotrwałego zaburzenia równowagi w obrębie mikrośrodowiska jest zwiększone ryzyko transformacji nowotworowej, która jest procesem wieloetapowym, wynikającym z deregulacji podstawowych mechanizmów kontrolujących najważniejsze funkcje komórek, takie jak proliferacja, różnicowanie, odpowiedź na stres czy programowana śmierć komórkowa [9]. Transformacja nowotworowa może mieć element losowości, ale nie jest wyłącznie przypadkowa. W biologii nowotworów mówi się raczej o współdziałaniu przypadku i selekcji, w którym losowe zmiany pojawiają się w sprzyjającym środowisku – obejmującym m.in. przewlekły stan zapalny, stres oksydacyjny oraz zaburzoną odpowiedź immunologiczną – a następnie ulegają utrwaleniu na drodze selekcji biologicznej [10]. Częściej jednak transformacja nowotworowa stanowi konsekwencję nagromadzenia zmian molekularnych i adaptacyjnych, które umożliwiają unikanie mechanizmów kontroli wzrostu oraz nabywanie cech sprzyjających przeżyciu i ekspansji [9]. Proces zachodzi w ścisłej interakcji z mikrośrodowiskiem tkankowym, które aktywnie uczestniczy w inicjacji, utrwalaniu i progresji fenotypu nowotworowego. Szczególną rolę przypisuje się, wspomnianym wcześniej, przewlekłym stanom zapalnym, które poprzez długotrwałe oddziaływanie na komórki sprzyjają niestabilności genetycznej oraz przeprogramowaniu odpowiedzi komórkowych [11]. Współczesne podejście do badań nad biologią nowotworów coraz wyraźniej odchodzi od postrzegania choroby wyłącznie jako efektu pojedynczych mutacji genetycznych, które w warunkach fizjologicznych byłyby zwalczane poprzez naprawę uszkodzonego DNA, zahamowanie proliferacji komórek z nieprawidłowym genomem lub ich selektywną eliminację [12]. Komórki dysponują rozbudowanymi mechanizmami radzenia sobie z mutacjami, które powstają w sposób ciągły, jednak tylko niewielka część z nich zostaje utrwalona i prowadzi do nieprawidłowej transformacji. Tkanka nowotworowa przypomina raczej system dynamiczny, w którym kluczowe znaczenie mają nie tylko zmiany w obrębie samych komórek, ale także ich zdolność adaptacji do zmiennych warunków, w tym pojawiających się sygnałów zapalnych, metabolicznych

i stresowych [9]. Złożoność biologii nowotworów, ich heterogeniczność oraz umiejętność nabywania oporności stanowią wyzwanie dla strategii terapeutycznych ukierunkowanych na pojedyncze cele molekularne, które w praktyce klinicznej okazują się być często niewystarczające do trwałej kontroli choroby. W konsekwencji poszukiwanie związków zdolnych do modulowania wielu elementów sieci regulacyjnych determinujących los komórki wydaje się być szczególnie uzasadnione. Na znaczeniu zyskują cząsteczki o wielokierunkowym profilu aktywności biologicznej, zdolne oddziaływać zarówno na procesy zachodzące w obrębie komórek nowotworowych, jak i na ich mikrośrodowisko.

Badania eksperymentalne oparte o dwu- i trójwymiarowe modele komórkowe stanowią podstawowe narzędzie umożliwiające analizę wpływu testowanych związków na kluczowe cechy komórek nowotworowych, takie jak zdolność proliferacji, przeżycia, indukcji programowanej śmierci komórkowej i odpowiedzi na stres. Wśród związków pochodzenia roślinnego szczególną uwagę zwracają triterpenoidy – liczna i strukturalnie zróżnicowana grupa cząsteczek, którym przypisuje się zdolność modulowania fundamentalnych procesów komórkowych. W farmakologii budzą ogromne zainteresowanie ze względu na niską toksyczność wobec zdrowych komórek oraz szerokim działaniem terapeutycznym. Coraz więcej danych wskazuje, że triterpenoidy charakteryzują się wielokierunkowym profilem aktywności biologicznej, wykraczającym poza pojedyncze cele molekularne, co lokuje je w centrum zainteresowania współczesnych badań nad nowymi strategiami terapeutycznymi [13,14]. Jednym z przedstawicieli tej grupy chemicznej jest kwas roburowy (ang. *roburic acid*; skrót: RA) – związek występujący w literaturze naukowej najczęściej pod anglojęzyczną nazwą i nieposiadający dotychczas ugruntowanego odpowiednika w języku polskim. RA jest tetracyklicznym triterpenoidem, należącym do grupy metabolitów wtórnych roślin, wyizolowanym z galasów dębowych oraz identyfikowanym w korzeniach roślin z rodzaju *Gentiana*, w tym *Gentiana macrophylla* oraz *Gentiana dahurica* (Ryc. 1A-1C) [15–17]. Dotychczas związek ten opisywany był głównie w kontekście właściwości przeciwzapalnych i antyoksydacyjnych [18–21]. Wczesne doniesienia literaturowe wskazywały na jego szerokie spektrum aktywności biologicznej, sugerujące potencjał wielokierunkowego oddziaływania na funkcjonowanie komórek. Biorąc pod uwagę ścisłe powiązania między przewlekłymi stanami zapalnymi,

a transformacją nowotworową, mechanizmy te, mogące mieć istotne znaczenie dla regulacji odpowiedzi komórkowej, pozostają jednak słabo scharakteryzowane. Dotychczasowe doniesienia dotyczące działania przeciwnowotworowego RA są nieliczne, ograniczają się do wybranych modeli eksperymentalnych oraz wąskiego zakresu analiz biologicznych [22–24]. Uzasadnia to potrzebę dalszych badań ukierunkowanych na ocenę jego wpływu na kluczowe mechanizmy determinujące los komórek nowotworowych.



Ryc. 1. (A) Goryczka dahurska (łac. *Gentiana dahurica*), autor fotografii Robert Pavlis¹; (B) Goryczka wielkolistna (łac. *Gentiana macrophylla*), autor fotografii Todd Boland²; (C) Galasy dębowe (kuliste narośla na liściach dębu), autor Getty Images³.

¹ <https://onrockgarden.com/index.php/plant-of-the-month?view=article&id=112:gentiana-dahurica&catid=22>

² <https://www.flickr.com/photos/toddboland/7856360082>

³ <https://murator-dom.pl/ogrod/pielęgnacja-roślin/galas-na-lisciu-co-to-jest-czy-galasy-sa-szkodliwe-jak-je-zwalczac-aa-r5ZN-wp2k-anor.html>

Cel pracy

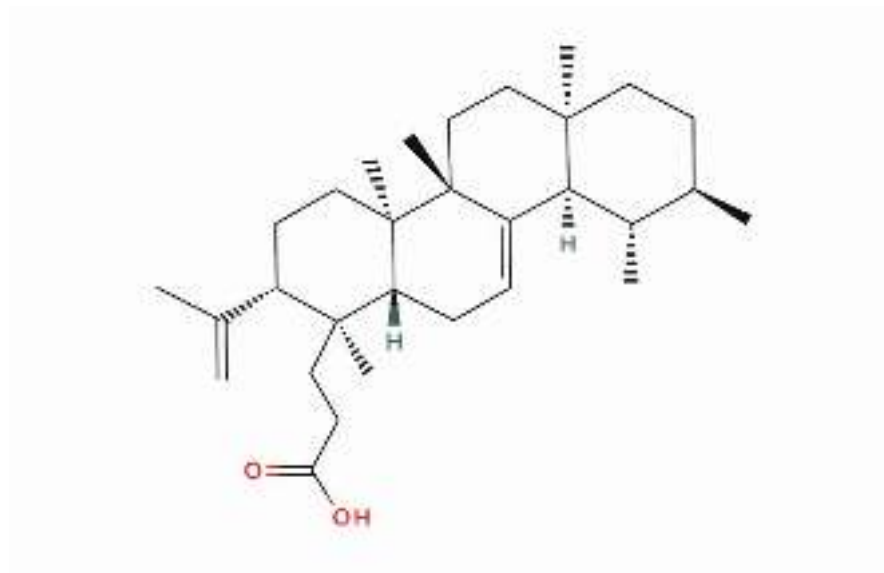
Celem niniejszej pracy była wieloaspektowa ocena aktywności biologicznej kwasu roburowego (RA), należącego do grupy tetracyklicznych triterpenoidów, w komórkach nowotworowych i prawidłowych człowieka. Szczegółowe badania przeprowadzone w układach modelowych *in vitro* miały na celu określenie wpływu RA na kluczowe mechanizmy komórkowe i wyjaśnienie/wskazanie molekularnych mechanizmów leżących u podstaw jego działania.

Hipoteza badawcza

Kwas roburowy wywiera zróżnicowany wpływ na komórki nowotworowe i prawidłowe człowieka w układzie *in vitro*, oddziałując na mechanizmy regulujące przeżycie i proliferację komórek. Właściwości te są istotne z punktu widzenia badań nad biologią nowotworów.

Materiały i metody

Badania przeprowadzono z wykorzystaniem chemicznie zdefiniowanego wzorca kwasu roburowego ($C_{30}H_{48}O_2$) o wysokiej czystości (Ryc.2). Związek rozpuszczono w dimetylosulfotlenku (DMSO), uzyskując roztwór (stock) macierzysty o stężeniu 50 mM, z którego następnie przygotowano 10 mM roztwór roboczy. W doświadczeniach zastosowano stężenia odpowiadające wartościom konsensusu 0,5 IC_{50} , IC_{50} oraz $2 \times IC_{50}$, wyznaczone na podstawie wyników testu MTT, testu z użyciem fioletu krystalicznego i testu wychwyty czerwieni obojętnej. Końcowe stężenie DMSO w podłożu hodowlanym nigdy nie przekroczyło 0,5% (v/v) [25].



Ryc. 2. Struktura chemiczna kwasu roburowego (RA). Rycina opracowana na podstawie PubChem (CID: 5317800) (publikacja nr 3).

W pracy wykorzystano komórki następujących linii nowotworowych człowieka:

- BxPC-3 (komórki gruczolaka trzustki, ang. *pancreas adenocarcinoma*, ATCC® CRL-1687™),
- DLD-1 (komórki gruczolaka jelita grubego, ang. *colorectal adenocarcinoma*, ATCC® CCL-221™),
- HCT-116 (komórki raka jelita grubego, ang. *colorectal carcinoma*, ATCC® CCL-247™),
- HT-29 (komórki gruczolaka jelita grubego, ang. *colorectal adenocarcinoma*, ATCC® HTB-38™),
- PC-3 (komórki raka prostaty, ang. *prostate cancer*, ATCC® CRL-1435™).

Z kolei ludzkie komórki prawidłowe należały do linii:

- CCD-841 CoN (komórki nabłonkowe jelita grubego, ang. *colon epithelial*, ATCC® CRL-1790™),
- NF-κB-TIME (ludzka śródbłonkowa linia reporterowa NF-κB, ang. *NF-κB-TIME human endothelial reporter cell line*, ATCC® CRL-4049™),
- WI-38 (prawidłowe fibroblasty płuc, ang. *human lung fibroblasts*, ATCC® CCL-75™).

Cel pracy został zrealizowany z wykorzystaniem następujących metod badawczych:

1. Do oceny właściwości cytotoksycznych kwasu roburowego (RA) zastosowano:
 - test MTT,
 - test z użyciem fioletu krystalicznego,
 - test wychwytu czerwieni obojętnej.
2. Potencjał genotoksyczny RA oceniono z wykorzystaniem alkalicznej wersji testu kometowego (elektroforezy pojedynczych komórek).
3. Analizę aktywności antyproliferacyjnej RA w komórkach linii nowotworowych wykonano z wykorzystaniem:
 - testu inkorporacji bromodeoksyurydyny (BrdU),
 - barwienia fluorescencyjnego z zastosowaniem przeciwciał anti-Ki67,
 - testu klonogenności (ang. *clonogenic assay*),
 - analizy gęstości i żywotności komórek z użyciem barwienia błękitem trypanu.
4. Właściwości proapoptotyczne RA oraz udział szlaków regulowanej śmierci komórkowej oceniono z wykorzystaniem:
 - analizy zmian mitochondrialnego potencjału błonowego ($\Delta\Psi_m$),
 - detekcji fosfatydyloseryny na powierzchni błony komórkowej metodą cytometrii przepływowej,
 - metody podwójnego barwienia oranżem akrydyny i bromkiem etydyny,
 - analizy aktywności kaspazy 9,
 - inhibitorów śmierci komórkowej: A-VD-OPh (apoptoza), nekrostatyna-1 (nekroptoza) oraz ferrostatyna-1 (ferroptoza).
5. Zdolności RA do indukcji stresu oksydacyjnego w badanych komórkach przeprowadzono z wykorzystaniem sondy fluorescencyjnej H₂DCF-DA (dioctanu dihydrodichlorofluoresceiny).

6. Do analizy przebiegu cyklu komórkowego i jego poszczególnych faz wykorzystano cytometr przepływowy.
7. Wpływ RA na aktywność transkrypcyjną czynnika NF- κ B oceniono z wykorzystaniem testu reporterowego lucyferazy w ludzkiej linii reporterowej NF- κ B-TIME po stymulacji prozapalnej.
8. Analizę ekspresji genów związanych z regulacją proliferacji i cyklu komórkowego przeprowadzono z zastosowaniem techniki RT-PCR.
9. Zmiany w intensywności sygnału oraz subkomórkowej lokalizacji białek regulatorowych p21 i p53 oceniono metodą immunofluorescencji.
10. Zdolność RA do indukcji senescencji komórkowej oceniono z wykorzystaniem barwienia SA- β -galaktozydazy (SA- β -Gal).
11. Do określenia parametrów fizykochemicznych, właściwości ADMETox oraz potencjalnych celów molekularnych RA wykorzystane zostały metody *in silico*.

Omówienie publikacji wchodzących w skład rozprawy doktorskiej

Rozprawę doktorską, złożoną z cyklu trzech artykułów naukowych oraz jednego manuskryptu, otwiera autorska praca przeglądowa zatytułowana ***Substances of Natural Origin in Medicine: Plants vs. Cancer*** (publikacja nr 1), której głównym celem było kompleksowe omówienie roli związków pochodzenia roślinnego w terapii przeciwnowotworowej oraz identyfikacja najbardziej perspektywicznych kierunków dalszych badań. Praca ma charakter pogłębionego i krytycznego przeglądu aktualnej literatury naukowej, obejmującego dane przedkliniczne i kliniczne, co pozwoliło na skonsolidowanie dotychczasowej wiedzy oraz opracowanie spójnej bazy teoretycznej dla realizacji celów niniejszej pracy.

W pierwszej części publikacji nr 1 omówiono problematykę chorób nowotworowych jako jedno z kluczowych wyzwań współczesnej medycyny, analizując skalę zachorowalności oraz wskazując na bariery ograniczające skuteczność obecnych schematów terapeutycznych. Dalej dokonano charakterystyki głównych czynników ryzyka rozwoju chorób nowotworowych, obejmujących zarówno predyspozycje genetyczne, jak i czynniki środowiskowe, co pozwoliło na osadzenie dalszych rozważań w szerokim kontekście biologicznym i klinicznym. Następnie opisane zostały podstawowe strategie leczenia systemowego nowotworów, koncentrując się na chemioterapii, oraz przedstawiono główne grupy leków cytostatycznych, takie jak związki alkilujące, antymetabolity, inhibitory topoizomeraz oraz inhibitory wrzeciona podziałowego. Wskazano zarówno na ich skuteczność terapeutyczną, jak i istotne ograniczenia wynikające z niskiej selektywności, toksyczności wobec prawidłowych tkanek oraz rozwoju oporności komórek nowotworowych.

W dalszej części opracowania zwrócono uwagę, że związki pochodzenia roślinnego są istotnym źródłem substancji bioaktywnych o wielokierunkowym mechanizmie działania. Naturalne metabolity roślinne mogą modulować jednocześnie wiele kluczowych szlaków sygnałowych zaangażowanych w transformację nowotworową, w tym komórkowy szlak sygnalizacyjny zależny od jądrowego czynnika transkrypcyjnego NF- κ B, szlak kinazy 3-fosfatydyloinozytolu/kinazy białkowej B (PI3K/AKT), szlak kinaz aktywowanych

mitogenami (MAPK) oraz mechanizmy odpowiedzi na uszkodzenia DNA. Wskazano na ich potencjał do indukcji różnych form programowanej śmierci komórkowej, w tym apoptozy i autofagii związanej z regulacją losu komórki, a także na możliwość wpływu na mikrośrodowisko guza i odpowiedź immunologiczną. Podkreślono również, że ponad 60% leków przeciwnowotworowych stosowanych obecnie w praktyce klinicznej wywodzi się bezpośrednio lub pośrednio ze związków pochodzenia roślinnego, co świadczy o ich ważnym znaczeniu w rozwoju nowoczesnej onkologii.

Kluczową część artykułu poświęcono analizie wybranych związków pochodzenia roślinnego. Omówiono substancje już wykorzystywane w onkologii, takie jak paklitaksel i irynotekan, skupiając się na molekularnych mechanizmach ich działania oraz strategiach zwiększania ich skuteczności, w tym zastosowaniu nowoczesnych systemów dostarczania leków. Równolegle przeanalizowano związki o wysokim potencjale terapeutycznym znajdujące się na etapie badań przedklinicznych i klinicznych, takie jak kwas betulinowy czy resweratrol, opisując ich właściwości biologiczne, aktywność przeciwnowotworową oraz aktualny status badań. Szczególną uwagę poświęcono kwasowi roburowemu (RA) jako związkowi relatywnie słabo scharakteryzowanemu, lecz wykazującemu zdolność modulowania odpowiedzi zapalnej oraz szlaków sygnałowych związanych z procesem nowotworzenia.

RA jest naturalnym triterpenoidem o szkielecie lanostanowym, czyli czteropierścieniowym układzie węglowym wywodzącym się z lanosterolu i charakterystycznym dla związków steroidopodobnych [26]. Triterpenoidy lanostanowe stanowią ważną klasę metabolitów wtórnych roślin i grzybów, którym przypisuje się szeroki zakres aktywności biologicznej, w tym właściwości przeciwzapalne, przeciwbakteryjne oraz przeciwnowotworowe [27]. Cząsteczka RA zawiera końcową grupę karboksylową oraz rozbudowany hydrofobowy szkielet węglowodorowy, co nadaje jej wyraźnie lipofilowy charakter. Taka budowa chemiczna sprzyja oddziaływaniom z dwuwarstwą fosfolipidową, ułatwiając przenikanie przez błony komórkowe oraz oddziaływanie z białkami zlokalizowanymi w ich obrębie. Dotychczasowe doniesienia wskazują, że RA charakteryzuje się działaniem przeciwwirusowym, przeciwzapalnym i hipolipemicznym. W badaniach eksperymentalnych związek powodował ograniczenie produkcji mediatorów zapalnych, takich jak tlenek azotu czy

interleukina-6, a także zahamowanie aktywności cyklooksygenaz, co potwierdza jego zdolność do modulowania kluczowych elementów odpowiedzi zapalnej [18,20]. Ponieważ przewlekła aktywacja szlaków zapalnych odgrywa istotną rolę w procesie nowotworzenia, związki wpływające na regulację tych procesów są często rozpatrywane jako potencjalne czynniki o działaniu przeciwnowotworowym. Jednocześnie dane dotyczące aktywności przeciwnowotworowej RA pozostają ograniczone. W momencie rozpoczęcia niniejszych badań, w 2022 roku, w literaturze dostępnych było zaledwie 9 publikacji oryginalnych dotyczących RA, z których jedynie 2 odnosiły się do jego potencjalnego działania przeciwnowotworowego. Pozostałe prace koncentrowały się na właściwościach przeciwzapalnych związku w kontekście leczenia takich schorzeń jak reumatoidalne zapalenie stawów, choroba Alzheimera czy nadmierna osteoklastogeneza. Przeprowadzona analiza literatury pozwoliła na identyfikację istotnych luk w wiedzy dotyczących mechanizmów działania RA, w szczególności jego wpływu na procesy proliferacji i przeżycia komórek nowotworowych oraz regulację szlaków sygnałowych w tych komórkach. Z tego względu RA został wytypowany jako główny obiekt badań eksperymentalnych, które stanowią przedmiot kolejnych publikacji wchodzących w skład niniejszej rozprawy doktorskiej. Obecnie (stan na marzec 2026) liczba prac poświęconych temu związkowi wzrosła do 19, co odzwierciedla rosnące zainteresowanie jego potencjałem biologicznym i terapeutycznym.

Badania nad podstawowymi mechanizmami działania RA opisano w oryginalnym artykule zatytułowanym ***Anticancer Activity of Roburic Acid: In Vitro and In Silico Investigation*** (publikacja nr 2). Praca poświęcona została analizie właściwości przeciwnowotworowych związku w modelach *in vitro*, ze szczególnym uwzględnieniem wpływu na proliferację komórek nowotworowych oraz modulację kluczowych szlaków sygnałowych. Badania prowadzono etapowo, co umożliwiło stopniowe pogłębianie charakterystyki działania związku, od ogólnej odpowiedzi komórkowej na RA po wielowymiarowe analizy *in silico* dotyczące właściwości farmakokinetycznych oraz identyfikacji potencjalnych celów molekularnych na podstawie modelowania interakcji ligand–białko.

W pierwszej kolejności oceniono wpływ RA na aktywność metaboliczną komórek nowotworowych (linie BxPC-3, DLD-1, HCT-116, HT-29, PC3) i prawidłowych (linie CCD-841 CoN, WI-38) z wykorzystaniem testu MTT. Analizę

przeprowadzono w dwóch układach czasowych (24- oraz 72-godzinnej ekspozycji), co umożliwiło porównanie odpowiedzi komórkowych zależnych od czasu inkubacji. Wybór panelu linii komórkowych oparto na dostępnych danych literaturowych dotyczących aktywności biologicznej związków o zbliżonej strukturze chemicznej, przy czym przedmiotem weryfikacji eksperymentalnej była selektywność działania RA. Uzyskane wyniki wskazały na zróżnicowaną, zależną od linii komórkowej oraz czasu inkubacji, odpowiedź komórek na działanie związku. RA powodował spadek intensywności sygnału (mierzony absorbancją, czyli zdolnością pochłaniania światła) korelujący ze wzrostem stężenia, co odzwierciedlało obniżenie aktywności metabolicznej komórek. Największą wrażliwość zaobserwowano w komórkach nowotworowych przewodu pokarmowego, podczas gdy komórki gruczolakoraka trzustki i raka prostaty wykazywały względnie niższą podatność na działanie związku. Na tej podstawie do dalszych analiz wybrano komórki linii raka jelita grubego DLD-1, HCT-116 oraz HT-29, a także linie komórek prawidłowych. W celu uniknięcia interpretacji opartej na pojedynczym parametrze metabolicznym, analogiczne badania przeprowadzono z wykorzystaniem testu wychwytu czerwieni obojętnej oraz barwienia fioletem krystalicznym. Na podstawie wyników każdego z zastosowanych testów wyznaczono wartości IC_{50} (stężenie związku chemicznego, które powoduje zahamowanie aktywności metabolicznej komórek o 50% w stosunku do grupy kontrolnej (komórek nietraktowanych lekiem) w określonym czasie), stanowiące ustandaryzowany parametr ilościowej oceny siły działania związku oraz umożliwiające porównywanie uzyskanych danych z doniesieniami literaturowymi. Indywidualne wartości zestawiono, a następnie wyznaczono konsensusowe IC_{50} jako średnią arytmetyczną uzyskaną z trzech niezależnych testów (Tabela 1). W kolejnych eksperymentach stosowano stężenia odpowiadające wielokrotnościom parametru IC_{50} ($0,5 IC_{50}$, IC_{50} oraz $2 \times IC_{50}$).

Tabela 1. Konsensusowe wartości IC_{50} ($\pm$ odchylenie standardowe; SD, ang. *standard deviation*), otrzymane po 24- i 72-godzinnej ekspozycji testowanych komórek linii nowotworowych i prawidłowych na kwas roburowy (RA), opracowane na podstawie wyników testu MTT, testu z użyciem fioletu krystalicznego i testu wychwytu czerwieni obojętnej.

Czas inkubacji	Linia komórkowa				
	Konsensusowe IC ₅₀ [μM] RA ± SD				
	DLD-1	HCT-116	HT-29	WI-38	CCD-841 CoN
24-h	16,23 ± 1,1	20,33 ± 2,18	22,39 ± 2,96	35,21 ± 3,23	35,25 ± 3,08
72-h	9,21 ± 1,74	12,06 ± 1,63	10,6 ± 0,77	15,32 ± 0,96	17,5 ± 2,15

Przeprowadzony etap pełnił funkcję badań przesiewowych, dobór modeli komórkowych oparto na wynikach testu MTT, natomiast wykorzystanie dodatkowych metod umożliwiło doprecyzowanie zakresu stężeń, tj. wyznaczenie konsensusowych wartości IC₅₀. Łączna analiza wykazała, że RA w sposób zależny od stężenia i czasu ekspozycji obniża sygnał w testach kolorymetrycznych, odzwierciedlających aktywność metaboliczną, integralność lizosomalną oraz masę komórkową komórek adherentnych. Jednocześnie należy zwrócić uwagę, że zastosowane metody nie pozwalały na rozróżnienie, czy obserwowane zmiany wynikały z indukcji śmierci komórek (efekt cytotoksyczny), czy z zahamowania ich proliferacji (efekt cytostatyczny).

W związku z powyższym rozszerzono zakres analiz o metody pozwalające na jednoznaczne określenie charakteru obserwowanego efektu. Zastosowano trzy uzupełniające się podejścia eksperymentalne: analizę gęstości komórek połączoną z testem wykluczenia błękitu trypanu, test inkorporacji bromodeoksyurydyny oraz test klonogenności. Badania przeprowadzono na liniach DLD-1, HCT-116 oraz HT-29, które w poprzednich analizach wykazały najwyższą responsywność na działanie badanego związku. W pierwszej kolejności oceniono żywotność komórek po ekspozycji na RA z wykorzystaniem testu wykluczenia barwnika błękitu trypanu, połączonego z oszacowaniem gęstości zawiesiny komórek. Wykazano, że związek nie powodował spadku przeżywalności komórek, ale istotnie ograniczał przyrost ich liczby, co sugerowało, że obserwowane wcześniej zmiany aktywności metabolicznej nie były konsekwencją masowej cytotoksyczności, lecz najprawdopodobniej wynikały z działania cytostatycznego. Dalszego potwierdzenia tej hipotezy dostarczyły wyniki testu inkorporacji bromodeoksyurydyny, uznawanej za „złoty standard” w badaniach nad proliferacją, które wskazywały na zahamowanie syntezy DNA w komórkach nowotworowych oraz ograniczenie ich zdolności do

podziałów. Ponadto, w przeprowadzonym eksperymencie jako kontrolę pozytywną zastosowano oksaliplatynę (15 μ M), której działanie okazało się być porównywalne, a w przypadku komórek linii HT-29 słabsze niż obserwowane dla RA. Uzupełnieniem krótkoterminowych analiz proliferacyjnych była ocena długoterminowego (10-dniowego) potencjału wzrostowego w teście klonogenności. Wykazano w nim istotne ograniczenie zdolności pojedynczych komórek do tworzenia kolonii po ekspozycji na RA, świadczące o upośledzeniu ich potencjału proliferacyjnego. Uzyskane wyniki pozwoliły jednoznacznie potwierdzić cytostatyczny charakter działania związku, który wynika z zahamowania podziałów komórkowych, a nie z bezpośredniej indukcji śmierci komórkowej. Uzasadnia to dalsze ukierunkowanie badań na identyfikację molekularnych mechanizmów odpowiedzialnych za obserwowane efekty antyproliferacyjne.

Ze względu na kluczową rolę szlaku NF- κ B w regulacji proliferacji i przeżycia komórek nowotworowych oraz odpowiedzi zapalnej, w kolejnym etapie oceniono wpływ RA na aktywność transkrypcyjną tego szlaku z wykorzystaniem testu reporterowego opartego na aktywności lucyferazy. Analizę przeprowadzono z użyciem dedykowanej linii komórkowej stabilnie transfekowanej konstrukcjami reporterowymi, umożliwiającymi ilościowy pomiar aktywności NF- κ B. Wykazano, że RA hamuje aktywację NF- κ B indukowaną czynnikiem martwicy nowotworów α (TNF- α), co może częściowo wyjaśniać jego cytostatyczny charakter działania. Wynik ten pozostaje spójny z wcześniejszymi doniesieniami literaturowymi sugerującymi możliwość oddziaływania RA z TNF- α , co potencjalnie mogłoby prowadzić do zaburzenia jego interakcji z receptorem i ograniczenia aktywacji NF- κ B.

Ostatnim etapem pracy była ocena właściwości farmakokinetycznych RA oraz jego potencjału jako kandydata terapeutycznego z wykorzystaniem narzędzi *in silico*. W badaniach uwzględniono parametry absorpcji, dystrybucji, metabolizmu, wydalania oraz toksyczności (ADMET), oceniono zgodność z kryteriami *drug-likeness* (podobieństwa do leków), a także przeprowadzono predykcję potencjalnych celów molekularnych wraz z dokowaniem molekularnym oraz symulacją dynamiki molekularnej. Analiza ADMET wskazała na korzystny profil farmakokinetyczny badanego związku, w tym przewidywaną akceptowalną biodostępność oraz brak istotnych sygnałów toksykologicznych w modelach

predykcyjnych. Zgodność z regułą Lipińskiego oraz dodatkowymi kryteriami *drug-likeness* sugeruje, że RA spełnia podstawowe wymagania stawiane małącząsteczkowym kandydatom na leki.

Potencjalne cele molekularne wytypowano na podstawie dostępnych online narzędzi predykcyjnych wykorzystujących podobieństwo chemiczne do znanych ligandów. Algorytmy porównały strukturę RA z bazami danych zawierającymi informacje o cząsteczkach o znanym powinowactwie do określonych białek, umożliwiając wskazanie potencjalnych interakcji. Następnie zweryfikowano znaczenie biologiczne wytypowanych białek w kontekście patogenezy nowotworów, co pozwoliło na wybór kilkunastu struktur do dalszej analizy. Dokowanie molekularne stanowiło komputerową symulację procesu wiązania cząsteczki RA w obrębie miejsca aktywnego wybranych białek. Metoda ta pozwoliła na ocenę przewidywanego powinowactwa ligandu do receptora, jego orientacji w kieszeni wiążącej oraz rodzaju tworzonych oddziaływań, takich jak wiązania wodorowe czy interakcje hydrofobowe. Spośród wszystkich analizowanych białek najkorzystniejsze wyniki wiązania (najniższe przewidywane energie wiązania) uzyskano dla anhidrazy węglanowej II (CAII), karboksylesterazy 1 (CES1), receptora nabłonkowego czynnika wzrostu (EGFR) oraz fosfolipazy A2 (PLA2G2A). Należy podkreślić, że dokowanie nie stanowi dowodu rzeczywistego wiązania RA z analizowanymi białkami, ale jest narzędziem predykcyjnym, pozwalającym ocenić, czy dana interakcja jest strukturalnie i energetycznie możliwa. W niniejszej pracy analiza ta miała na celu zawężenie przestrzeni hipotez co do potencjalnych celów molekularnych oraz zbudowanie mechanistycznej spójności pracy, wskazując, czy takie interakcje są strukturalnie możliwe w kontekście obserwowanych efektów biologicznych. Dokowanie dało racjonalną podstawę świadczącą o tym, że wybór celów do walidacji nie jest przypadkowy.

Spośród analizowanych białek szczególną uwagę zwrócił kompleks RA-CES1, który charakteryzował się najkorzystniejszą wartością energii wiązania w analizie dokowania molekularnego. Przewidywana orientacja ligandu w obrębie kieszeni aktywnej wskazywała na możliwość tworzenia stabilnych interakcji z kluczowymi resztami aminokwasowymi miejsca katalitycznego. Symulacja dynamiki molekularnej również wykazała wysoką stabilność kompleksu, bez istotnych zaburzeń konformacyjnych białka ani utraty kontaktu

ligandu z miejscem wiążącym. Uzyskane parametry stabilności strukturalnej sugerują, że interakcja ta może być energetycznie korzystna w warunkach modelowych. CES1 jest enzymem hydrolitycznym zaangażowanym w metabolizm ksenobiotyków oraz regulację przemian lipidowych [28–30]. Choć nie należy do klasycznych regulatorów proliferacji komórkowej ani bezpośrednich komponentów szlaków sygnałowych związanych z transformacją nowotworową, jego rola w modulowaniu metabolizmu lipidów oraz farmakokinetyki związków bioaktywnych może pośrednio wpływać na środowisko metaboliczne komórek nowotworowych. W tym kontekście stabilność kompleksu RA-CES1 uzyskana w analizach *in silico* wskazuje na potencjalny, choć wymagający dalszej weryfikacji, kierunek badań mechanistycznych.

Publikacja nr 2 stanowi pierwszą wielopoziomową ocenę przeciwnowotworowej aktywności RA w modelach *in vitro*. Zastosowane komplementarne testy biologiczne wykazały, że związek działa w sposób zależny od stężenia i czasu ekspozycji, a jego cytostatyczny charakter związany jest z zahamowaniem proliferacji komórek nowotworowych. Analiza wartości IC₅₀ ujawniła zróżnicowaną responsywność badanych linii komórkowych, przy czym szczególną wrażliwość obserwowano w komórkach raka jelita grubego. Działanie RA może być związane z modulacją szlaków sygnałowych regulujących proliferację komórek, w tym szlaku NF-κB, którego aktywność ulegała redukcji w warunkach stymulacji TNF-α. Badania eksperymentalne uzupełniono analizami *in silico*, mającymi charakter hipotezotwórczy, które pozwoliły na wstępne wskazanie potencjalnych celów molekularnych oraz ocenę właściwości farmakokinetycznych badanego związku. Wyniki te poszerzają dotychczasową wiedzę na temat przeciwnowotworowej aktywności RA oraz stanowią podstawę do dalszych badań nad molekularnymi mechanizmami jego działania.

Trzecia publikacja zatytułowana ***Roburic Acid as a Therapeutic Candidate: Antiproliferative Activity and Secondary Cell Death Response in Colorectal Cancer Cells*** (publikacja nr 3) stanowi pogłębioną analizę mechanizmu działania RA w komórkach raka jelita grubego, rozwijając wnioski sformułowane w poprzednim opracowaniu. Po wykazaniu jego cytostatycznego charakteru oraz zdolności do modulowania aktywności szlaku NF-κB, kolejnym krokiem było określenie, które procesy i komponenty białkowe są bezpośrednio

zaangażowane w odpowiedź komórkową na działanie związku oraz w jaki sposób przekłada się to na obserwowane ograniczenie proliferacji.

Badania rozpoczęto od immunofluorescencyjnej analizy markera proliferacji, antygenu Ki-67 (Ki-67), obecnego wyłącznie w komórkach przechodzących przez fazy G₁, S, G₂ i M, a nieobecnego w fazie spoczynkowej G₀. Ekspozycja badanych komórek na RA prowadziła do istotnego obniżenia poziomu białka Ki-67, co wskazuje na zmniejszenie frakcji komórek aktywnie się dzielących. Równocześnie analiza cytometryczna rozkładu poszczególnych faz cyklu komórkowego wykazała akumulację komórek w fazie G₀/G₁ oraz redukcję populacji komórek w fazie S, potwierdzając zahamowanie progresji cyklu komórkowego na etapie przejścia przez punkt restrykcyjny G₁/S, czyli przed rozpoczęciem syntezy DNA. Aby powiązać obserwowane zmiany z regulacją na poziomie molekularnym, przeanalizowano ekspresję kilku genów związanych z proliferacją i kontrolą cyklu komórkowego metodą RT-qPCR. Do badania wybrano: gen kodujący marker proliferacji Ki-67 (*MKI67*), jądrowy antygen komórek proliferujących (*PCNA*), gen inhibitora kinaz zależnych od cyklin 1A (*CDKN1A*) oraz gen supresorowy kodujący białko p53 (*TP53*). Analiza RT-qPCR wykazała istotne obniżenie poziomu transkryptów *MKI67* oraz *PCNA*, potwierdzające ograniczenie aktywności proliferacyjnej i kompetencji replikacyjnej komórek inkubowanych z RA. Podkreśla to spójność otrzymanych wyników zarówno na poziomie transkryptu, jak i białka. Kolejnym istotnym elementem odpowiedzi molekularnej był wzrost ekspresji genu *CDKN1A*, którego białko inhibitor kinaz zależnych od cyklin p21 (p21) wiąże się z kompleksem kinaza zależna od cykliny 2 (CDK2)-cyklina E/A, blokując jego aktywność i uniemożliwiając przejście przez punkt kontrolny G₁/S, w konsekwencji powodując „areszt” w fazie G₀/G₁ [31–33]. Zmiany w ekspresji genu *TP53* miały charakter zależny od linii komórkowej i nie korelowały bezpośrednio z indukcją *CDKN1A*, co sugeruje możliwość częściowej aktywacji p21 niezależnie od *TP53*. Warto podkreślić, że analizowane linie komórkowe różniły się statusem mutacyjnym *TP53* – HCT-116 zachowuje allel typu dzikiego, podczas gdy DLD-1 i HT-29 posiadają mutacje w obrębie tego genu – co może tłumaczyć zróżnicowany profil odpowiedzi transkrypcyjnej [34,35]. Brak jednoznacznej korelacji pomiędzy zmianami w ekspresji *TP53* a indukcją *CDKN1A* sugeruje, że synteza białka p21 może zachodzić przynajmniej częściowo niezależnie od

klasycznej osi *TP53*. W tym kontekście mechanistycznie istotna wydaje się zdolność RA do hamowania szlaku NF- κ B, który promuje sygnały pro-proliferacyjne i pro-przeżyciowe. Supresja aktywności NF- κ B może zatem sprzyjać utrwaleniu p21-zależnego zatrzymania cyklu komórkowego, niezależnie od statusu *TP53*.

Uzyskane wyniki pozostają zgodne z doniesieniami innych badaczy wskazującymi, że RA obniża ekspresję cyklin D1, E1 i B1 oraz czynnika transkrypcyjnego Myc (c-Myc), ograniczając sygnały promujące proliferację [22]. Xu i in. wykazali także, że RA hamuje aktywację szlaków MAPK i PI3K/AKT w warunkach stymulacji TNF- α , zmniejszając fosforylację ERK, kinazy białkowej aktywowanej stresem białka p38 (p38), kinazy AKT oraz przekaźnika sygnału i aktywatora transkrypcji 3 (STAT3), a także blokuje fosforylację inhibitora κ B α (I κ B α) przez kinazę inhibitorową κ B (IKK), co prowadzi do zahamowania aktywacji osi IKK/I κ B α /NF- κ B [22]. Choć w literaturze istnieją rozbieżności dotyczące wpływu RA na modulację szlaku ERK, hamowanie sygnalizacji NF- κ B oraz indukcja *CDKN1A* stanowią bardziej konsekwentnie opisywany element mechanizmu ograniczania sygnałów proliferacyjnych i zatrzymania cyklu komórkowego [18,22].

Niezależnie od dominującego efektu cytostatycznego, w analizie rozkładu faz cyklu komórkowego zaobserwowano również obecność frakcji sub-G₁, szczególnie widoczną po dłuższej ekspozycji i przy wyższych stężeniach RA. Jej pojawienie się sugerowało możliwość występowania fragmentacji DNA w części populacji komórek. W związku z tym przeprowadzono ocenę indukcji apoptozy z wykorzystaniem testu wiązania aneksyny V znakowanej izotiocyanianem fluoresceiny (FITC)/jodkiem propidyny (PI), umożliwiającego detekcję wczesnej eksternalizacji fosfatydyloseryny, oraz metodą podwójnego barwienia oranżem akrydyny/bromkiem etydydy (AO/EB), pozwalającą na ocenę morfologicznych cech apoptozy. Analiza cytometryczna wykazała istotny wzrost odsetka komórek apoptotycznych w liniach nowotworowych DLD-1 i HCT-116 po 24 godzinach wyłącznie przy najwyższym stężeniu ($2 \times IC_{50}$), odpowiadającym warunkom subtoksycznym, wykraczającym poza zakres typowo cytostatyczny. Po wydłużeniu czasu ekspozycji efekt ten ulegał nasileniu przy stężeniu odpowiadającym $2 \times IC_{50}$ ponad 50% komórek w każdej z analizowanych linii nowotworowych wykazywało cechy apoptozy, a w wybranych modelach wzrost

frakcji apoptotycznej obserwowano również przy stężeniu równym wyznaczonej wartości IC_{50} . Wyniki uzyskane metodą podwójnego barwienia również potwierdziły występowanie morfologicznych cech apoptozy w badanych komórkach głównie w warunkach długotrwałej ekspozycji i przy najwyższych stężeniach, przy czym odsetek komórek apoptotycznych był niższy niż w poprzedniej analizie. Rozbieżność ta wynika z odmiennych procesów biologicznych wykrywanych przez zastosowane metody. Podczas gdy test aneksyny V/FITC pozwala na detekcję wczesnej eksternalizacji fosfatydyloseryny, barwienie AO/EB identyfikuje komórki znajdujące się w bardziej zaawansowanych stadiach procesu i charakteryzujące się wyraźnymi zmianami morfologicznymi. Tym samym obecność komórek dodatnich dla aneksyny V nie musi oznaczać nieodwracalnego zaangażowania w egzekucję apoptozy.

Dodatkowo, przeprowadzona analiza zmian potencjału błony mitochondrialnej wykazała jego istotne obniżenie po zastosowaniu praktycznie wszystkich analizowanych stężeń RA, co wskazuje, że zaburzenia homeostazy mitochondrialnej stanowią wczesny element odpowiedzi komórkowej na związek. Wyraźna indukcja apoptozy obserwowana była przy najwyższych zastosowanych stężeniach RA i po dłuższej ekspozycji komórek na jego działanie, sugerując, że sama depolaryzacja mitochondriów nie jest równoznaczna z natychmiastowym uruchomieniem programu śmierci komórkowej. Co istotne, nawet w warunkach długotrwałego działania RA na badane komórki poziom frakcji nekrotycznej pozostawał niski i ten rodzaj śmierci komórkowej nie dominował w komórkach żadnej z badanych linii, co wskazuje, że RA nie wywołuje niespecyficznego uszkodzenia błon komórkowych. Warto też uwzględnić opisywaną wcześniej zdolność RA do modulowania sygnalizacji $TNF-\alpha$, który aktywuje zarówno $NF-\kappa B$, jak i mechanizmy zależne od receptorów śmierci. Jego hamowanie przez RA może ograniczać udział procesu apoptozy poprzez szlak zewnętrzny. Jednocześnie obserwowany spadek potencjału błony mitochondrialnej oraz pojawienie się cech apoptozy pozwalają przypuszczać, że w analizowanych warunkach dominującą rolę mogła odgrywać ścieżka mitochondrialna ww. procesu. Uzyskane wyniki sugerują, że RA działa przede wszystkim cytostatycznie, natomiast cechy apoptozy pojawiają się wtórnie —

w warunkach przekraczających adaptacyjne możliwości komórek — jako konsekwencja utrzymującego się stresu proliferacyjnego i mitochondrialnego.

Do nieco odmiennych wniosków doszli Xu i wsp., którzy w komórkach raka jelita grubego (linie nowotworowe HCT-116 oraz HCT-115) zaobserwowali istotny wzrost odsetka komórek apoptotycznych już przy stężeniu 4 μM RA (odpowiadającym w naszym modelu około 0,25 IC_{50}) i interpretowali działanie związku jako cytotoksyczne [22]. Warto jednak podkreślić, że w cytowanym badaniu eksperymenty prowadzono w innych warunkach hodowli, w tym pozbawionym surowicy medium, co może ograniczać dostęp do czynników wzrostowych i sygnałów pro-przeżyciowych, tym samym obniżając próg aktywacji apoptozy. Dodatkowo w linii nowotworowej HCT-115 odsetek komórek apoptotycznych w warunkach kontrolnych był relatywnie wysoki (prawie 20%), co wskazuje na podwyższony poziom bazowego stresu komórkowego i co może wpływać na dynamikę odpowiedzi na RA. Jednak z uwagi na różnice w profilu biologicznym analizowanych linii komórkowych oraz zastosowanych warunkach eksperymentalnych bezpośrednie zestawianie wyników obu badań nie jest w pełni zasadne. Dane te proponujemy traktować jako komplementarne, odzwierciedlające kontekstową zmienność odpowiedzi komórkowej na związek.

Dotychczasowe doniesienia dotyczące aktywności przeciwnowotworowej RA koncentrowały się głównie na jego wpływie na tempo proliferacji komórek oraz indukcję apoptozy. W literaturze brakowało jednak danych dotyczących potencjalnego działania genotoksycznego oraz wpływu związku na równowagę oksydacyjno-redukcyjną komórek. W celu uzupełnienia tej luki przeprowadzono analizę uszkodzeń DNA z wykorzystaniem alkalicznej wersji elektroforezy pojedynczych komórek, nazywanej też testem kometowym. Badanie to pozwoliło ocenić, czy związek może bezpośrednio uszkadzać materiał genetyczny. Równolegle oceniono poziom powstawania reaktywnych form tlenu z zastosowaniem fluorescencyjnej sondy umożliwiającej detekcję zmian w potencjale oksydacyjnym komórek. Wyniki wykazały, że w analizowanych warunkach RA nie działał genotoksycznie na materiał genetyczny badanych komórek ani nie powodował wyraźnego efektu prooksydacyjnego, co jedynie utwierdza przekonanie, że jego aktywność przeciwnowotworowa nie wynika z bezpośredniego uszkodzenia DNA, lecz z modulacji procesów proliferacyjnych i sygnalizacyjnych. Zgodnie z dostępną wiedzą jest to pierwsze badanie

ocenijące potencjał genotoksyczny oraz prooksydacyjny RA w modelach raka jelita grubego.

Ostatni etap badań, stanowiący przedmiot manuskryptu zatytuowanego **Revisiting Apoptosis: What Lies Behind the Antiproliferative Activity of Roburic Acid in Colorectal Cancer Cells? (manuskrypt nr 1)**, koncentruje się na dalszym pogłębieniu analizy mechanizmu działania RA poprzez uwzględnienie dodatkowych aspektów odpowiedzi komórkowej, w tym indukcji senescencji, udziału różnych form regulowanej śmierci komórkowej oraz zmian w poziomie i lokalizacji białek regulatorowych p21 i p53.

W pierwszej kolejności oceniono zdolność RA do indukcji senescencji, uwzględniając fakt, że związki o działaniu cytostatycznym mogą prowadzić do trwałego zatrzymania cyklu komórkowego i przejścia komórek w stan starzenia, któremu towarzyszą zmiany funkcjonalne i fenotypowe [36]. W tym celu zastosowano barwienie SA- β -galaktozydazy (SA- β -Gal), które umożliwia identyfikację komórek senescentnych na podstawie zwiększonej aktywności lizosomalnej β -galaktozydazy w warunkach suboptymalnego pH 6,0 [37–39]. Uzyskane wyniki wykazały, że RA nie indukował fenotypu starzeniowego, podczas gdy w populacji komórek traktowanych dokсорubicyną, zastosowaną jako kontrola pozytywna, obserwowano wyraźny wzrost odsetka komórek dodatnich dla SA- β -Gal. Co istotne, po usunięciu RA komórki odzyskiwały zdolność do podziału, co wskazuje, że obserwowane zatrzymanie cyklu komórkowego ma charakter odwracalny i nie prowadzi do trwałego upośledzenia funkcji proliferacyjnych.

Wcześniejsze obserwacje wskazujące na obecność zmian o charakterze apoptotycznym, nie pozwalały jednoznacznie określić znaczenia tego procesu w mechanizmie działania RA. Skłoniło nas to do podjęcia próby bardziej szczegółowej weryfikacji, w tym zaangażowania różnych form regulowanej śmierci komórkowej. W tym celu zastosowano podejście farmakologiczne z wykorzystaniem inhibitorów apoptozy (Q-VD-OPh), nekroptozy (nekrostatyna-1) oraz ferroptozy (ferrostatyna-1) w kombinacji z RA w stężeniu odpowiadającym wartości IC₅₀. Uzyskane wyniki wykazały, że zahamowanie poszczególnych szlaków nie prowadziło do istotnego zniesienia obserwowanego efektu spadku aktywności metabolicznej komórek, sugerując, że żadna z analizowanych form śmierci komórkowej nie stanowi dominującego mechanizmu odpowiedzi na

działanie RA. Brak wpływu inhibitorów ferroptozy i nekroptozy pozostaje spójny z wcześniejszymi obserwacjami wskazującymi na modulację sygnalizacji TNF- α przez RA oraz brak istotnego wzrostu poziomu reaktywnych form tlenu. Jednak wyniki uzyskane z zastosowaniem inhibitora apoptozy również nie potwierdziły jego udziału w mechanizmie działania RA. Równocześnie analiza aktywności kaspazy 9 wykazała jej wzrost w warunkach odpowiadających wartości IC₅₀ w komórkach linii HCT-116 i HT-29, wskazując na zaangażowanie szlaku wewnętrznego apoptozy. W kontekście obserwowanego spadku potencjału błony mitochondrialnej sugeruje to, że sygnalizacja mitochondrialna może uczestniczyć w inicjacji odpowiedzi apoptotycznej, jednak jej aktywacja ma charakter ograniczony i zależny od warunków ekspozycji.

Uzupełnieniem wcześniejszych analiz na poziomie transkryptu, obejmujących m.in. geny *CDKN1A* oraz *TP53*, była analiza immunofluorescencyjna białek p21 i p53, pozwalająca nie tylko na ocenę intensywności sygnału, lecz również dystrybucji tych białek pomiędzy jądrem a cytoplazmą. Zróżnicowany status mutacyjny *TP53* stanowił istotny kontekst interpretacyjny dla zaobserwowanych zmian, przekładając się na wyraźną zależność odpowiedzi od linii komórkowej. W komórkach linii HCT-116, posiadającej funkcjonalny allel *TP53* typu dzikiego, mimo obserwowanego spadku białka w lokalizacji jądrowej, można zakładać częściowe zachowanie aktywności p53. Natomiast w komórkach linii DLD-1 oraz HT-29, charakteryzujących się obecnością mutacji w obrębie *TP53*, widoczne było wyraźne przesunięcie sygnału w kierunku cytoplazmatycznym, zgodne z zaburzoną funkcją p53. Co istotne, w komórkach HT-29 w warunkach działania doksorubicyny również nie obserwowano typowej akumulacji jądrowej p53, co dodatkowo potwierdza ograniczoną funkcjonalność szlaku w tej linii. Odmienne profile odpowiedzi dotyczyły białka p21. W komórkach linii HT-29 obserwowano wzrost udziału sygnału jądrowego po ekspozycji na RA, podczas gdy w warunkach działania doksorubicyny dominowała jego lokalizacja cytoplazmatyczna. W pozostałych liniach komórkowych dystrybucja p21 była bardziej zrównoważona lub przesunięta w kierunku cytoplazmatycznym. Uzyskane wyniki wskazują, że odpowiedź komórkowa na RA przejawia bardzo złożony charakter. W szczególności brak jednoznacznej, spójnej aktywacji osi p53–p21 w ujęciu jądrowym sugeruje, że działanie RA nie polega na indukcji

klasycznej odpowiedzi typu „DNA damage response”. Jednocześnie obserwowane zmiany lokalizacji p21, zwłaszcza jego jądrowa akumulacja w komórkach linii HT-29, wskazują na alternatywne mechanizmy regulacji cyklu komórkowego, częściowo niezależne od funkcjonalnego p53.

Podsumowując, proponowany mechanizm działania RA obejmuje wielopoziomową modulację szlaków proliferacyjnych i pro-przeżyciowych. Po pierwsze, RA ogranicza aktywację osi $\text{TNF-}\alpha/\text{IKK/I}\kappa\text{B}\alpha/\text{NF-}\kappa\text{B}$, co zmniejsza sygnalizację sprzyjającą proliferacji komórek. Po drugie, zgodnie z doniesieniami literaturowymi, RA może tłumić aktywację wybranych szlaków MAPK, a w części modeli także ERK, oraz modulować elementy pośredniczące w przekazywaniu sygnału, co dodatkowo ogranicza ekspresję genów proliferacyjnych. Po trzecie, na poziomie efektorowym skutkuje to zatrzymaniem cyklu komórkowego w fazie G_0/G_1 , które, jak wskazują uzyskane wyniki, może zachodzić częściowo niezależnie od osi p53-p21 i wynikać zarówno z zaburzenia aktywności kompleksów CDK-cyklina, jak i z osłabienia sygnałów proliferacyjnych. Jednocześnie brak jednoznacznej, spójnej aktywacji jądrowej p53 i zróżnicowana, zależna od linii komórkowej lokalizacja p21 wskazują, że klasyczna odpowiedź na uszkodzenie nie stanowi dominującego mechanizmu działania RA. W szczególności obserwowana w części modeli przewaga cytoplazmatycznej lokalizacji tych białek sugeruje raczej ograniczenie ich funkcji transkrypcyjnej i udział alternatywnych szlaków regulacyjnych. Po czwarte, zauważa się istotne zaangażowanie mitochondriów jako wczesnego elementu odpowiedzi komórkowej na RA, co potwierdzają obserwowany spadek potencjału błony mitochondrialnej i aktywacja kaspazy 9. Jednakże brak efektu ochronnego po zastosowaniu inhibitorów regulowanej śmierci komórkowej oraz ograniczona aktywacja apoptozy w warunkach odpowiadających IC_{50} sugerują, że zaburzenia funkcji mitochondriów nie przekładają się bezpośrednio na natychmiastowe uruchomienie programu śmierci komórkowej. Dalej, analizy *in silico* wskazują na możliwe oddziaływanie RA z CES1, enzymem związanym z metabolizmem lipidów, co hipotetycznie mogłoby zaburzać utlenianie kwasów tłuszczowych i dostępność energii, wzmacniając efekt antyproliferacyjny [29,40,41]. Wreszcie, obserwowany profil odpowiedzi, obejmujący dominującą cytostazę oraz ujawnianie się cech apoptozy przy wyższych stężeniach i dłuższej ekspozycji, wskazuje, że RA działa bardziej jako modulator szlaków regulacyjnych

i metabolizmu komórki niż jako klasyczny związek o ostrej cytotoksyczności. Mechanizm jego działania można zatem opisać jako „pierwotna cytostaza, wtórna apoptoza”, co może wynikać z osłabienia sygnałów pro-przeżyciowych oraz narastającej dysfunkcji mitochondrialnej, a nie z bezpośredniego uszkodzenia DNA. Tym samym działanie RA wykazuje podobieństwo do związków o charakterze cytostatycznym, tj. metforminy czy rapamycyny [42–45].

Wnioski

1. Kwas roburowy wykazuje istotną aktywność antyproliferacyjną wobec komórek raka jelita grubego *in vitro*, działając w sposób zależny od stężenia i czasu ekspozycji.
2. Przy stężeniach odpowiadających konsensusowym wartościom IC₅₀ RA wywiera przede wszystkim efekt cytostatyczny, a nie cytotoksyczny.
3. Kwas roburowy powoduje zatrzymanie cyklu komórkowego w fazie G₀/G₁, czemu towarzyszy spadek ekspresji markerów proliferacji *MKI67*, *PCNA* oraz obniżenie poziomu białka Ki-67.
4. Wzrost ekspresji *CDKN1A* oraz zmiany poziomu i lokalizacji białka p21 stanowią molekularny korelat zatrzymania cyklu komórkowego, przy czym mechanizm ten może zachodzić częściowo niezależnie od funkcjonalnej osi p53.
5. Analiza immunofluorescencyjna wykazała, że subkomórkowa lokalizacja p21 i p53 ma charakter zależny od linii komórkowej i statusu mutacyjnego *TP53*, co wpływa na ich funkcję biologiczną. Dominacja sygnału cytoplazmatycznego wskazuje na brak klasycznej aktywacji jądrowej i ograniczenie funkcji transkrypcyjnych tych białek.

6. Kwas roburowy hamuje aktywność transkrypcyjną szlaku NF- κ B, co wskazuje na jego udział w ograniczaniu sygnałów pro-proliferacyjnych i pro-przeżyciowych.
7. Zaburzenie potencjału błony mitochondrialnej stanowi wczesny element odpowiedzi komórkowej na RA, jednak depolaryzacja mitochondriów nie prowadzi do natychmiastowej aktywacji apoptozy.
8. Apoptoza indukowana przez RA ma charakter wtórny, zależny od czasu i dawki, pojawia się głównie przy wyższych stężeniach ($2 \times IC_{50}$) lub po wydłużonej ekspozycji i obejmuje jedynie część populacji komórek.
9. Brak istotnego efektu ochronnego po zastosowaniu inhibitorów apoptozy, nekroptozy i ferroptozy wskazuje, że żadna z tych form regulowanej śmierci komórkowej nie stanowi dominującego mechanizmu działania RA, a obserwowana apoptoza ma charakter ograniczony.
10. Aktywacja kaspazy 9 sugeruje zaangażowanie mitochondrialnego (wewnętrznego) szlaku apoptozy, jednak jego rola jest wtórna i nie determinuje głównego efektu biologicznego związku.
11. Kwas roburowy nie wykazuje istotnego działania genotoksycznego ani prooksydacyjnego w analizowanych warunkach, co wskazuje, że jego aktywność przeciwnowotworowa nie wynika z bezpośredniego uszkodzenia DNA, ani indukcji stresu oksydacyjnego.
12. Mechanizm działania RA ma charakter wielopoziomowy i obejmuje przede wszystkim zahamowanie sygnalizacji pro-przeżyciowej (m.in. NF- κ B), zatrzymanie cyklu komórkowego oraz wtórne zaburzenie funkcji mitochondrialnych, prowadzące do ograniczonej aktywacji apoptozy.

Streszczenie w języku polskim

Związki pochodzenia roślinnego stanowią bogate źródło substancji o potencjale terapeutycznym, zdolnych do wielokierunkowej modulacji szlaków regulujących proliferację, przeżycie oraz procesy zapalne. Ponieważ przewlekły stan zapalny pozostaje w ścisłym związku z transformacją nowotworową, jego regulacja może odgrywać istotną rolę w kontroli nowotworzenia. Kwas roburowy (RA), tetracykliczny triterpenoid pochodzenia roślinnego, był dotychczas analizowany głównie w kontekście aktywności przeciwzapalnej, podczas gdy jego potencjał przeciwnowotworowy pozostaje słabo poznany. Celem niniejszych badań była zatem kompleksowa ocena właściwości biologicznych RA w modelach raka jelita grubego oraz identyfikacja mechanizmów molekularnych odpowiedzialnych za jego działanie.

Analizy żywotności *in vitro* wykazały, że związek w sposób zależny od stężenia i czasu ekspozycji obniża aktywność metaboliczną komórek nowotworowych. Uzupełniające testy proliferacji, obejmujące test inkorporacji bromodeoksyurydyny oraz analizę klonogenności, potwierdziły ograniczenie syntezy DNA i potencjału klonalnego, wskazując na cytostatyczny, a nie cytotoksyczny charakter działania RA. Zatrzymaniu proliferacji towarzyszyła akumulacja komórek w fazie G₀/G₁ cyklu komórkowego, spadek ekspresji markerów proliferacji (*MKI67*, *PCNA*) oraz obniżenie poziomu białka Ki-67. Jednocześnie obserwowano wzrost ekspresji *CDKN1A* oraz zmiany poziomu i lokalizacji białka p21, częściowo niezależne od osi p53, co wskazuje na alternatywne mechanizmy regulacji cyklu komórkowego. Wykazano również hamowanie aktywności transkrypcyjnej szlaku NF-κB indukowanej TNF-α, co wskazuje na udział osi TNF-α/NF-κB w ograniczaniu sygnałów pro-proliferacyjnych i pro-przeżyciowych.

Analiza potencjału błony mitochondrialnej ujawniła jego obniżenie już we wczesnych etapach ekspozycji, jednak wyraźna indukcja apoptozy występowała głównie przy najwyższych stężeniach i po wydłużonym czasie inkubacji, bez istotnego wzrostu nekrozy. Dodatkowo brak jednoznacznego efektu po zastosowaniu inhibitorów regulowanej śmierci komórkowej wskazuje, że apoptoza nie stanowi dominującego mechanizmu działania RA, a jego aktywacja ma charakter wtórny. Obserwowana aktywacja kaspazy 9 sugeruje udział mitochondrialnego szlaku apoptozy. Badania genotoksyczności oraz analiza

poziomu reaktywnych form tlenu nie wykazały istotnego uszkodzenia DNA ani działania prooksydacyjnego. Natomiast, analizy *in silico* wskazały na korzystny profil farmakokinetyczny RA oraz zidentyfikowały potencjalne cele molekularne związane z regulacją metabolizmu lipidów, co sugeruje możliwy udział mechanizmów metabolicznych w obserwowanym efekcie antyproliferacyjnym.

Uzyskane wyniki wskazują, że RA działa jako wielopoziomowy modulator szlaków regulujących proliferację i przeżycie komórek nowotworowych, wywołując przede wszystkim efekt cytostatyczny poprzez hamowanie osi TNF- α /NF- κ B oraz zatrzymanie cyklu komórkowego w fazie G₀/G₁, z wtórną aktywacją mechanizmów apoptotycznych zależnych od mitochondriów.

Streszczenie w języku angielskim

Plant-derived compounds constitute a rich source of substances with therapeutic potential, capable of multi-target modulation of pathways regulating cell proliferation, survival, and inflammatory processes. Since chronic inflammation is closely associated with tumorigenesis, its regulation may play a key role in cancer control. Roburic acid (RA), a plant-derived tetracyclic triterpenoid, has so far been investigated mainly for its anti-inflammatory activity, while its anticancer potential remains poorly characterized. The present study aimed to comprehensively evaluate the biological properties of RA in colorectal cancer models and to identify the molecular mechanisms underlying its activity.

In vitro viability assays demonstrated that RA reduced cancer cell metabolic activity in a concentration- and time-dependent manner. Complementary proliferation analyses, including bromodeoxyuridine incorporation and clonogenic assays, confirmed a reduction in DNA synthesis and clonogenic potential, indicating a cytostatic rather than cytotoxic mode of action. Inhibition of proliferation was associated with cell cycle arrest in the G₀/G₁ phase, decreased expression of proliferation markers (*MKI67*, *PCNA*), and reduced levels of the Ki-67 protein. At the same time, increased expression of *CDKN1A* and changes in the level and subcellular localization of p21 were observed, partially independent of the p53 axis, suggesting alternative mechanisms of cell cycle regulation. In addition, RA suppressed TNF- α -induced NF- κ B transcriptional activity,

suggesting the involvement of the TNF- α /NF- κ B axis in the suppression of pro-proliferative and pro-survival signaling.

Analysis of mitochondrial membrane potential revealed its early reduction upon RA exposure; however, pronounced induction of apoptosis was observed mainly at higher concentrations and after prolonged incubation, without a significant increase in necrosis. Furthermore, the lack of a clear protective effect following the application of inhibitors of regulated cell death suggests that apoptosis is not the dominant mechanism of RA action, but rather a secondary response. The observed activation of caspase 9 indicates the involvement of the mitochondrial apoptotic pathway. Genotoxicity assays and analysis of reactive oxygen species levels did not reveal significant DNA damage or pro-oxidative effects. Complementary *in silico* analyses indicated a favorable pharmacokinetic profile of RA and identified potential molecular targets associated with lipid metabolism, suggesting a possible contribution of metabolic mechanisms to the observed antiproliferative effect.

Collectively, the results indicate that RA acts as a multi-level modulator of signaling pathways regulating cancer cell proliferation and survival, primarily exerting a cytostatic effect through inhibition of the TNF- α /NF- κ B axis and induction of G₀/G₁ cell cycle arrest, with secondary activation of mitochondria-dependent apoptotic mechanisms.

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Sulfonamides in Normal and Cancer Cells In Vitro. *Int J Mol Sci.* 2023 Feb 17;24(4):4053. doi: 10.3390/ijms24044053. PMID: 36835469; PMCID: PMC9966268. IF: 6.208; MEiN: 140.

14. Kciuk M, Gielecińska A, Mujwar S, Mojzych M, Marciniak B, Drozda R, Kontek R. Targeting carbonic anhydrase IX and XII isoforms with small molecule inhibitors and monoclonal antibodies. *J Enzyme Inhib Med Chem.* 2022 Dec;37(1):1278-1298. doi: 10.1080/14756366.2022.2052868. PMID: 35506234; PMCID: PMC9090362. IF: 4.673; MEiN: 140.
15. Kciuk M, Mujwar S, Rani I, Munjal K, Gielecińska A, Kontek R, Shah K. Computational Bioprospecting Guggulsterone against ADP Ribose Phosphatase of SARS-CoV-2. *Molecules.* 2022 Nov 28;27(23):8287. doi: 10.3390/molecules27238287. PMID: 36500379; PMCID: PMC9739500. IF: 3.06; MEiN: 140.
16. Kciuk M, Gielecińska A, Kołat D, Kałuzińska Ż, Kontek R. Cancer-associated transcription factors in DNA damage response. *Biochim Biophys Acta Rev Cancer.* 2022 Jul;1877(4):188757. doi: 10.1016/j.bbcan.2022.188757. Epub 2022 Jun 30. PMID: 35781034. IF: 7.365; MEiN: 200.
17. Kciuk M, Gielecińska A, Budzinska A, Mojzych M, Kontek R. Metastasis and MAPK Pathways. *Int J Mol Sci.* 2022 Mar 31;23(7):3847. doi: 10.3390/ijms23073847. PMID: 35409206; PMCID: PMC8998814. IF: 4.183; MEiN: 140.
18. Kciuk M, Gielecińska A, Mujwar S, Mojzych M, Kontek R. Cyclin-Dependent Kinase Synthetic Lethality Partners in DNA Damage Response. *Int J Mol Sci.* 2022 Mar 24;23(7):3555. doi: 10.3390/ijms23073555. PMID: 35408915; PMCID: PMC8998982. IF: 4.183; MEiN: 140.
19. Kciuk M, Gielecińska A, Mujwar S, Mojzych M, Kontek R. Cyclin-dependent kinases in DNA damage response. *Biochim Biophys Acta Rev Cancer.* 2022 May;1877(3):188716. doi: 10.1016/j.bbcan.2022.188716. Epub 2022 Mar 7. PMID: 35271993. IF: 7.365; MEiN: 200.

Łączny IF wszystkich publikacji: 115.518

Punkty MEiN/MNiSzW: 3040

Raport Web of Science (z dnia 5.05.2026):

H-index: 14

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Liczba wszystkich cytowani bez autocytowań: 1194

Uśredniona ilość cytowań w przeliczeniu na publikacje: 55.09

Komunikaty zjazdowe

1. V Multidyscyplinarna Konferencja Doktorantów Uniwersytetu Szczecińskiego MKDUS 2.0, A. Gielecińska, R. Kontek; referat naukowy w języku angielskim „*Natural Strategies Against Cancer: Cytostatic Properties of Roburic Acid*”, 25-27.06.2025 (online).
2. XI Forum Młodych Naukowców „Nauki Przyrodnicze i Medyczne”, A. Gielecińska, R. Kontek; referat naukowy w języku angielskim „*Cytostatic Over Cytotoxic Effects of Roburic Acid: A Targeted Approach to Cancer Cell Growth Inhibitor*”, 6-7.12.2024 (online).
3. IX Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen, A. Gielecińska, R. Kontek; poster naukowy w języku polskim „*Aktywność przeciwnowotworowa kwasu roburowego*”, 11-12.04.2024 (online).
4. VIII Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen, A. Gielecińska, R. Kontek; poster naukowy w języku polskim „*Aktywność przeciwnowotworowa nowej trójcyklicznej pochodnej*”, 13-14.04.2023 (online).
5. Ogólnopolska Konferencja Genetyczna „Genomica” 2022, A. Gielecińska, R. Kontek; poster naukowy w języku polskim „*Badanie aktywności przeciwnowotworowej nowych trójcyklicznych pochodnych pyrazolo[4,3-e][1,2,4]triazyny – MM124 i MM125*”, Kraków, 22 maj 2022.
6. NATURE Polish Scientific Conference 2021, A. Gielecińska, M. Kciuk; poster naukowy w języku polskim „*Wpływ NAD⁺ zależnych sirtuin na*

starzenie komórkowe C. elegans”, Lublin, 12 czerwca 2021. Wystąpienie nagrodzone nagrodą za zajęcie I miejsca w konkursie na najlepszy poster.

Działalność organizacyjna i promocyjna

- Wygłoszenie referatu zatytułowanego „Różne oblicza śmierci komórkowej” w marcu 2025 roku na seminarium naukowym zorganizowanym przez Polskie Towarzystwo Biologii Komórki oraz Polskie Towarzystwo Fizjologiczne;
- Współprowadzenie warsztatów zatytułowanych „Genetyczne odkrycia: tworzymy i oglądamy preparaty chromosomowe” w ramach ogólnopolskiej akcji „Noc Biologów” promującej wiedzę biologiczną w formie popularnonaukowej (16.01.2026);
- Czynny udział w Studenckim Kole Naukowym Biologów sekcji Cytogenetycznej (2022-2026);
- Czynny udział w Polskim Towarzystwie Genetycznym (PTG) (2022-2026);
- Działalność w Kolegium Nadzorczym Doktorantów oraz Samorządowej Komisji Wyborczej Doktorantów UŁ (2022-2026).

Stáže naukowe

- Staż naukowy w Instytucie Biologii Naczyniowej i Badań nad Zakrzepicą na Uniwersytecie Medycznym w Wiedniu (Institute of Vascular Biology and Thrombosis Research, Center for Physiology and Pharmacology, Medical University of Vienna) w ramach programu PROM – krótkookresowej wymiany akademickiej na Uniwersytecie Łódzkim, zawartego z Narodową Agencją Wymiany Akademickiej (NAWA), finansowanego ze środków Unii Europejskiej w ramach projektu pn. „Krótkookresowa wymiana akademicka sposobem podniesienia jakości kształcenia w instytucjach szkolnictwa wyższego i nauki”, 1-30.04.2025;
- Praktyki zagraniczne w Instytucie Biologii Naczyniowej i Badań nad

Zakrzepicą na Uniwersytecie Medycznym w Wiedniu w ramach realizowanego przez UŁ projektu Erasmus+ Mobilność edukacyjna. Temat realizowanego projektu : „Molecular Links Between MYC and Tissue Factor in Cancer Progression”, 1.07–30.09.2025.

Kursy

- „Hodowla komórek adherentnych i w zawiesinie. Transfekcja.”, Centrum Medycznego Kształcenia Podyplomowego, 24-26.03.2026.
- „Sekwencjonowanie następnej generacji w technologii Ion Torrent”, Centrum Medycznego Kształcenia Podyplomowego, 16–18.03.2026.
- „Rola mikroRNA w procesie nowotworzenia”, Centrum Medycznego Kształcenia Podyplomowego, Warszawa, 19.02.2026.
- „Wybrane zagadnienia z molekularnych podstaw onkogenezy”, Centrum Medycznego Kształcenia Podyplomowego, Warszawa, 12.02.2026.
- „Molecular modeling techniques for drug design”, Łódź, 5-9.09.2024.

Nagrody i wyróżnienia

- I Nagroda Naukowa Rektora Uniwersytetu Łódzkiego dla doktorantek i doktorantów Szkół Doktorskich UŁ za osiągnięcia w zakresie pracy naukowej i badawczej, publikacji i umiędzynarodowienia kształcenia (2025).
- Wyróżnienie za prezentację referatu naukowego w języku angielskim zatytułowanego „*Cytostatic Over Cytotoxic Effects of Roburic Acid: A Targeted Approach to Cancer Cell Growth Inhibitor*” podczas XI Forum Młodych Naukowców „Nauki Przyrodnicze i Medyczne”, 6-7.12.2024 (online).
- Nagroda za zajęcie I miejsca w konkursie na najlepszy poster podczas ogólnopolskiej konferencji NATURE Polish Scientific Conference 2021; Lublin, 12 czerwca 2021, „*Wpływ NAD⁺ zależnych sirtuin na starzenie komórkowe C. elegans*”.

Oświadczenia współautorów

Vienna, June 2nd 2026
(place, date)

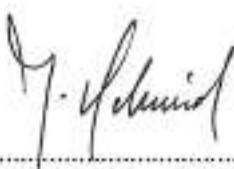
Prof. Johannes A. Schmid
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Medical University of Vienna

Contributor's Statement

Gleecińska A, Kciuk M, Mujwar S, Schmid JA, Kontek R. Anticancer Activity of Roburic Acid: In Vitro and In Silico Investigation. Int J Mol Sci. 2025 Jul 3;26(13):6420. doi: 10.3390/ijms26136420.

I declare that my participation in the publication included providing supervision. I assess my participation as 5%.




Prof. Johannes A. Schmid

Raport weryfikacji

Wynik
Wynik przetwarzania
Skrót pliku z podpisem
Nazwa pliku z podpisem
Identyfikator weryfikacji

WAŻNY/PRAWIDŁOWY
Zakończono
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J. Schmid Ada contribution_signiert.pdf
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Lista podpisów

Status weryfikacji podpisu
Data i czas wykonania podpisu
Data i czas weryfikacji podpisu
Certyfikat podpisującego

WAŻNY/PRAWIDŁOWY
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2026-06-02 14:40:32 GMT+02:00
kwalifikowany

Ścieżka certyfikacji

Certyfikat podpisującego

Walidacja certyfikatu
Status certyfikatu
Wystawiony dla

Wystawiony przez

Ważny od
Ważny do
Data wystawienia CRL/OCSP
Numer seryjny

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Alfred Schmid
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Certyfikat wystawcy

Walidacja certyfikatu
Status certyfikatu
Wystawiony dla

Wystawiony przez
Ważny od
Ważny do
Numer seryjny

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4e4f0766

Chandigarh, India 14 February 2026
(place, date)

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Contributor's Statement

Giełecińska A, Kciuk M, Mujwar S, Celik I, Kolat D, Kaluzińska-Kolat Z, Kontek R. Substances of Natural Origin in Medicine: Plants vs. Cancer. *Cells*. 2023 Mar 23;12(7):986. doi: 10.3390/cells12070986.

I declare that my participation in the publication included providing supervision. I assess my participation as 5%.

Giełecińska A, Kciuk M, Mujwar S, Schmid JA, Kontek R. Anticancer Activity of Roburic Acid: In Vitro and In Silico Investigation. *Int J Mol Sci*. 2025 Jul 3;26(13):6420. doi: 10.3390/ijms26136420.

I declare that my participation in the publication was associated with *in silico* studies – molecular docking and molecular dynamics simulations. I assess my participation as 5%.



.....
Dr Somdutt Mujwar

OŚWIADCZENIE

Działając na podstawie § 4 ust. 4 Regulaminu postępowania w sprawie nadania stopnia doktora na Uniwersytecie Łódzkim, oświadczam, że przedłożenie oświadczenia współautora dr. Somdutta Mujwara jest nadmiernie utrudnione. Współautor jest zatrudniony i na stałe przebywa w Indiach, co wiąże się ze znaczną odległością geograficzną oraz utrudnieniami organizacyjnymi w zakresie obiegu dokumentów. Ponadto nie posiada kwalifikowanego podpisu elektronicznego umożliwiającego złożenie dokumentu w formie akceptowanej przez Uniwersytet Łódzki, a dostarczenie podpisanego własnoręcznie oryginału dokumentu drogą pocztową w terminie wymaganym dla przeprowadzenia postępowania doktorskiego nie jest możliwe.

W związku z powyższym samodzielnie określám wkład dr. Somdutta Mujwara w publikacje stanowiące część rozprawy doktorskiej:

1. Gielecińska A., Kciuk M., Mujwar S., Schmid J.A., Kontek R. *Anticancer Activity of Roburic Acid: In Vitro and In Silico Investigation*. International Journal of Molecular Sciences. 2025;26(13):6420. DOI: 10.3390/ijms26136420.

Wkład współautora: udział w badaniach in silico, obejmujących dokowanie molekularne oraz symulacje dynamiki molekularnej. Udział w przygotowaniu publikacji: 5%.

2. Gielecińska A., Kciuk M., Mujwar S., Celik I., Kotat D., Katuzińska-Kołat Ż., Kontek R. *Substances of Natural Origin in Medicine: Plants vs. Cancer*. Cells. 2023;12(7):986. DOI: 10.3390/cells12070986.

Wkład współautora: sprawowanie nadzoru merytorycznego nad realizacją pracy (superwizja). Udział w przygotowaniu publikacji: 5%.

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Dane kontaktowe współautora:

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W przypadku konieczności potwierdzenia powyższych informacji współautor pozostaje dostępny pod wskazanym adresem poczty elektronicznej.

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(data i podpis doktorantki)

02.06.2026

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Contributor's Statement

Gielecińska A, Kciuk M, Mujwar S, Celik I, Kotat D, Kaluzińska-Kotat Z, Kontek R. Substances of Natural Origin in Medicine: Plants vs. Cancer. Cells. 2023 Mar 23;12(7):986. doi: 10.3390/cells12070986.

I declare that my participation in the publication included providing supervision. I assess my participation as 5%.


.....
dr Ismail Celik

Łódź, 12.02.2026 r.
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Oświadczenie współautora

Gielecińska A, Kciuk M, Mujwar S, Celik I, Kołat D, Kałuzińska-Kołat Z, Kontek R. Substances of Natural Origin in Medicine: Plants vs. Cancer. Cells. 2023 Mar 23;12(7):986. doi: 10.3390/cells12070986.

Oświadczam, że mój udział w pracy polegał na nadzorze merytorycznym oraz wprowadzaniu poprawek do tekstu. Wkład w przygotowanie publikacji wynosił: 5%.



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Dr Żaneta Kałuzińska-Kołat



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Raport weryfikacji

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Nazwa pliku z podpisem

Identyfikator weryfikacji

WAŻNY/PRAWIDŁOWY

Zakończono

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Oswiadczenie wspolautora_, Kaluzinska_Kolat.pdf

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Lista podpisów

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Data i czas wykonania podpisu

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Format podpisu

Poziom podpisu

Certyfikat podpisującego

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BES (Podpis w postaci podstawowej)

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Certyfikat podpisującego

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Numer seryjny

WAŻNY/PRAWIDŁOWY

WAŻNY

surname=Kałużyńska-Kołat, givenName=Żaneta, CN=Żaneta Kałużyńska-Kołat, serialNumber=PNOP-95022605768, C=PL

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Certyfikat wystawcy

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Gielecińska A, Kciuk M, Mujwar S, Celik I, Kołat D, Kałuzińska-Kołat Z, Kontek R. Substances of Natural Origin in Medicine: Plants vs. Cancer. Cells. 2023 Mar 23;12(7):986. doi: 10.3390/cells12070986.

Oświadczam, że mój udział w pracy polegał na nadzorze merytorycznym oraz wprowadzaniu poprawek do tekstu. Wkład w przygotowanie publikacji wynosił: 5%.

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2026-02-12 15:06

Dr Damian Kołat

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Wynik
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Skrót pliku z podpisem
Nazwa pliku z podpisem
Identyfikator weryfikacji

WAŻNY/PRAWIDŁOWY
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Oswiadczenie wspolautora_Kolat Damian.pdf
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Lista podpisów

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Ścieżka certyfikacji

Certyfikat podpisującego

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Status certyfikatu
Wystawiony dla

Wystawiony przez
Ważny od
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Data wystawienia CRL/OCSP
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Certyfikat wystawcy

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Status certyfikatu
Wystawiony dla
Wystawiony przez
Ważny od
Ważny do
Numer seryjny

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60f06092d2309c1892a9c147e186eaf8bcade302

10.03.2025 Łódź
(miejscowość, data)

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Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki

Oświadczenie współautora

Gielecińska A., Kciuk M., Gruszka R., Wawrocki S., Kontek R. Roburic Acid as a Therapeutic Candidate: Antiproliferative Activity and Secondary Cell Death Response in Colorectal Cancer Cells. Int J Mol Sci. 2026 Mar 8;27(5):2478. doi: 10.3390/ijms27052478.

Oświadczam, że mój udział w pracy polegał na współtworzeniu koncepcji pracy, określeniu jej głównych założeń badawczych oraz sprawowaniu superwizji nad przebiegiem realizacji. Wkład w przygotowanie publikacji wynosił: 5%.


Dr Renata Gruszka

Nida', 05.05.2026 r.
(miejscowość, data)

Dr Beata Marciniak
Katedra Biotechnologii Molekularnej i Genetyki
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Oświadczenie współautora

Oświadczam, że w pracy Giełecińska A., Wawrocki S., Marciniak B., Kcluk M., Wojtczak A. i Kontek R. (2026) „Revisiting Apoptosis: What Lies Behind the Antiproliferative Activity of Roburic Acid in Colorectal Cancer Cells?” mój udział polegał na tworzeniu koncepcji pracy, wsparciu w pracy laboratoryjnej oraz monitorowaniu postępów projektu. Wkład w przygotowanie publikacji wynosił 5%.

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Łódź, 10.03.2026
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Oświadczam, że mój udział w pracy polegał na współtworzeniu koncepcji pracy, wprowadzaniu poprawek do manuskryptu oraz sprawowaniu superwizji nad realizacją badań i przygotowaniem publikacji. Wkład w przygotowanie publikacji wynosił: 5%.

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Gielecińska A, Kciuk M, Mujwar S, Celik I, Kołat D, Kałuzińska-Kołat Ż, Kontek R. Substances of Natural Origin in Medicine: Plants vs. Cancer. Cells. 2023 Mar 23;12(7):986. doi: 10.3390/cells12070986.

Oświadczam, że mój udział w pracy polegał na współudziale w przygotowaniu manuskryptu, w tym na jego redakcji oraz wprowadzaniu poprawek po recenzjach. Wkład w przygotowanie publikacji wynosił: 5%.

Gielecińska A, Kciuk M, Mujwar S, Schmid JA, Kontek R. Anticancer Activity of Roburic Acid: In Vitro and In Silico Investigation. Int J Mol Sci. 2025 Jul 3;26(13):6420. doi: 10.3390/ijms26136420.

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Oświadczam, że w pracy Glebocka A., Wawrocki S., Mardiniak B., Kciuk M., Wojtczak A. i Kontek R. [2026] „Revisiting Apoptosis: What Lies Behind the Antiproliferative Activity of Roburic Acid in Colorectal Cancer Cells?” mój udział polegał na tworzeniu koncepcji pracy oraz monitorowaniu jej postępów. Wkład w przygotowanie publikacji wynosił 5%.

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Oświadczam, że w pracy Gielecińska A., Wawrocki S., Mardniak B., Kciuk M., Wojtczak A. i Kontek R. (2026) „Revisiting Apoptosis: What Lies Behind the Antiproliferative Activity of Roburic Acid in Colorectal Cancer Cells?” mój udział polegał na współtworzeniu koncepcji pracy, określeniu jej głównych założeń badawczych oraz sprawowaniu supervizji nad przebiegiem realizacji. Wkład w przygotowanie publikacji wynosił: 5%.

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Oświadczenie współautora

Gielecińska A, Kciuk M, Mujwar S, Celik I, Kołat D, Kałuzińska-Kołat Ż, Kontek R. Substances of Natural Origin in Medicine: Plants vs. Cancer. Cells. 2023 Mar 23;12(7):986. doi: 10.3390/cells12070986.

Oświadczam, że mój udział w pracy polegał na a współudziale w opracowaniu zarysu koncepcyjnego pracy oraz sprawowaniu superwizji nad jej realizacją. Wkład w przygotowanie publikacji wynosił: 5%.

Gielecińska A, Kciuk M, Mujwar S, Schmid JA, Kontek R. Anticancer Activity of Roburic Acid: In Vitro and In Silico Investigation. Int J Mol Sci. 2025 Jul 3;26(13):6420. doi: 10.3390/ijms26136420.

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Gielecińska A., Kciuk M., Gruszka R., Wawrocki S., Kontek R. Roburic Acid as a Therapeutic Candidate: Antiproliferative Activity and Secondary Cell Death Response in Colorectal Cancer Cells. Int J Mol Sci. 2026 Mar 8;27(5):2478. doi: 10.3390/ijms27052478.

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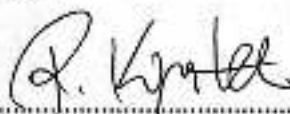

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Oświadczam, że w pracy Giełedzińska A., Wawrocki S., Marciniak B., Kciuk M., Wojtczak A. i Kontek R. (2026) „Revisiting Apoptosis: What Lies Behind the Antiproliferative Activity of Roburic Acid in Colorectal Cancer Cells?” mój udział polegał na współtworzeniu koncepcji pracy, określeniu jej głównych założeń badawczych oraz sprawowaniu supervizji nad przebiegiem realizacji. Wkład w przygotowanie publikacji wynosił: 5%.


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Oświadczam, że mój udział w pracy polegał na opracowaniu koncepcji pracy, przeglądzie literatury, opracowaniu całego manuskryptu oraz wprowadzaniu poprawek po recenzjach. Wkład w przygotowanie publikacji wynosił: 70%.

Gielecińska A, Kciuk M, Mujwar S, Schmid JA, Kontek R. Anticancer Activity of Roburic Acid: In Vitro and In Silico Investigation. Int J Mol Sci. 2025 Jul 3;26(13):6420. doi: 10.3390/ijms26136420.

Oświadczam, że mój udział w pracy polegał na opracowaniu koncepcji pracy, przeglądzie literatury, zaplanowaniu i przeprowadzeniu eksperymentów, współuczestniczeniu w badaniach *in silico*, analizie statystycznej, wizualizacji i interpretacji danych, opracowaniu całego manuskryptu oraz wprowadzaniu poprawek po recenzjach. Wkład w przygotowanie publikacji wynosił: 80%.

Gielecińska A., Kciuk M., Gruszka R., Wawrocki S., Kontek R. Roburic Acid as a Therapeutic Candidate: Antiproliferative Activity and Secondary Cell Death Response in Colorectal Cancer Cells. Int J Mol Sci. 2026 Mar 8;27(5):2478. doi: 10.3390/ijms27052478.

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Gielecińska Adrianna
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Gielecińska
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Adrianna Gielecińska

Kopie publikacji wchodzących w skład rozprawy

Review

Substances of Natural Origin in Medicine: Plants vs. Cancer

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Abstract: Continuous monitoring of the population's health is the main method of learning about disease prevalence. National and international data draw attention to the persistently high rates of cancer incidence. This necessitates the intensification of efforts aimed at developing new, more effective chemotherapeutic and chemopreventive drugs. Plants represent an invaluable source of natural substances with versatile medicinal properties. Multidirectional activities exhibited by natural substances and their ability to modulate key signaling pathways, mainly related to cancer cell death, make these substances an important research direction. This review summarizes the information regarding plant-derived chemotherapeutic drugs, including their mechanisms of action, with a special focus on selected anti-cancer drugs (paclitaxel, irinotecan) approved in clinical practice. It also presents promising plant-based drug candidates currently being tested in clinical and preclinical trials (betulinic acid, resveratrol, and roburic acid).

Keywords: cancer; paclitaxel; irinotecan; betulinic acid; resveratrol; roburic acid



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1. Introduction

Despite the advances in modern medicine and pharmacology, cancer is a global problem, posing one of the greatest challenges of the 21st century. It is assumed that one in six deaths is due to cancer [1]. The late diagnosis affects the rate of progression of pathological changes and minimizes the chances of remission of the disease. It is predicted that the burden of cancer is likely to intensify over the next several years [2]. High incidence and mortality rates are causing scientific and research centers to intensify their efforts to reduce the cancer burden, through endeavors aimed at developing effective oncopharmaceuticals. For decades, substances of natural origin have been the main reservoir of therapeutic agents, playing a key role in clinical use and prevention [3,4]. Their extraordinary diversity makes them a rich collection of active compounds with high therapeutic potential. These substances are isolated from microorganisms and animal organisms, but special merit is given to compounds of plant origin [5–7]. Their therapeutic properties have been particularly appreciated in oncology, where it is estimated that about 60% of all oncopharmaceuticals available on the drug market have been designed based on plant compounds [8–10]. Anthracyclines, taxanes, and *vinca* alkaloids are just some of the groups of compounds commonly used as anti-cancer drugs [11–13]. Many other promising compounds have been tested, reported, and validated, but have not entered clinical practice. This is attributed to the poor water solubility, low stability, and bioavailability of natural compounds. Another obstacle is the isolation of compounds on an industrial scale, which also limits future production possibilities. Moreover, most of the research conducted in the mid-twentieth century was based on phenotypic screening, which did not focus on the

specific mechanisms of action, but only on the effect caused by the substance [14]. This gave an incomplete depiction of the characteristics and therapeutic potential of the substances studied. As a result, many scientific research centers and pharmaceutical companies have reduced their work on natural compounds, thereby shifting their attention to chemically synthesized compounds [15]. Obtaining these compounds has proven to be faster and more efficient than extracting substances from natural raw materials.

Intensive technological and methodological development involves introducing information systems, developing computational methods, creating programs to study protein structures, and predicting both molecular targets and drug-target interactions. High-throughput experimental techniques that can be used to screen new chemical entities, combined with conceptual advancement, has led to the emergence of interdisciplinary fields of science, targeted at understanding the biological mechanisms and processes in living organisms. The available tools allow for the analysis of genes, genomes, protein expression, and signaling and metabolic pathways, but importantly, also allow for faster and more efficient study of chemical compounds and drug design. By using the achievements of nanotechnology in medicine as modern drug delivery systems, the so-called nanoparticles (liposomes, micelles, lipid nanoparticles, polymer nanoparticles, etc.) makes it possible to overcome the problem of low bioavailability of compounds, and also improves compounds' solubility and metabolic rate [16–18]. The possibility of overcoming the previous difficulties related to physicochemical properties, substance availability, and the complexity of pharmacological effects, can realistically influence the transfer of tested compounds from research laboratories to widespread use in clinics. It justifies the recently observed trend of reinventing and researching natural substances, which, combined with modern tools, gives an opportunity to better understand their properties and potential therapeutic utility [19]. At the same time, their multifaceted mechanisms of action are being discovered, as well as their tendency to simultaneously affect multiple oncogenic signaling pathways by modulating the expression of their molecular targets, which translates into the ability of cells to proliferate, migrate, metastasize or undergo apoptotic cell death. Due to the complexity of their properties, natural compounds can be used directly or as chemotherapeutic adjuvants, supporting anti-cancer activity, overcoming therapy resistance in cancer cells, and supporting cells' repair mechanisms [20,21]. In addition to their important therapeutic role, compounds of natural origin may be leading structures in the search and development of other pharmaceuticals. On their basis, modifications can be introduced and analogs with increased affinity to the target and higher selectivity to the enzyme isoforms can be synthesized. The selection of the leading structure and the synthesis of derivatives from natural compounds seems to be more effective when compared to the use of synthetic compounds. In the era of current efforts and opportunities, many natural compounds that are newly discovered or re-tested can be expected to reach the next stages of drug development and final clinical use. In this paper, we present an up-to-date review of both the best-studied and the new, potential plant compounds with anti-cancer properties.

2. Main Risk Factors

Cancer susceptibility often has a genetic basis. The disease can be triggered by a rare inherited gene mutation with high or low penetrance in carriers, and cause so-called familial syndromes, i.e., breast or ovarian cancer with breast cancer gene 1 (*BRCA1*) or breast cancer gene 2 (*BRCA2*) mutations, and Li-Fraumeni syndrome associated with rare tumor protein P53 (*TP53*) variants that lead to development of soft tissue sarcomas, breast cancer, colon cancer, gastric cancer, and brain tumors [22]. Diagnosis of hereditary cancer predisposition syndrome is based on pedigree analysis, clinical evaluation, and genetic testing. Alternatively, common genetic variants with the sporadic risk that do not have a clear hereditary syndrome may lead to cancer occurrence. Their role in shaping enzyme and protein activity is important in the context of mutations and DNA repair. In addition, the accumulation of low penetrance variants or susceptibility gene loci within target genes may affect pathways and mechanisms involved in cancer development [23].

Environmental etiological factors of cancer include poor eating habits, obesity, smoking, lack of physical activity, and exposure to air or water pollution, radiation, or infectious pathogens [24–27]. For instance, excess insulin levels, caused by high blood glucose often found in people with excessive body fat, will lead to lower insulin-like growth factor binding protein 3 (IGFBP-3) levels. Increased levels of unbound insulin-like growth factor-1 (IGF-1) promote cancer formation. Hyperinsulinemia also modifies the bioavailability of sex hormones, resulting in prolonged exposure to free estrogens and androgens, which, unopposed by hormones with anti-proliferative effects, can promote oncogenesis [27,28]. Cigarette smoke contains more than 50 carcinogens (e.g., nitrosamines and DNA methylating agents) that cause numerous types of DNA damage. About 60% of cases of malignant melanoma are caused by the genotoxic effects of UVA and UVB radiation, which is exacerbated not only by lack of protection from the sun's rays but also by the fashion of using tanning beds. Most of the presented environmental risk factors are modifiable. Thus, it is possible to influence the burden of cancer through actions that contradict these factors. If the disease progresses despite prophylactic measures, systemic treatment with cytostatic drugs (chemotherapy), hormonally active drugs (hormone therapy), drugs that stimulate the immune system (immunotherapy), or those that target specific pathways and signaling pathways (targeted therapy) is implemented.

3. Conventional Treatment

Chemotherapy remains the most common and effective method of cancer treatment. Its aim is to damage the genetic material of cancer cells. The presence of damage activates the DNA damage response pathway (DDR), which constitutes a detection, signaling, and repair system [29]. The DDR stimulates subsequent molecular mechanisms and triggers events leading to inhibition of the cell cycle progression and cell death [30]. The use of agents that induce DNA damage is a primer of the cancer treatment. Based on their mechanism of action, chemotherapeutics are divided into alkylating agents, antimetabolites, topoisomerase inhibitors, mitotic spindle inhibitors, and others [31,32]. These include substances of both natural (plant) and synthetic origin.

Alkylating agents act by alkylating DNA, causing structural changes in DNA through the attachment of alkyl groups to DNA bases, leading to inappropriate base pairing, depurination of DNA molecules, and formation of inter- or intrastrand cross-links. The versatility of DNA damage caused by a specific agent depends mainly on the number of its reactive sites (mono or bifunctional agents). Monofunctional agents contain a single chemical grouping that modifies a single base in DNA molecule, while bifunctional agents contain two groups that can bind to different sites in DNA [33]. Damage prevents the process of replication and transcription from taking place. Alkylating agents currently used in chemotherapy include several groups of substances, including oxazaphosphorines (cyclophosphamide, ifosfamide), nitrogen mustard derivatives (melphalan, chlorambucil), platinum derivatives (cisplatin, oxaliplatin), nitrosourea derivatives (carbamustine, lomustine), and triazines (dacarbazine, temozolomide) [31,33].

Antimetabolites are chemical compounds that, due to their structural similarity to metabolites, can displace them from specific metabolic pathways, thereby disrupting their course. Purine or pyrimidine analogs (6-mercaptopurine, 5-fluorouracil) are incorporated into nucleic acids, disrupting their proper synthesis and the fidelity of the genetic material further transferred to progeny [34]. Some antifolates (methotrexate) interfere with the activity of enzymes, i.e., dihydrofolate reductase, which supply cells with tetrahydrofolate and carry mono-carbon groups that act as coenzymes in the synthesis of purines and pyrimidines and the metabolism of homocysteine, methionine, and other amino acids [35]. Among the best-studied antimetabolites are pyrimidine analogs 5-fluorouracil, tegafur, capecitabine, cytarabine, gemcitabine, azacitidine; purine analogs, including azathioprine, 6-mercaptopurine, thioguanine, cladribine, clofarabine, fludarabine; antifolates, including methotrexate, pralatrexate, pemetrexed; thymidylate synthase inhibitors (raltitrexed); ribonucleotide reductase inhibitors (hydroxyurea); and others [31,34,36].

Topoisomerases are a group of enzymes responsible for maintaining correct DNA topology during replication and transcription that pose a torsional threat to DNA molecules. Inhibition of their action causes fragmentation of the genetic material of cells through the formation of DNA strand breaks, which, if unrepaired, will lead to cell death [37]. One of the first known topoisomerase inhibitors was camptothecin, isolated from the plant *Camptotheca acuminata*. The formation of DNA-protein complexes at the ends of the DNA breaks prevents the restoration of the proper DNA structure. However, due to its high toxicity and poor solubility, the substance was withdrawn from clinical trials [10]. Based on the natural derivative, two semi-synthetic derivatives were created: irinotecan and topotecan. Other substances with inhibitory effects on topoisomerases include etoposide, teniposide, and anthracyclines [34].

Another effective approach in the fight against cancer cells is targeting cell division. Microtubules play a key role in the segregation of chromosomes during cell division. Structurally, microtubules consist of α -tubulin and β -tubulin, which are a network of protein filaments whose dynamics are responsible for the proper segregation of chromosomes [37,38]. *Catharanthus roseus* contains a broad variety of alkaloids, i.e., vincristine and vinblastine with proven anti-cancer activity. They are commonly used in lung and breast cancers or lymphomas due to their ability to inhibit microtubule movement or depolymerization, leading to disruption of their structure and arrest of cells in the M phase of the cell cycle [39]. However, their use is associated with high hepatotoxicity and neurotoxicity, which not only caused damage to internal organs, but also prevents the continuation of therapy. Due to the adverse side effects of the use of vincristine and vinblastine, their semi-synthetic analogs—vindesine and vinorelbine—have been created, which have a reduced toxicity profile [34]. Another group of compounds aimed at inhibiting mitosis are taxanes, which are isolated from the needles and bark of yew trees. The first drug developed from this group with anti-mitotic activity is paclitaxel. It prevents the reorganization of the cytoskeleton, thereby disrupting cellular activities and leading to its death [37]. As some types of cancer are resistant to paclitaxel, a semi-synthetic derivative called docetaxel has been created that has a similar chemical structure and action. A summary of the previously mentioned groups of compounds is shown in Figure 1.

Through their increased capacity for growth and proliferation, dedifferentiation, immortalization, metastasis, and overall devastating effects, cancer cells pose a threat to the entire body. Targeting the key features of cancer—limiting the growth and division of cancer cells—remains an effective method of fighting cancer. Therefore, the search for substances that act on different stages of the cell cycle and prevent its progression is one of the primers in anti-cancer drug discovery. Inhibition of this process results from the effect of drug molecules on diverse pathways and mechanisms, i.e., interference with transcription (actinomycin D), induction of free radical formation (bleomycin), and inhibition of growth factor signaling (imatinib), that ultimately induce cancer cell death [40–42].

However, the cytostatics currently used in clinical setting have non-selective effects and affect not only cancer cells but also normal cells, especially rapidly dividing cells and those with limited repair capacity [43,44]. Therefore, the characteristic side effects of received chemotherapy are alopecia, pruritic skin, hand–foot syndrome, anemia, myelosuppression, vomiting, diarrhea, inflammation, damage to hair follicle cells, gastrointestinal tract, blood, and oral mucosa. Chemotherapy also carries a high risk of drug adverse effects due to acute toxicity of oncopharmaceuticals, causing damage to the liver (hepatotoxicity), kidneys (nephrotoxicity), nerves (neurotoxicity), and lungs (pulmonary toxicity) [45,46]. Long-term drug use can have distant consequences on the patient's subsequent life and health, causing infertility, hypogonadism, or metabolic syndrome [47]. Side effects vary depending on the treatment regimen adopted, including the type of chemotherapy used, route of administration, and frequency of dosage. Therefore, there is an urgent need to combine conventional chemotherapeutics with non-toxic adjuvant compounds that can contribute to a better response to therapy and mitigate side effects.

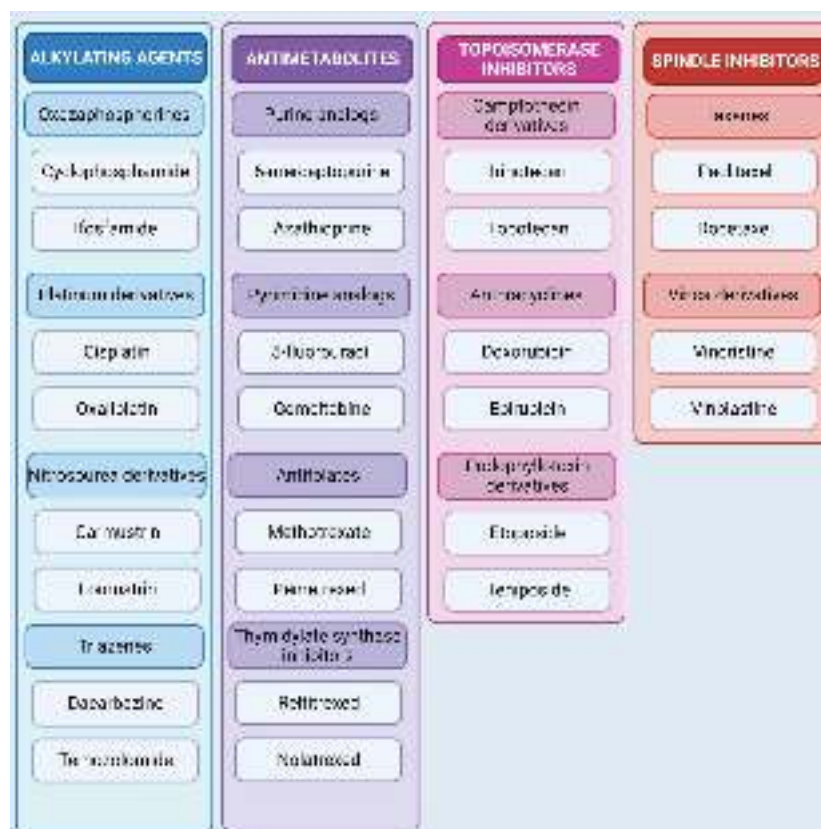


Figure 1. The main types of chemotherapeutics sorted by their mechanism of action. Among the most popular are alkylating compounds, antimetabolites, topoisomerase inhibitors, and mitotic spindle inhibitors. Created with BioRender.com.

4. Natural Compounds

Plants are a rich source of natural substances with medicinal properties. Their use dates back to ancient times, and in modern times, they are an undeniable staple of medicine and pharmacology. Most secondary metabolites do not have an essential effect on plant growth; they play specific functions in the protection against pathogens and potential predators, or they may serve as signaling substances responsible for interaction with symbionts [48,49]. These compounds protect plants but can be toxic to other organisms. Unlike primary metabolites, there are many of them, and their activity is relatively poorly understood. They may have a complex biosynthetic pathway accompanied by complex regulation. The synthesis of these substances can be limited to a species or even individuals. Their specific properties justify their use in various fields of medicine, including oncology. More than half of the oncopharmaceuticals currently used clinically are of plant origin, and estimates suggest that up to 3000 plants may have anti-cancer properties [8–10]. At the same time, ample evidence is emerging on the role of plant compounds as inhibitors of key steps in oncogenesis and related processes [9,50]. This underscores their importance as potential preventive and therapeutic agents.

The use of substances of natural origin is not without drawbacks. Some plant-based compounds, due to their high toxicity, have been withdrawn from further studies during clinical trials, or their use has been restricted because of the number of side effects, i.e., paclitaxel and vinblastine. Nevertheless, they are considered more biologically friendly than synthetic compounds. Another obstacle is low bioavailability and poor water solubility of these compounds, which significantly limit their use. Therefore, efforts are being made to develop semi-synthetic analogs with improved physicochemical parameters and higher efficacies compared with the precursor natural compounds [51]. However, natural compounds of plant origin do not have to serve as stand-alone drugs. They can also be

used as supportive or auxiliary substances that contribute to the reduction of side effects and enhance safety and pharmacokinetic profiles. The toxic burden on the body can be reduced by replacing part of the dose of a standard chemotherapeutic agent with a natural substance with a specific effect [52]. Natural compounds constitute a base for the design and development of new pharmaceuticals.

Among the secondary metabolites of plant origin with proven therapeutic properties, several can be distinguished, including alkaloids, terpenoids (diterpenes, triterpenes), and polyphenols, such as flavonoids and lignans. Some of them have established and documented anti-cancer activity, as discussed in this review.

4.1. Paclitaxel

Paclitaxel is undoubtedly one of the leading compounds with anti-cancer activity. Since the introduction of paclitaxel to the pharmaceutical market, under the name Taxol[®], it has been consistently popular due to its efficacy in the treatment of many types of cancer.

Paclitaxel belongs to the group of tricyclic diterpenoids. Structurally, paclitaxel encompasses the taxane ring system composed of a 6,8,6-tricyclic condensed scaffold connected to a four-membered ring of oxetane. It occurs naturally in the bark and needles of the short-leaf yew *Taxus brevifolia*. However, due to low availability or ecological reasons, alternative options for obtaining paclitaxel are used, i.e., plant cell culture [53], extraction from genetically modified fungi [54], chemical synthesis, or semi-synthesis [55]. The multi-step natural synthesis of this compound involves the cooperation of a variety of enzymes and makes the paclitaxel biosynthesis pathway complex and difficult to harness for the production of the drug [56].

The mechanism of action of paclitaxel differs compared to most compounds belonging to the class of division spindle inhibitors. During the initial stages of mitosis (prophase), a karyokinetic spindle, composed of microtubules, is formed. These fiber-like structures are responsible for the migration of chromosomes to opposite poles of the cell. Their dynamic instability, i.e., the ability to rapidly shorten or lengthen, is associated with the binding and hydrolysis of guanosine-5'-triphosphate (GTP). In the free state, GTP-bound $\alpha\beta$ -tubulin dimers are attached to the growing microtubule by polymerization [57]. If the process is slower than GTP hydrolysis, microtubules are broken down and shortened. This situation occurs in anaphase, when tubulin molecules depolymerize, leading to the shortening of spindle elements and the separation of chromosomes. Paclitaxel increases the percentage of folding tubulin subunits, stabilizing microtubules and preventing spindle breakdown [58]. In this way, it disrupts the normal course of mitosis. However, the pathway from stopping cell division to cell death has not been thoroughly elucidated. Prevention of mitosis can lead to cell death, but also to mitotic slippage that is understood as an exit from the cell cycle without chromosome segregation or without cytoplasm division [58]. The mitotic checkpoint plays a key role in the process of paclitaxel-mediated mitotic arrest as it is one of the main mechanisms of cell cycle control during division; activation of this process promotes the sensitivity of cells to the drug and is required for their elimination. Both cells with normal and weakened mitotic checkpoints show similar sensitivity to the drug [59,60]; some theorized that cell fate may also depend on the length of time of mitosis arrest, meaning that the consequences should be the same for all or most cells. When attempts were made to verify this view, however, its credibility was shattered, and sister cells were shown to have a different fate. It was therefore assumed that the response of cells to mitosis arrest is stochastic [61].

During numerous studies on paclitaxel, it was observed that, at certain concentrations, the compound induced the formation of multipolar spindles [62,63]. Cells arrested in metaphase tended to have bipolar spindles, and chromosomes aligned in the equatorial plane assumed a typical position. However, when using clinically relevant intratumor concentrations, paclitaxel did not lead to mitotic arrest. Instead, it caused cell death due to the abnormal segregation of multipolar spindles [64].

Currently, paclitaxel is one of the most potent anti-cancer drugs, mainly used as a first-line treatment for breast, ovarian, gastric, and non-small cell lung cancer [65]. It is also used in the treatment of other conditions, i.e., psoriasis [66], and acts as a neurotoxin inhibitor [67]. However, its utility is limited due to poor water solubility, the development of multidrug resistance (MDR) in the treated cells, or clinical neurotoxicity [68,69]. This has prompted the development of paclitaxel analogs that have a similar structure but improved pharmacokinetic parameters and reduced side effects. Various modifications of paclitaxel structure resulted in the introduction of docetaxel and cabazitaxel in clinical settings [70], and further led to the development of the paclitaxel liposomes, which involves encapsulating the drug in a nanocarrier to improve its distribution and solubility, as well as reducing the drug's toxicity [65]. The generation of nab-paclitaxel, a nanoparticle drug bound to albumin, allowed increases in drug solubility and reduction of post-therapy neuropathy symptoms [71].

The effect that conventional chemotherapy has on the body's immune system is currently gaining more and more attention. This is because the majority of studies examining the effectiveness of such drugs against cancer cells are carried out *in vitro* or on immunocompromised animals, and they do not include immunological follow-ups. In contrast, chemotherapy can provide the foundation for the immune system mobilization and reorganization, providing a tumor-targeted immune environment [72,73]. Paclitaxel and other compounds in the mitotic spindle inhibitor group have been shown to have immunomodulatory properties. Their effect on the stimulation of an adaptive anti-tumor immune response is manifested by the promotion of (a) the ability of cells to present antigens, (b) the activation of T and B lymphocytes, (c) the increase in the number and activity of natural killer effector cells, and (d) the production of antibodies and active substances, i.e., tumor necrosis factor α (TNF- α) and interleukins (IL-6, IL-8, and IL-12) [56]. Paclitaxel's effects on the immune system were studied in C57BL/6 mice with Lewis lung cancer (3LL) [72]. Paclitaxel was shown to selectively reduce the number of regulatory T cells (Treg cells), impairing their ability to produce pro-inflammatory cytokines without affecting CD4+Foxp3- effector T cells (Teff) [72]. Treg cells are considered key players in immune suppression, capable of blocking the immune response, inhibiting T cell proliferation, and maintaining a state of immune tolerance. However, in neoplastic diseases, they show enhanced immunosuppressive activity, facilitating tumor growth. Therefore, reducing their engagement may provide a more tumor-oriented immune microenvironment. In addition, paclitaxel reduces the production of transforming growth factor β (TGF- β), which in advanced tumors, promotes epithelial–mesenchymal transition (EMT), angiogenesis, and immunosuppression [56,74,75]. Analyses of peripheral blood samples from patients with advanced non-small cell lung cancer (NSCLC) showed that paclitaxel treatment increases the percentage of Teff cells, and decreases Treg cells levels, but did not affect the number of CD8+ T cells and CD19+ B cells [76]. At the same time, an increase in the number of memory T cells, which are the basis for the development of long-term immune memory, was also noted; this may be a guarantee of sustained remission of the disease. Chemotherapy with paclitaxel in combination with carboplatin is commonly used to treat melanoma. Both drugs can stimulate innate and acquired immune responses [56]. The anti-mitotic effect of paclitaxel promotes apoptotic death of tumor cells, accelerating the release of antigens and their presentation to immune cells. When used in this scheme, carboplatin can reduce the activity of programmed death receptor-ligand 2 (PD-L2), expressed in cancer cells, which plays a key role in their ability to evade the immune system by inhibiting T cells [74]. Blocking the interaction of the programmed death receptor (PD-1) with its ligand PD-L2 increases the detection of cancer cells, preventing them from escaping the anti-tumor immune response. A number of clinical trials are currently underway to estimate the effects of paclitaxel–carboplatin in combination with various cytostatics and/or monoclonal antibodies for the treatment of lung cancer and other cancers [77]. Examples of preclinical studies with paclitaxel and its derivatives and ongoing clinical trials are presented in Table 1.

Table 1. Preclinical and clinical studies with paclitaxel and its derivatives in monotherapy and combination therapies.

Agent	Type of Study	Eligible Disease Site(s) and Organism	Combinational Agent	Ref.
Paclitaxel Nab-paclitaxel	In vivo	Mouse model of B16F10 melanoma	Albumin-coated nanocrystal formulation of Paclitaxel and Abraxane (Nab-paclitaxel)	[78]
Docetaxel	In vivo	Rats' circulation system	Docetaxel nanocrystal-loaded micelles	[79]
Cabazitaxel	In vitro and in vivo	Mouse xenograft model PC346C-DCCCK	Cabazitaxel and enzalutamide	[80]
Agent	Phase of Clinical Trial	Eligible Disease Site(s)	Combinational Agent	Nct Clinical Trial Number
Paclitaxel	Phase 1	Ovarian cancer	Fostamatinib and Paclitaxel	NCT03246074
Paclitaxel	Phase 2	Lung cancer	Carboplatin, Paclitaxel, and AZD1775	NCT02513563
Paclitaxel	Phase 1/2	Gastric cancer	Varlitinib and Paclitaxel	NCT05400915
Paclitaxel	Phase 2	Breast cancer	Cisplatin vs. Paclitaxel	NCT01982448
Paclitaxel	Phase 1	Breast neoplasms	Cirmtuzumab and Paclitaxel	NCT02776917
Nab-paclitaxel	Phase 1/2	Pancreatic cancer	Gemcitabine, Nab-paclitaxel, and Paricalcitol	NCT03520790
Nab-paclitaxel	Phase 1	Breast cancer	Pembrolizumab and Nab-paclitaxel	NCT02999477
Nab-paclitaxel Paclitaxel	Phase 3	Breast cancer	Abraxane (Nab-paclitaxel) and Paclitaxel	NCT01822314
Nab-paclitaxel	Phase 2	Breast cancer	Mifepristone and Nab-paclitaxel	NCT02788981
Nab-paclitaxel	Phase 2	Breast cancer	Single agent	NCT04192331
Docetaxel	Phase 3	Prostate cancer	Single agent	NCT00653848
Docetaxel	Phase 2	Breast cancer	Docetaxel and Carboplatin	NCT02547987
Cabazitaxel	Phase 2	Prostate cancer	Abiraterone acetate, Cabazitaxel, and Prednisone	NCT02218606
Cabazitaxel	Phase 3	Adenocarcinoma of prostate/progression of prostate cancer	Cabazitaxel and radiotherapy	NCT01952223

4.2. Irinotecan

Irinotecan is one of the most popular compounds in the topoisomerase inhibitor group used in oncology. Although it is a semi-synthetic compound, it was developed based on a natural alkaloid, camptothecin, which showed significant anti-cancer activity, but was withdrawn from clinical trials due to its high toxicity and poor water solubility [37]. Many analogs have been developed based on this natural compound, including topotecan and irinotecan [81]. Of all the derivatives known to date, irinotecan (also known as CPT-11) is believed to have the highest therapeutic efficacy, and there are high hopes for its usefulness.

Irinotecan is a pentacyclic compound with an embedded bis-piperidine side chain [82]. It can undergo structural changes when exposed to the pH of the cellular environment. One of the key elements of irinotecan is the lactone ring, responsible for its metabolic activity and anti-cancer properties. Opening of the ring can lead to the inactivation of the compound because it is converted to a carboxylate that is impermeable through cell membranes [37]. Irinotecan exists in the form of a pro-drug, a substance that requires metabolic activation to exert biological activity. As a result of the hydrolysis of the compound by hepatic enzymes of the carboxylesterase group, irinotecan is converted to the active and highly toxic metabolite 7-ethyl-10-hydroxycamptothecin (SN38) [83]. Although the bioactivation process occurs rapidly, a significant portion of irinotecan is excreted

without being pre-metabolized. The resulting SN38 is transported to the liver, where it is inactivated and converted to SN38-glucuronide (SN38-G) by the microsomal enzyme uridine 5'-diphosphate-glucuronosyltransferase (UGT) [84]. This step is designed to detoxify the metabolite and facilitate its elimination from the body. The metabolites of irinotecan are excreted mainly in the feces, except for SN38-G, which is excreted with the bile [85]. In the final step, SN38-G is transported to the intestine, where it can be reactivated by bacterial β -glucuronidase (β G). Local reactivation of SN38 results in its reabsorption [86].

The main target of irinotecan's action is the nuclear enzyme topoisomerase I, which is responsible for maintaining the proper topology of DNA during the replication and transcription of genetic material [87,88]. Its task is to alleviate torsional tensions formed on the DNA molecule by the movement of multiprotein replication or transcription complexes along the strand. As proteins move along the strand, they unravel the double helix, causing buildup of positive and negative supercoils [89]. Supercoils negatively affect the availability of DNA for transcription factors and prolong the duration of the process. In addition, they disrupt the correct shape of DNA, which is unable to rotate around its axis. Therefore, enzymes are needed to enable the regulation of torsions exerted on the DNA molecule. The resolution of torsional stress is carried by topoisomerase enzymes, which are responsible for the addition of supercoils and their removal (DNA relaxation). Under physiological conditions, topoisomerase I cleaves a single ester bond in a DNA strand by covalently binding a tyrosyl group in the active site of the enzyme to a phosphoryl group of DNA molecule [90]. This is a fully controlled and reversible transesterification reaction, resulting in DNA relaxation [82]. The topoisomerase-DNA crosslink is hydrolyzed as soon as the supercoil is removed, and the DNA strands are ligated again. The attachment of irinotecan to the DNA-bound topoisomerase blocks the dissociation of the enzyme from the DNA molecule, leading to the exposition of the ternary complex for collision with replication forks followed by the formation of double-strand breaks (DSBs) in the DNA molecule. The resulting damage is detected by DDR, which forms a signaling cascade and activates subsequent molecular mechanisms [29], such as activation of cell cycle checkpoints, DNA repair, or apoptosis [91].

Topoisomerase I remains the main but not the only target of action of irinotecan and its metabolites. Other topoisomerase-independent interactions of SN38 with protein factors were reported. For example, the active metabolite of irinotecan has been shown to inhibit the DNA binding activity of human far upstream element binding protein 1 (FUBP1) [92]. The FUBP1 protein is important for the normal development of the nervous system and lungs [93,94]. Its overexpression is found in many cancers, where it plays an anti-apoptotic role, promoting the expansion of cancer cells. The interaction of SN38 with FUBP1 prevents the oncoprotein from binding to molecular targets. Other proposed targets include the mouse homolog of double minute 2 (MDM2) that works as the E3 ligase of the tumor suppressor *TP53* and the anti-apoptotic B-cell lymphoma-extra large (BCL-xL) in human hepatocellular carcinoma cells. These interactions result in the reduction in cell proliferation and cell-cycle arrest in the G2/M phase [92]. Takeba et al. demonstrated that SN38 promotes apoptosis in a *TP53*-dependent manner [95]. However, apoptosis is not the only cellular response to irinotecan treatment. Cells exposed to SN-38 exhibit characteristic features of senescent cells including growth arrest, increased size and granularity, polyploidization or accumulation of P21 and cyclin D1 proteins [96]. These and other findings demonstrate that camptothecin derivatives are still an under-explored source of therapeutic agents with multidirectional effects.

For more than 2 decades, irinotecan has been used to treat colorectal cancer [97], lung cancer [98], stomach cancer [99], ovarian cancer [100], and others. Approved by the US Federal Drug Administration (FDA) under the name Campto[®], it is used both as monotherapy and in combination with other chemotherapeutics. Combinations with kinase inhibitors [101,102], platinum-based drugs [103], or immunotherapy [104] are also common. Efforts are also undertaken to improve drug stability and reduce gastrointestinal toxicity by introducing prophylactic agents that inhibit SN38 reabsorption (alkylating drugs, plant

extracts, probiotics), as well as nanoparticles, peptides, and carbohydrates with the goal of improving the clinical utility of the drug. Oncoral[®] has recently been developed as an enteric-coated tablet that is released in the duodenum, improving tolerance for the drug. In addition, liposomal formulations of irinotecan are also being introduced to protect against structural changes and chemical degradation and to improve the cytotoxic effect of irinotecan [105,106]. Improving quality and prolonging patient survival are overarching goals in efforts to develop effective formulations based on irinotecan and other camptothecin derivatives. Examples of preclinical studies with irinotecan and ongoing clinical trials are presented in Table 2.

Table 2. Preclinical and clinical studies with irinotecan and its modification in monotherapy and combination therapies.

Agent	Type of Study	Eligible Disease Site(s) and Organism	Combinational Agent	Ref.
Irinotecan	In vitro and in vivo	Mouse H22 tumor model	Irinotecan-loaded PLGA microspheres	[107]
Irinotecan	In vivo	Rabbit VX2 liver tumor model	Irinotecan-loaded QuadraSphere microspheres	[108]
Irinotecan	In vitro and in vivo	Wistar rats and potentially colon cancer	Irinotecan-loaded solid lipid nanoparticles	[109]
Agent	Phase of Clinical Trial	Eligible Disease site(s)	Combinational Agent	NCT Clinical Trial Number
Irinotecan	Phase 1/2	Small cell lung cancer	DS-3201b and Irinotecan	NCT03879798
Irinotecan	Phase 3	Small cell lung cancer	Irinotecan liposome injection vs. Topotecan	NCT03088813
Nanoliposomal Irinotecan	Phase 1	Refractory solid tumors	Nanoliposomal Irinotecan and TAS-102	NCT03810742
Nanoliposomal Irinotecan	Phase 1/2	Biliary tract cancer	Nivolumab plus nanoliposomal Irinotecan, 5-Fluorouracil, and Leucovorin	NCT03785873
Irinotecan Hydrochloride	Phase 1	Metastatic colorectal carcinoma	Utomilumab, catuximab, and irinotecan hydrochloride	NCT03290937

4.3. Betulinic Acid

Betulinic acid (BA) is a pentacyclic compound that belongs to the triterpenoid group [110]. It occurs naturally in many plant species, including birch, eucalyptus, and plane trees. It exhibits a broad spectrum of biological properties, including anti-inflammatory and anti-cancer activities, making it an interesting candidate for pharmacological utility. Several of the BA derivatives known to date have entered clinical trials (NCT00701987, NCT00346502, www.clinicaltrials.gov).

The main method of obtaining betulinic acid is extraction from plant materials, with bark being the most commonly used material, and leaves and stems being less common. However, unlike betulin, the content of betulinic acid in extracts is low, usually about 4–12% [111]. These amounts are not sufficient to cover the market demand and allow free large-scale research. In addition, methods of sublimation, ultrasonic extraction, or extraction with organic solvents are not very environmentally friendly and involve the generation of hazardous waste [112,113]. Therefore, efforts have focused on other methods of obtaining BA, i.e., chemical synthesis. Since betulin is a natural precursor of betulinic acid, it is the primary substrate for its synthesis [111]. One method of BA synthesis involves the oxidation reaction of betulin using the Jones reagent, followed by the reduction of the resulting betulinic acid [114] or the chemoselective oxidation of betulin's hydroxyl group directly to a carboxyl group at the C17 position [115]. The described BA synthesis reactions are time-consuming, and the reagents used in them are both toxic and expensive. Other

techniques include biotransformation catalyzed by the fungi *Aspergillus foetidus* ZU-G1, *Aspergillus oryzae* [116], *Armillaria luteovirens* Sacc ZJUQH100-6 [117], and the expression of the compound in transgenic yeast [118]. Similar to other methods, biotransformation processes and genetic engineering tools have limitations and drawbacks, including the deep understanding of the betulinic acid biosynthetic pathways. Despite these facts, knowledge regarding its activity is steadily increasing.

Compounds belonging to the triterpene group often exhibit anti-diabetic and anti-inflammatory properties. The anti-inflammatory activity of the compounds is particularly important in the context of cancer development. Chronic inflammation leads to the formation of an inflammatory tumor microenvironment (TME) that is conducive to escape from the supervision of the immune system and promoting progression as well as metastasis [119]. The microenvironment is created by cells of the immune system, i.e., macrophages, B lymphocytes, subpopulations of T lymphocytes, dendritic cells, and natural killer (NK) cells, which act against cancer. The response generated by them, however, can be effectively inhibited by immunosuppressive cells (Tregs cells, type 2 macrophages), which drive the state of chronic inflammation and pro-angiogenic environment [120]. The constant presence of immune system cells and the proteolytic enzymes they produce can start to work in favor of the tumor, promoting survival, angiogenesis, and impairment of the function of anti-cancer lymphocyte populations. The anti-inflammatory and anti-diabetic properties of betulinic acid have been confirmed in vitro and in vivo. In a study by Khataylou et al., mice with autoimmune diabetes treated with BA exhibited a decrease in the inflammatory cytokines IL-1 and TNF- α in the serum and kidneys, with an increase in anti-inflammatory cytokines IL-10, C-peptide, and insulin, as well as an improvement in the histopathological changes of the kidneys. Betulinic acid may thus show efficacy in the treatment of inflammatory and autoimmune diseases [121]. In a mouse model of endotoxemia, the administration of BA protected mice from a lethal dose of lipopolysaccharide (LPS) [122]. It also caused an increase in IL-10 levels, which inhibited LPS-induced TNF- α release. In in vitro experiments, the same authors showed that BA treatment inhibited TNF- α and nitric oxide (NO) levels in LPS-activated macrophages, accompanied by an increase in IL-10 production [122]. The nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) is one of the main factors responsible for the regulation of gene expression and the production of proteins associated with proliferation, inflammation, tumorigenesis, and cell death. It is present in the cytosol in an inactive state, with the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitory protein [123]. As a result of incoming stimuli (including TNF family ligands), the I κ B Kinase (IKK β) is activated; this phosphorylates I κ B and leads to its ubiquitination and degradation [123]. The released NF- κ B moves to the nucleus, where it binds to DNA and induces the expression of pro-inflammatory factors [124]. Viji et al. showed that inhibition of I κ B α by betulinic acid prevents nuclear translocation of NF- κ B and, therefore, up-regulation of pro-inflammatory factors [125]. Furthermore, by inhibiting the cell signaling cascade of the NF- κ B, extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase (MAPK), and protein kinase B (AKT) pathways, betulinic acid contributes to the inhibition of the synthesis of critical pro-inflammatory mediators and enzymes, i.e., cyclooxygenase 2 (COX-2) and pro-inflammatory prostaglandin E2 (PGE2). Blockage of the ERK/Mitogen-activated protein kinase (MEK) signaling pathway was also observed, as well as a decrease in tumor cell migration and invasion [126] (Figure 2).

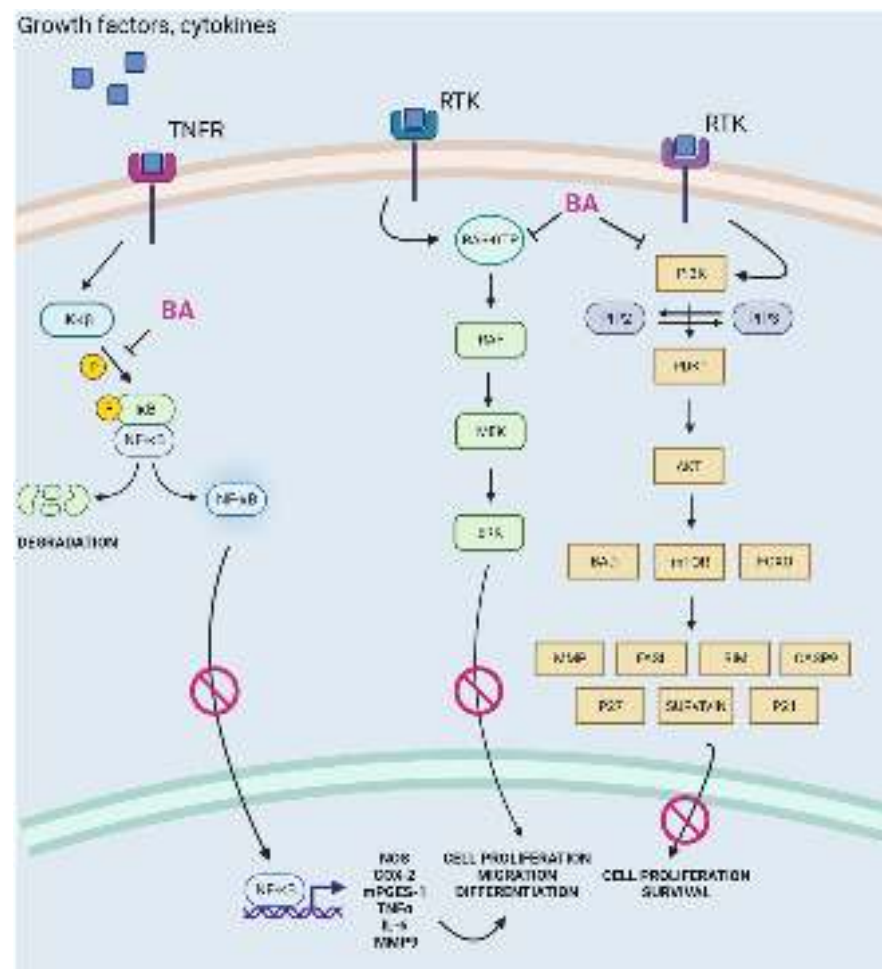


Figure 2. The activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathways that support the development of the tumor and the pro-inflammatory cellular environment. Phosphorylation of B-cells inhibitor (IκB) by IκB kinase (IKKβ) as a result of cellular signals (growth factors, cytokines) received by receptor (tumor necrosis factor receptors (TNFR)), results in heterodimer disconnection, release of NF-κB, and degradation of IκB complex component. The released NF-κB translocates from the cytoplasm to the nucleus, where it acts as a transcription factor responsible for the expression of proteins involved in cell proliferation, migration, and inflammation. The activation of receptor tyrosine kinases (RTKs) initiates activation of ERK/MAPK and PI3K/AKT pathways, promoting survival, migration, and invasion of cancer cells. Binding of the ligand to the RTKs causes the “turning on” of the rat sarcoma virus (RAS) protein through GDP-to-GTP exchange, which activates the RAF proto-oncogene serine/threonine-protein kinase (RAF), mitogen-activated protein kinase (MEK) and ERK kinase, leading to the growth of cancer cells and supporting the transcription of pro-inflammatory factors. Similarly, PI3K is activated via RTKs, which catalyze the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3). As a result of the interaction of PIP3 and phosphatidylinositol kinase (PDK-1), AKT is activated. Being a serine-threonine kinase, AKT phosphorylates numerous proteins involved in the regulation of cell proliferation, growth, and survival. The key substrates for AKT are the mammalian target of rapamycin (mTOR) and the pro-apoptotic factor BCL-2-associated death promoter (BAD), which are capable of inhibiting apoptosis, and nuclear transcription factors fork head box O (FOXO). The use of betulinic acid (BA) prevents the phosphorylation of IκB, and thus, the release and translocation of NF-κB into the nucleus. As a consequence, the expression of inflammatory mediators and enzymes is not induced. BA also inhibits the activation of ERK/MAPK and PI3K/AKT signaling pathways, suppressing mechanisms oriented toward tumor growth and development. Created with BioRender.com.

Untreated chronic inflammation can lead to the development of cancer. Numerous studies have confirmed that BA exhibits not only anti-inflammatory but also anti-cancer properties. It acts in multiple ways, including topoisomerases inhibition (anti-proliferative activity) and suppression of angiogenesis, but its ability to induce the intrinsic apoptotic pathway is the major mechanism of BA action [111]. Since its anti-cancer properties in melanoma were described in 1995 [127], there have been regular reports of its use in other types of cancer. Lee et al. reported that BA induced apoptosis in A2780 ovarian cancer cells by increasing the expression of caspases -3, -8, -9, and proapoptotic BCL-2-associated X protein (BAX) protein [128]. Treatment of BA ovarian cancer cells followed by Hoechst 33342 staining showed bright blue condensed nuclear chromatin, which is a morphological feature typical of apoptotic cells, suggesting that betulinic acid induces this type of cell death. Luo et al. showed that BA inhibits topoisomerases and decreases cyclin expression, leading to cell cycle arrest and apoptotic death [129]. Betulinic acid inhibits the binding of NF- κ B to specific sequences of NF- κ B response elements (NRE) and proteasome-dependent degradation of transcription factors, i.e., transcription factor Sp1 (SP1), transcription factor Sp3 (SP3), and down-regulation of vascular endothelial growth factor (VEGF) expression. SP1 and SP3 are transcription factors involved in chromatin remodeling; depending on transcript modification, by binding to GC-BOXes sequences in promoter regions, they may act as activators or repressors of gene expression of numerous of genes, including oncogenes and tumor suppressors as well as proteins involved in cell cycle regulation, damage response, or apoptosis [130]. Similarly, overexpression of VEGF in tumor cells correlates with an increase in tumor aggressiveness due to its potent pro-angiogenic functions. By activating various signaling pathways, i.e., phosphoinositide 3-kinase (PI3K)/AKT and MAPK/ERK, VEGF plays role in the chemotaxis and migration of cancer cells, and also stimulates the secretion of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6) and proteolytic enzymes (matrix metalloproteinase-9 (MMP-9)) [131] (Figure 3). MMP-9 has a proteolytic cleavage activity for the extracellular matrix (ECM) enabling its remodeling and increasing the metastatic potential of cells. Degradation of basement membrane components (i.e., type IV collagen) by MMP-9 promotes tumor invasion and metastasis [131]. It has been observed that treatment of cancer cells with BA causes a decrease in the expression of MMPs, indicating that BA may be a protective factor in cancer progression [127]. As a consequence of BA treatment, human gastric cancer MGC-803 cells showed changes in mitochondrial membrane permeability (MiMP) with the cytochrome C release and activation of caspase -3 and -9 enzymes and nuclear DNA degradation [132]. These changes indicated the induction of death via the mitochondrial pathway of apoptosis.

Apoptosis is reportedly not the only BA-initiated cell death-related process. Autophagy is a catabolic process involving the massive degradation of cytoplasm and organelles through fusion with lysosomes [133]. Although the process physiologically mediates cell survival under stress conditions, it has been suggested that it may also be exploited to specifically target cancer cells [133]. Several different types of autophagy involve complex pathways and multiple protein factors. Among the best-studied proteins that trigger autophagy are mammalian target of rapamycin complex 1 (mTORC1) and 5' AMP-activated protein kinase (AMPK). mTORC1 is a protein complex composed of mTOR kinase and mTOR associated protein LST8 homolog (MLST8) [134]. Acting as a negative regulator, mTORC1 phosphorylates proteins essential for the occurrence of autophagy, i.e., autophagy-associated protein 13 (ATG13) and Unc-51 like autophagy activating kinase (ULK1), inhibit their activity [133–135]. The role of AMPK is to promote autophagy by inducing ULK1 [134]. Inactivation of mTORC1 combined with simultaneous activation of AMPK drives the formation of a phagophore structure from intracellular membranes. The formation of the phagophore membrane is mediated by autophagy-associated protein 5 (ATG5), autophagy-associated protein 12 (ATG12), and autophagy-associated protein 16 (ATG16). The vesicular protein sorting kinase 34 (VPS34), in complex with beclin 1, stimulates the production of PIP3, which is necessary for phagophore elongation and recruitment of other execution proteins. Then, the process of conjugation involving autophagy-related

protein 3 (ATG3), autophagy-related protein 4 (ATG4), and autophagy-related protein 7 (ATG7) to protein light chain (LC3) is initiated, during which the LC3 protein is processed to LC3-I, LC3-II, and LC3-III [136]. Meanwhile, an autophagosome is formed from the phagophore, containing part of the cytoplasm along with proteins destined for degradation. The LC3-II form participates in the selection of transported material by binding to p62/sequestosome 1 (SQTM 1) [135,136]. This is followed by the fusion of the autophagosome with the lysosome, resulting in the formation of the autolysosome, in which the encapsulated material undergoes enzymatic digestion. The general concept of autophagosome formation is shown in Figure 4. Wang et al. reported that BA induces autophagy of SGC-7901 gastric cancer cell line, indicating a more versatile mode of action of BA [126]. This was indicated by increased levels of LC3-II and decreased expression of LC3-I and p62 [126]. A decrease in the expression levels of phosphorylated ERK and MEK proteins was also observed. This suggests that the anti-tumor effect of BA on SGC-7901 gastric cancer cells is related to the induction of cell death with autophagy and blockage of the ERK/MEK signaling pathway [126]. To circumvent cellular mechanisms of drug resistance, as well as to increase the solubility and bioavailability of betulinic acid, new formulations of BA are developed. Wang et al. compared the efficacy of free betulinic acid with the effects of betulinic acid-loaded nanoliposomes on colon cancer cells [137]. Both formulations showed significant anti-cancer effects; however, the nanoliposomal formulation showed superior anti-cancer activity in vitro and in vivo as indicated by increased cellular uptake and proliferation inhibition compared with free BA. Zhao et al. used betulinic acid nanoparticles on lung cancer cells of HKULC2, H1299, and H23 lines [138]. The results showed a decrease in proliferation in all cell lines tested. HKULC2 proved to be particularly sensitive to BA treatment, where a decrease in migratory potential, invasiveness, and metastatic capacity were also observed [138].

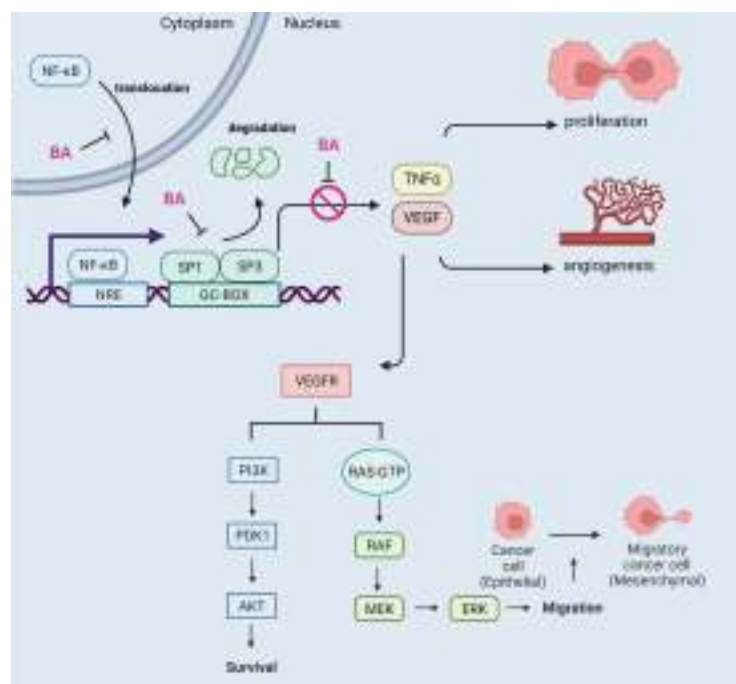


Figure 3. Tumor necrosis factor α (TNF- α) and vascular endothelial growth factor (VEGF) with pro-angiogenic function are overexpressed in tumors depending on transcription factors, i.e., transcription factor Sp1 (SP1) and transcription factor Sp3 (SP3). They are supported by the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), which bind to specific sequences known as NF- κ B response elements (NRE) and GC-BOXes. The presence of VEGF in the cell enhances the activation of the rat sarcoma virus-GTP (RAS-GTP)/RAF proto-oncogene serine/threonine-protein kinase (RAF)/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) and

phosphoinositide 3-kinase (PI3K)/phosphatidylinositol kinase (PDK-1)/protein kinase B (AKT) pathways, promoting invasive potential and tumor metastases. Betulinic acid (BA) prevents the translocation of NF- κ B to the nucleus and promotes the degradation of SP1 and SP3, down-regulating VEGF. Created with BioRender.com.

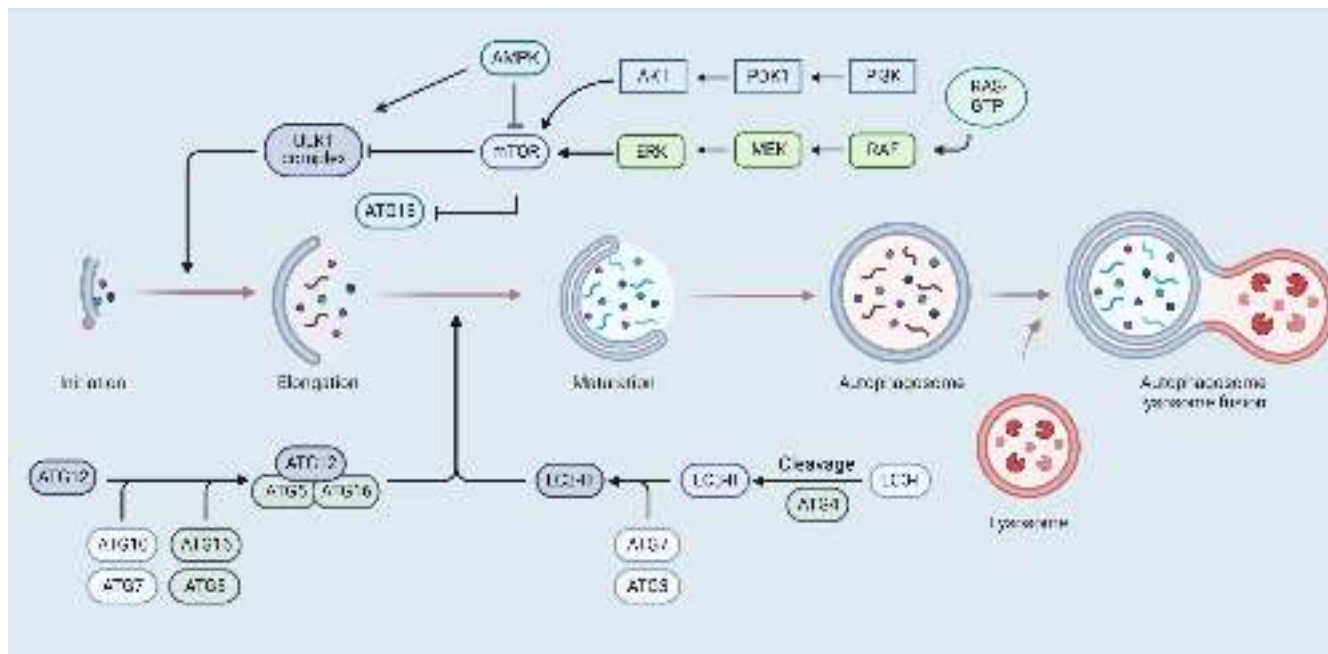


Figure 4. The process of autophagolysosome formation. Stimulated by the mitogen-activated protein kinase (MAPK) and protein kinase B (AKT) pathways, mammalian target of rapamycin (mTOR) and 5' AMP-activated protein kinase (AMPK) regulate autophagy by interacting with Unc-51 like autophagy activating kinase (ULK1) complex and autophagy-related protein 13. ULK1, activated by AMPK, initiates autophagosome formation from intracellular membranes and its elongation. Then the autophagosome enters the elongation phase. Under the influence of the ATG3, ATG4 and ATG7 proteins, protein light chain (LC3) protein is converted to the LC3-I, LC3-II and LC3-III forms. The autophagy-related proteins (ATGs) (like ATG5, ATG7, ATG10, ATG12, ATG16) together with LC3-III contribute to the maturation of autophagosome that, through fusion with the lysosome, forms an autophagolysosome, which is a structure capable of enzymatic digestion of proteins. Created with BioRender.com.

The first phase I/II clinical trials on BA were implemented in 2006 (NCT00346502, University of Illinois, USA, www.clinicaltrials.gov). Their objective was to evaluate the safety and efficacy of an ointment containing 20% BA in the treatment of dysplastic melanocytic nevi (DMN), which in many cases can be a precursor to melanoma. Unfortunately, due to a lack of funding, the study was suspended in 2013, and the results were not published. Another phase I clinical trial began in 2008, where BA (ALS-357) was administered as an ointment for 4 weeks to patients with metastatic melanoma. In addition to efficacy and safety, histopathological features and apoptotic markers were evaluated. Unfortunately, the results were never published. Information on the range of concentrations tested was also not provided.

Interest has shifted from betulinic acid to its natural and synthetic derivatives, which, in addition to their anti-cancer activity, are also characterized by high cytotoxicity and better water solubility. Liebscher et al. studied the effects of BA and its derivatives, NVX-207 and B10, on melanoma cells in horses [139]. All examined compounds exhibited cytotoxic effects, promoting death through activation of the intrinsic apoptotic pathway. NVX-207 showed the highest efficacy and was then encapsulated in 2-hydroxypropyl- β -cyclodextrin and used in in vivo studies in melanoma-affected horses. The substance was well-tolerated. The safety and efficacy of NVX-207 were also evaluated in phase I/II clinical trials in dogs

with spontaneous tumors [140]. Treatment consisting of topical administration of NVX-207 and NVX-207 in combination with cisplatin resulted in a favorable response to treatment in the form of a reduction in tumor size in all subjects. The therapy was not accompanied by systemic side effects [140].

Despite the many benefits of BA in vitro and in vivo, clinical trials examining its anti-cancer properties seem to have stalled long ago. Clinical trials focusing on other properties of this compound, i.e., anti-anxiety and stress-reducing properties, have been undertaken (NCT03904511, University of Ottawa, Canada, www.clinicaltrials.gov). Nevertheless, there has been a recent resurgence of interest in compounds of natural origin. Nowadays, the number of studies on natural compounds are increase rapidly, including research on BA. Perhaps this the aspect of betulinic acid's anti-cancer properties will also return in clinical trials. Examples of preclinical studies with betulinic acid and its derivatives and clinical trials are presented in Table 3.

Table 3. Preclinical and clinical studies with betulinic acid and its derivatives.

Agent	Type of Study	Eligible Disease Site(s) and Organism	Combinational Agent	Ref.
Betulinic acid	In vitro and in vivo	Murine models with TNBS- and DSS-induced inflammatory bowel disease	Hydroxamate of Betulinic Acid	[141]
Betulinic acid	In vitro and in vivo	Mouse model with colorectal cancer	Betulinic Acid-loaded liposomes coated with Eudragit S100	[142]
Betulinic acid analogue (2c)	In vitro and in vivo	Mice and rats with colorectal cancer	Nanoencapsulated betulinic acid analogue	[143]
Betulinyll-bis-sulfamate NVX-207	In vitro	Equine sarcoid cells, equine malignant melanoma, and fibroblasts	Betulinyll-bis-sulfamate NVX-207	[144]
Betulinic acid NVX-207	In vivo	Horses with melanocytic tumors	Betulinic acid and NVX-207	[145]
Agent	Phase of Clinical Trial	Eligible Disease Site(s)	Combinational Agent	NCT Clinical Trial Number
Betulinic acid	Phase 1 (unknown)	Melanoma	ALS-357 (Betulinic Acid)	NCT00701987
Betulinic acid	Phase 1/2 (withdrawn)	Dysplastic Nevus Syndrome	20% Betulinic Acid Ointment	NCT00346502

4.4. Resveratrol

Resveratrol is a natural organic compound, belonging to the group of polyphenols and based on a stilbene scaffold. It is synthesized by an enzyme, stilbene synthase, as a secondary plant metabolite [146,147]. Its synthesis and accumulation usually occur in response to stress conditions, i.e., pathogen infection, mechanical damage, or unfavorable abiotic conditions. Resveratrol was first isolated in the late 1930s from the root of *Veratrum album*. Subsequently, it was also discovered in *Polygonum cuspidatum*. It is now known to occur in more than 70 plant species, including grapes and red wine, blueberries, bilberries, black currants, raspberries, peanuts, and products containing cocoa [148–150]. Due to the complicated and inefficient extraction process from plant tissues, alternative methods of production are used, including synthesis in recombinant yeast [151].

Resveratrol is a natural anti-oxidant used in the fight against free radicals and photoaging. It exists in two isomeric forms: an intractable *cis*- and a highly active *trans*- form. Conversion from *trans*- to *cis*- isomer occurs due to UV radiation and/or under high pH conditions [152]. Due to its stability, research has focused mainly on *trans*-resveratrol. Its structure features two phenol rings, and unlike, the stilbene from which it is derived, the

phenyl groups at the 3', 4', and 5' positions of the ring have been substituted by hydroxyl groups, which influence its anti-oxidant properties. Stivala et al. showed that the presence of the 4' hydroxyl group is necessary for its activity, while the presence of other 3' and 5' enhance its biological effects [153]. In addition to anti-oxidant properties, resveratrol also exhibits anti-inflammatory and anti-cancer activities. It acts pleiotropically by modulating the activity of multiple signaling pathways, including the DDR pathway [154]. Resveratrol leads to the stimulation of the TP53 via phosphorylation by the phosphatidylinositol 3-kinase-related kinases (PIKK) family kinases, including ataxia teleangiectasia mutated (ATM) and ataxia teleangiectasia and Rad3 related (ATR). ATM/ATR phosphorylates checkpoint kinase 1 (CHK1) and checkpoint kinase 2 (CHK2) therefore stabilize TP53 and prevent its interaction with inhibitory factor MDM2 [155] (Figure 5A). The TP53 gene product TP53 works as a transcription factor for many target genes and is a critical tumor suppressor protein [156]. Shang et al. described the anti-inflammatory and cardioprotective properties of resveratrol in a rat model of cardiomyopathy and sepsis [157]. The authors investigated the relationship between the PI3K/AKT/mTOR signaling pathway and NF- κ B activity, and the effect of resveratrol on both pathways. Rats treated with resveratrol showed inhibition of myocardial tissue damage and a decrease in the number of apoptotic cells. This was accompanied by an increase in the expression of PI3K, AKT, and mTOR proteins, with down-regulation of factors associated with the NF- κ B pathway i.e., TNF- α , IL-6, IL-1 β , and toll-like receptor 4 (TLR4) [157]. Mohapatra et al. evaluated the effects of resveratrol on MCF-10A mammary gland epithelial cells transformed with cigarette smoke condensate (CSCs) in a mouse xenograft model [158]. Treatment of MCF-10A with resveratrol-caused cell death via the apoptotic pathway, which is related to resveratrol's ability to trigger damage to DNA and affect the DNA repair processes occurring in cells. A dose-dependent decrease in the expression of the main protein components of the base excision repair (BER) pathway, including β , δ , ϵ polymerases, proliferating cell nuclear antigen (PCNA), and Flap endonuclease 1 (FEN-1), were demonstrated in MCF-10A, while no changes in gene expression were observed in P21-depleted MCF-10A cells. This is consistent with previous reports on the role of P21 in interference with DNA repair factors, such as DNA polymerase δ [159] and FEN-1 [158,160]. In the in vivo model, a reduction in tumor size and a decrease in the expression of anti-apoptotic markers PI3K/AKT and NF- κ B were observed. The results suggest that resveratrol-induced impairment of BER activity may trigger apoptosis in a P21-dependent manner [158]. The above studies also demonstrate the dualistic nature of resveratrol, which can protect cells from apoptosis and inflammation but can also induce the death of cancer cells. Another important process involved in cancer metastasis is the EMT. In this transition, cells lose their epithelial characteristics in favor of mesenchymal characteristics. Until recently, this process was understood to include two separate cell populations and to be accompanied by a decrease in the expression of epithelial markers (E-cadherin, cytokeratins) and an increase in mesenchymal markers (N-cadherin, vimentin, fibronectin, MMPs) [161]. However, recent research suggests that EMT is gradual process and is manifested by varying cellular states and fluctuating levels of epithelial and mesenchymal markers [162,163]. Thus, cells may show intermediate or hybrid EMT features, taking into account morphological, transcriptional, and epigenetic biological aspects [161]. EMT is triggered by many factors, the most prominent of which is TGF- β , which binds to its cognate receptor transforming growth factor β receptor (TGF- β R) [164]. As a result of promoting EMT, the cell initiates the expression of mesenchymal markers and transcription factors: zinc finger protein SNAI1 (SNAIL), zinc finger protein SNAI2 (SLUG), and zinc finger E-box binding $\frac{1}{2}$ eobox 1/2 (ZEB1/2) [165]. During EMT, the loss of connection with the basement membrane and other cells, digestion of the extracellular matrix, and increased cell motility are observed [161]. The process of MAPK and AKT pathways activation that promotes EMT is shown in Figure 5B. Resveratrol has been shown to inhibit TGF- β -induced EMT in LoVo colon cancer cells in vitro and in vivo, accompanied by a decrease in the expression of EMT-related factors, i.e., MMPs, extracellular matrix-degrading proteins, and vascular basement membrane proteins that normally

promote metastasis [166]. Li et al. have also reported that resveratrol caused a therapeutic effect by inhibiting the viability, proliferation, and EMT of prostate cancer cells by reducing the level of TNF receptor-associated factor 6 (TRAF6) [167]. Overexpression of TRAF6 may lead to increased cell migration and promote the transcription of genes encoding EMT-related proteins. TRAF6 undergoes K63-linked ubiquitination induced by TGF- β , which, unlike K48-linked ubiquitination, does not degrade the protein, but instead serves as a regulatory signal and a scaffold for the assembly of protein complexes responsible for the activation of other protein mediators [168]. Polyubiquitinated TRAF6 is required for the activation of the tertiary kinase cascade, which activates the N-terminal kinase c-Jun (JNK) and p38. Increased activation of JNK and p38, which belong to the MAPK pathway, is accompanied by overexpression of the transcription factors SNAIL and SLUG and promotes tumor suppression and EMT transition [168]. The above examples underline the effects of resveratrol on key signaling pathways involved in the etiopathogenesis and development of cancer. Due to the plethora of publications in this review, we did not focus on a specific type of cancer, but rather on the mechanism of action. This topic was reviewed elsewhere [169].

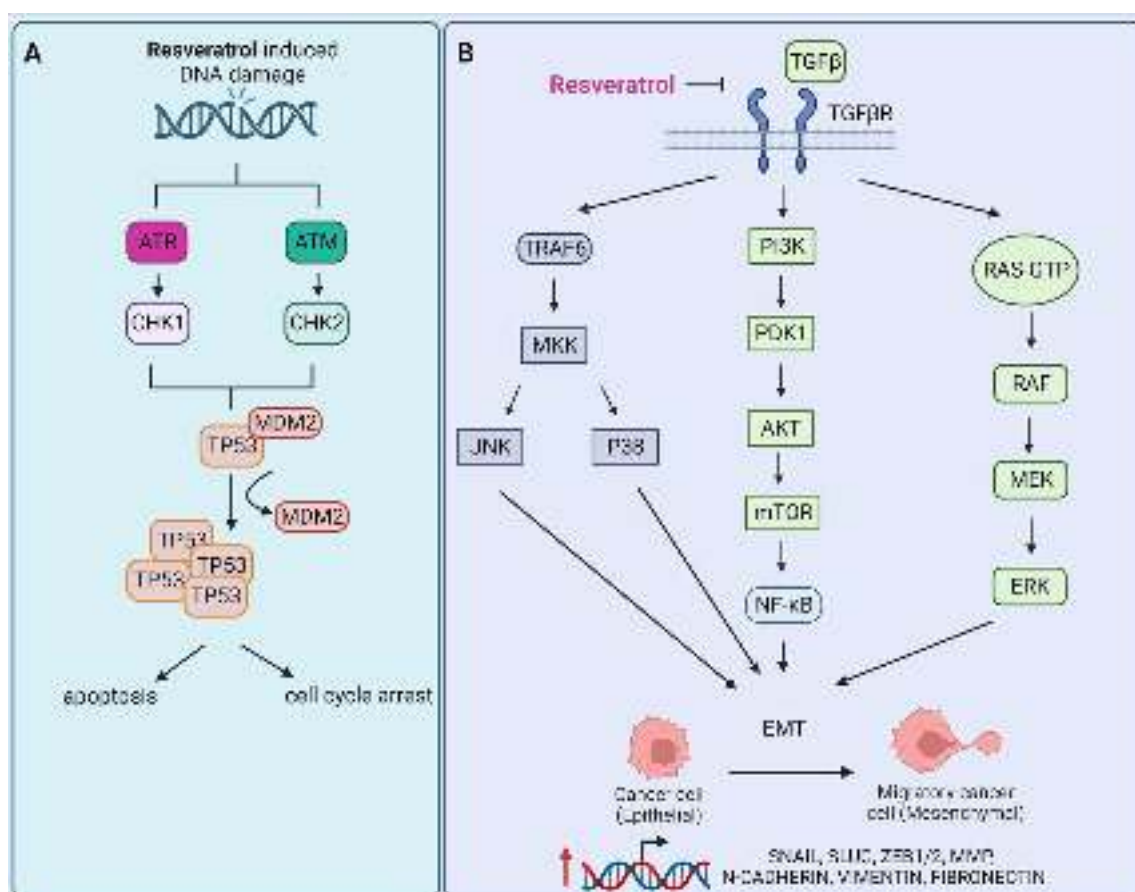


Figure 5. (A) Resveratrol can induce DNA damage, resulting in the recruitment of ataxia telangiectasia mutated (ATM)/ataxia telangiectasia and RAD3 related (ATR) kinases that phosphorylate checkpoint kinase 1 (CHK1)/checkpoint kinase 2 (CHK2) serine-threonine kinases. This prevents the interaction of tumor protein P53 (*TP53*) with mouse homolog of double minute 2 (MDM2), thereby protecting the *TP53* protein from degradation. *TP53* induces the expression of genes involved in cell cycle arrest and apoptosis. (B) Epithelial–mesenchymal transition (EMT) is initiated by many factors, including the activation of transforming growth factor β receptor (TGF- β R) by the native ligand transforming growth factor β (TGF- β). This event initiates the signaling through rat sarcoma

virus-GTP (RAS-GTP)/RAF proto-oncogene serine/threonine-protein kinase (RAF)/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K)/phosphatidylinositol kinase (PDK-1)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR)/ nuclear factor kappa-light-chain-enhancer of activated B Cells (NF- κ B) pathways, resulting in the expression of EMT-related factors. EMT can also be promoted by c-Jun N-terminal kinase (JNK) and p38 kinase, which are members of the mitogen-activated protein kinase (MAPK) pathway. They are activated by MAP kinase (MKK) and TNF receptor-associated factor 6 (TRAF6) with K63-linked polyubiquitin chains by TGF- β -dependent polyubiquitinylation. Resveratrol inhibits TGF- β and TRAF6, preventing EMT of cancer cells. Created with BioRender.com.

Due to its high therapeutic potential and success in preclinical studies, resveratrol has moved into clinical trials. The first reports *in vitro* and *in vivo*, focusing on the pharmacokinetics and safety of the substance, showed that the concentrations used in tests are not achievable in humans [170]. The low systemic availability is most likely attributed to the compound's rapid metabolism. Moreover, resveratrol exhibited a non-linear dose-response relationship. In phase I clinical trial (NCT0098969, University of Michigan Comprehensive Cancer Center, Ann Arbor, Michigan, USA, www.clinicaltrials.gov), Brown et al. administered resveratrol to 40 healthy subjects at a dose of 0.5, 1, 2.5, or 5 g daily for 29 days [170]. All doses used were safe, although the two highest doses (2.5 and 5 g) reported mild gastrointestinal symptoms. The most effective inhibitory effect on the expression of plasma IGF-1 and IGFBP-3 was observed for a dose of 2.5 g/day. In contrast, at a dose of 5 g/day, the highest circulating levels of the parent agent were observed; these levels were close to the concentrations achieved in cells *in vitro* and were intended to cause pharmacological activity. Cai et al. compared the tissue distribution and efficacy of different doses of resveratrol *ex vivo* and *in vivo* [171]. The results showed that resveratrol at a low dose of 5 mg/day had higher efficacy compared to the dose used by Brown et al. The authors also suggested that the anti-neoplastic properties of resveratrol result from autophagy and senescence induction of cancer cells [171]. Other studies investigated resveratrol used in milligram doses; however, they failed to achieve the target IC₅₀ concentration in human plasma needed for chemopreventive effects [172,173]. In an attempt to circumvent the compound's bioavailability problem, a micronized formulation of resveratrol was used in phase I clinical trials in patients with colorectal cancer (NCT00920803, GSK Investigational Site, Leicester, Leicestershire, United Kingdom, www.clinicaltrials.gov). This allowed for a three-times higher maximum concentration compared to non-micronized resveratrol. The authors reported a pro-apoptotic effect of the compound manifested by an increase in the expression of cleaved caspase-3 [174]. Resveratrol together with other anti-oxidant compounds is being investigated for its epigenetic influence and reduction of DNA damage (NCT05306002, Institute for Social Security and Services for State Workers, Merida, Yucatán, Mexico, www.clinicaltrials.gov) and in phase II study in combination with sirolimus in patients with lymphangioleiomyomatosis (LAM) (NCT03253913, University of Cincinnati, Ohio, United States, www.clinicaltrials.gov).

The results obtained so far show that resveratrol is a thriving preventive and/or therapeutic agent against many types of cancer. Like most natural compounds, it has some limitations, like low bioavailability, poor solubility, and rapid metabolism. Work is underway to overcome the described problems with available tools, i.e., nanoparticles, biodegradable formulations, use of resveratrol derivatives or metabolites, and others [175]. Resveratrol is one of the hot topics in the field of oncology and the development of new anti-cancer agents. Examples of preclinical studies with resveratrol and clinical trials are presented in Table 4.

Table 4. Preclinical and clinical studies with resveratrol.

Agent	Type of Study	Eligible Disease Site(s) and Organism	Combinational Agent	Ref.
Resveratrol	In vivo	Athymic BALB/C nude mice injected with HeLa cells (cervical cancer)	Single agent	[176]
Resveratrol	In vivo	Mouse xenograft tumor model cervical cancer	Hydroxypropyl- β -cyclodextrin-complexed Resveratrol	[177]
Resveratrol	In vitro and in vivo	Mouse xenograft tumor model cervical cancer	Single agent	[178]
Agent	Phase of Clinical Trial	Eligible Disease Site(s)	Combinational Agent	NCT Clinical Trial Number
Resveratrol	Phase 1 (completed)	Colon cancer	Single agent	NCT00256334
Resveratrol	Phase 1 (completed)	Colorectal cancer	Single agent	NCT00433576
Resveratrol	Phase 1 (completed)	Healthy participants	Single agent	NCT00098969
Resveratrol (SRT501)	Phase 1 (completed)	Neoplasms, colorectal	Single agent	NCT00920803
Resveratrol	Phase 2 (completed)	Lymphangiomyomatosis	Resveratrol and Sirolimus	NCT03253913
Resveratrol	Not applicable (active)	HBOC Syndrome, nutrition therapy	Resveratrol with anti-oxidant therapy dietary components	NCT05306002

4.5. Roburic Acid

Roburic acid (RA) is a tetracyclic triterpenoid and a terpene derivative, encompassing the combination of five-carbon isoprene units. It was first isolated from oak galls, which are growths developed from injury to plant tissues caused by insects feeding on them. It was also found in the plants *Gentiana macrophylla* and *Gentiana dahurica*, used in traditional Chinese herbal medicine. The first studies on this compound showed its broad biological activity, including anti-oxidant, anti-microbial, anti-atherosclerotic, and anti-inflammatory properties [179–181].

Roburic acid and its derivatives have been categorized as inhibitors of microsomal prostaglandin E2 synthase-1 (mPGES-1), which prevent the formation of PGE2, a key mediator in inflammation and tumorigenesis [182]. Triterpenic acids, including RA, are found in acidic fractions of resin gums (frankincense extracts) of *Boswellia* spp. plants. These compounds interfere with the activity of mPGES-1 and are involved in the conversion of arachidonic acid (AA) by COX-1 to PGE2 [182]. In addition, RA has also been identified as a COX-1 and COX-2 inhibitor, which may explain its anti-inflammatory properties [183]. Chen et al. showed that roburic acid reduced NO and IL-6 production in LPS-stimulated macrophages. In addition, it inhibited phosphorylation and prevented the degradation of I κ B α , which acts as an inhibitor of NF- κ B [179]. The biological activity of RA was associated with the increase in the expression of cytosolic P65. This constitutes evidence of inhibition of NF- κ B translocation from the cytoplasm to the nucleus. In the same study, the authors also assessed the activation of the MAPK pathway, which, like NF- κ B, regulates gene transcription, protein biosynthesis, and the processes of cell division, differentiation, and survival. RA inhibited the phosphorylation of two major effector kinases of this pathway: JNK and p38. No effect on ERK1/2 kinases was observed [179]. In another study, the authors showed that inhibition of NF- κ B signaling occurs through direct interaction of RA with TNF- α , which prevents the native ligand from binding the target tumor necrosis factor receptor 1 (TNF-R1) [180]. Changes in the expression of cell cycle marker proteins after

RA treatment of HCT-116 and HCT-15 cells were also studied. The regulation of the cell cycle takes place with the participation of cyclin-dependent kinases (CDKs), which, when activated as a result of association with cyclins, stimulate the progression of the cell cycle. For instance, cyclins D and E promote transition to the G1 phase (D-CDK4/6; E-CDK2), cyclin B1 to M phase (B-CDK1), and cyclin A to S and G2 phase (A-CDK2) [184]. Their synthesis in cell takes place *de novo*, and the concentrations of specific cyclins change as the cell cycle progresses; an increase in the concentration leads to the activation of the partner CDK and is followed by a decrease due to cyclin proteasomal degradation, which restores CDK to an inactive state. Disturbances in the expression of cyclins may lead to the development of neoplasia. It was shown that RA exerted an anti-proliferative effect by reducing the expression of cyclins B, D, and E, which led to cell cycle arrest in the G0/G1 phase [180]. In addition, it reduced the expression of the c-MYC oncoprotein, which also promotes the transition of cells from the G1 to the S phase. In the same study, the level of apoptosis regulatory proteins of the BCL-2 family was also evaluated. These proteins are divided into two subgroups that promote or block apoptotic signals by interacting with other proteins involved in the process of cell death. Pro-apoptotic proteins composed of a single BH3 domain (BH3 interacting-domain death agonist (BID); BCL-2 interacting mediator of cell death (BIM); P53 upregulated modulator of apoptosis (PUMA); phorbol-12-myristate-13-acetate-induced protein 1 (NOXA)) play the role of activators, receiving and transmitting the apoptotic signal to pro-apoptotic proteins of multi-domain proteins with an effector function (BAX and BCL-2 homologous antagonist killer (BAK)) [185]. These proteins cause changes in the permeability of the outer mitochondrial membrane, which leads to the release of cytochrome C and the formation of the apoptosome, which is needed for autocatalytic maturation and activation of caspases. Caspases occur in the form of zymogens that undergo proteolytic activation [186]. The role of the resulting “caspase cascade” is to initiate events related to apoptotic death. HCT-116 and HCT-115 cells treated with RA showed a decrease in anti-apoptotic proteins, i.e., B-cell lymphoma 2 (BCL-2), BCL-xL, X-linked inhibitor of apoptosis protein (XIAP), myeloid leukemia cell 1 (MCL-1), and survivin. Moreover, RA promoted proteolytic cleavage of effector caspases -3 and -7, leading to their activation [180]. The anti-inflammatory and anti-oxidant properties of RA were also investigated by Wang et al., where they caused attenuation of receptor activator of nuclear factor Kappa-B ligand (RANKL)-induced osteoclastogenesis. RA not only reduced the number of osteoclasts, but also reduced hydroxyapatite resorption and calcium oscillation [181]. Moreover, it down-regulated TRAF6 expression and inhibited related signaling cascades, including ERK1/2 phosphorylation and I κ Ba degradation. It also promoted the activation of the nuclear factor-erythroid 2 related factor 2 (NRF2)/kelch-like ECH-associated protein 1 (KEAP1)/anti-oxidant response element (ARE) pathway, which is responsible for the production of anti-oxidants in opposition to reactive oxygen species (ROS) production promoting TRAF6 effects [181]. With promising anti-inflammatory and pro-apoptotic properties, roburic acid has emerged as an interesting candidate anti-cancer agent. Wang et al. tested RA in combination with docetaxel and a proton pump inhibitor esomeprazole (ESOM) on human PC3 prostate cancer cells. The authors noted the benefits of the synergistic action of the two substances manifested by inhibition of tumor cell viability, suppression of the NF- κ B pathway, and induction of apoptosis. In addition, a reduced tendency to form spheres was observed, indicating inhibition of the ability of cancer stem cells to self-renew [187]. In addition to its important anti-inflammatory and anti-tumor effects, roburic acid has also shown therapeutic effects in Alzheimer’s disease due to its ability to reduce the levels of neurotoxic A β peptide accumulation in the brain by breaking down β/γ secretase complexes without inhibiting secretase’s proteolytic activities [188]. Examples of preclinical studies with roburic acid are presented in Table 5.

Table 5. Preclinical studies with roburic acid.

Agent	Type of Study	Eligible Disease Site(s) and Organism	Combinational Agent	Ref.
Roburic acid	In vitro and in vivo	Nude mouse xenograft tumor model of colorectal cancer	Single agent	[180]
Roburic acid	In vitro and in vivo	Ovariectomized mice model for treating osteoporosis	Single agent	[181]
Roburic acid	In vitro	Prostate cancer cells	Roburic acid, Docetaxel, and Esomeprazole	[187]
Roburic acid	In vitro	RAW264.7 macrophage cells	Single agent	[179]

Roburic acid is one of the latest discovered and the poorest studied substances of natural origin, which, in addition to its strong anti-inflammatory properties, also exhibits anti-tumor activity. Hence, it can be considered a stand-alone or adjunctive therapeutic agent for further studies. This topic, however, requires further research.

4.6. Other Substances

In addition to the compounds discussed here, several other substances are being studied as anti-cancer agents. Of note are natural plant polyphenols, such as epigallocatechin-3-gallus (EGCG) that are found in green tea extracts. EGCG has proven anti-cancer, anti-oxidant, and anti-inflammatory properties, providing many chemotherapeutic as well as chemopreventive benefits [189,190]. EGCG disrupted the growth of SH-SY5Y immature neuroblastoma by targeting the dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A) in vitro [191]. It has also been reported to play a crucial role in the promotion of apoptotic cell death through inhibition of the epidermal growth factor receptor (EGFR), signal transducer and activator of transcription 3 (STAT3), and NF- κ B transcription factor pathways in breast cancer cells and head and neck squamous cell carcinoma cells [192,193]. In vivo, it demonstrated inhibition of tumor growth, accompanied by suppression of metastasis and prolongation of survival time in a 4T1 mouse model of breast cancer [194]. Among patients with advanced precancerous lesions of the head and neck, treatment with the combination of EGCG and erlotinib was well-tolerated and had a high pathological response rate (NCT01116336, www.clinicaltrials.gov) [195]. Phase I clinical trials with EGCG for colon cancer patients (NCT02891538, www.clinicaltrials.gov), for cirrhosis-free liver cancer participants (NCT03278925, www.clinicaltrials.gov), and even studies of the role of EGCG for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-infected cancer patients (NCT05758571, www.clinicaltrials.gov) are currently underway (recruitment phase). The clinical trial using EGCG, quercetin, zinc, and metformin as adjuvant compounds in add-on therapy for early metastatic breast cancer and triple-negative breast cancer is also planned (NCT05680662).

Other polyphenols include the calebin A and curcumin components of turmeric, which have shown anti-inflammatory and anti-tumor properties, particularly in colorectal cancer [196,197]. These substances can re-sensitize resistant colorectal cancer cells to commonly used chemotherapeutics. Furthermore, they can regulate growth factors, transcription factors, inflammatory cytokines, and proteins of the BCL-2 family [198,199]. The potential of curcumin has also been tested in conjugation with antibodies and adjuvants to increase the selectivity and improve the biological activity of the compound in future clinical trials [200,201]. When combined with conventional anti-cancer therapy, the benefits of multidirectional activity and reduced toxicity have been observed. Several reviews have been published describing the effect of curcumin in preclinical models and clinical studies [196,197,202].

Also, other natural substances belonging to the group of alkaloids (berberine [203], nephrine [204]), xanthonoids (kaempferol [205]), phenols (capsaicin [206]), and flavonoids

(salvigenin [207]) show interesting chemoprotective and chemotherapeutic properties, which require further research. Due to the vastness of the topic, this paper does not discuss all of them.

5. Conclusions and Future Prospects

Plants are a rich source of substances that have been used in herbalism and natural medicine for centuries. Many of them constitute the blueprint for the development of synthetic compounds for the treatment of cancer. The most commonly used natural, plant-derived anti-cancer agents include paclitaxel, *vinca* alkaloids, camptothecin derivatives, and podophyllotoxins. An important advantage of these compounds is their multidirectional action, manifested by modulation of the activity of key signaling pathways and multiple molecular targets involved in cancer cell proliferation, migration, and apoptosis. Increased research into the abnormalities of cellular signaling pathways, as well as an understanding of the function and cooperation of oncogenes and anti-oncogenes, boosts the search for new substances and development of analogs with enhanced anti-cancer activity, as well as refined safety profile circumventing problems related to delivery, metabolism, and bioavailability of natural compounds. The natural compounds described in this review are currently being tested in both clinical and preclinical studies and are potential candidates for clinical use. However, despite the high level of interest, the complex nature of cellular response triggered following their use means that they require comprehensive research.

Each of the compounds described in this review that has entered the clinical trial phase has shown side effects or toxicities that limit its use. Among the main side effects of paclitaxel are myalgia, arthralgia, nausea, diarrhea, mucositis, hypotension, bradycardia, and others [46]. The described cases of neutropenia necessitated changes in the dosing schedule and shortened the drug's infusion time. The appearance of thrombocytopenia or anemia has been observed with less severity [208]. The most commonly reported infections during paclitaxel treatment include urinary tract and upper respiratory tract infections, as well as episodic pneumonia and peritonitis. Neurotoxic events, which result in numbness, autonomic system dysfunction, and movement disorders, are also observed in a dose- and time-dependent manner [209]. Severe toxicities occur in 5–10% of patients treated with paclitaxel [210]. Palliative therapy options include dose reduction and discontinuation of treatment. Hypersensitivity to paclitaxel can also be minimized with corticosteroids and antihistamines [46]. Another of the drugs mentioned in this review, irinotecan, is known mainly for gastrointestinal complaints and neutropenia, limiting the dose of the drug [211]. Both monotherapy and the combination regimen of 5-fluorouracil, leucovorin, and irinotecan (FOLFIRI) show severe diarrhea and neutropenia in approximately 50% of patients [212]. Their duration is correlated with the length of drug exposure. Attempts have been made to reduce toxicity using cyclosporine and phenobarbital to modulate the pharmacokinetics of irinotecan, with positive results in patients [213]. Other studies have suggested a reduction in severe diarrhea after combining irinotecan with antibiotics or probiotics [211,214]. Similarly, the severity of side effects depends on the dose and duration of exposure to resveratrol. Mild and short-term side effects include gastrointestinal complaints, headache, and rash, which occur at doses greater than 2.5 g/day [170,215]. At doses greater than 5 g/day, patients have been found to exhibit signs of nephrotoxicity [216]. Cases of renal failure were observed only with monotherapy, while this severe side effect is scarce when administered in combination with bortezomib [175]. There are still few results from human studies of resveratrol, so both the distribution in tissues and the observed toxicity leave many open questions. There is a lack of information on monotherapy and combination therapies based on resveratrol or betulinic acid.

The described examples of drug toxicity and side effects can be overcome with specific combinations of substances, either through their delivery or the use of nontoxic prodrugs metabolized to their active form. In 2022, Nasibullin et al. published the results of a project in which they designed a retrosynthetic prodrug that was converted using artificial metalloenzymes [217]. The authors put emphasis on identifying useful chemical reactions that

lead to the formation of the pharmacophore, and thus designed a naphthylclombretastatin-based prodrug that forms highly active cytostatic agents through sequential ring-closing metathesis and aromatization. Based on this mechanism, the substance showed excellent anti-cancer activity in vivo [217]. The described methodology may allow for the retesting of drugs whose use has been restricted due to high toxicity. Drug efficacy and bioavailability can also be improved by designing and synthesizing new derivatives with an improved pharmacokinetic profile. Currently, drug design is refined with the use of bioinformatics and cheminformatics. Computer-aided drug design involves identifying potential targets, searching databases for drug candidates, further optimizing candidate compounds, and assessing their potential toxicity. Following these analyses, the compounds are subjected to in vitro and in vivo experiments. Natural substances or plant-based compounds can provide excellent examples of lead structures that can be used as a starting scaffold for the development of new structures with improved physicochemical parameters. Current experimental approaches involve the review and re-testing of currently known substances for new indications in a process of drug-repurposing. Natural substances are particularly useful for this given their wide biological activity and the multiple molecular pathways they target. Both toxicity and bioavailability limitations can be addressed through appropriately selected carriers, delivery systems, and pro-drug design. In addition, there is an increased focus on personalizing each patient's treatment strategy based on the results of a screening to identify genes that confer resistance to specific chemotherapy.

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Abbreviations

3LL	Lewis lung cancer
AA	Arachidonic acid
AKT	Protein kinase B
AMPK	5' AMP-activated protein kinase
ARE	Anti-oxidant response element
ATG13	Autophagy-related protein 13
ATG3	Autophagy-related protein 3
ATG4	Autophagy-related protein 4
ATG5	Autophagy-related protein 5
ATG7	Autophagy-related protein 7
ATG10	Autophagy-related protein 10
ATG12	Autophagy-related protein 12
ATG16	Autophagy-related protein 16
ATM	Ataxia teleangiectasia mutated
ATR	Ataxia teleangiectasia and Rad3 related
BA	Betulinic acid
BAD	BCL-2-associated death promoter
BAK	BCL-2 homologous antagonist killer
BAX	BCL-2-associated X protein
BCL-2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-extra large

BER	Base excision repair
BID	BH3 interacting-domain death agonist
BIM	BCL-2 interacting mediator of cell death
<i>BRCA1</i>	Breast cancer type 1 susceptibility protein
<i>BRCA2</i>	Breast cancer type 2 susceptibility protein
CDKs	Cyclin-dependent kinases
CHK1	Checkpoint kinases 1
CHK2	Checkpoint kinases 2
COX	Cyclooxygenase
CPT-11	Irinotecan
CSCs	Cigarette smoke condensate
DDR	DNA damage response
DSB	Double-strand breaks
DYRK1A	Dual specificity tyrosine-phosphorylation-regulated kinase 1A
ECM	Extracellular matrix
EGCG	Epigallocatechin-3-gallus
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
ESOM	Esomeprazole
FDA	Federal drug administration
FEN1	Flap endonuclease 1
FOXO	Fork head box O
FUBP1	Far upstream element binding protein 1
GTP	Guanosine-5'-triphosphate
IGF-1	Insulin-like growth factor-1
IGFBP-3	Insulin-like growth factor binding protein 3
IKK β	I κ B kinase
ILs	Interleukins
I κ B	B-cells inhibitor
JNK	c-Jun N-terminal kinase
KEAP1	Kelch-like ECH-associated protein 1
LAM	Lymphangioleiomyomatosis
LC3	Protein light chain
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCL-1	Myeloid leukemia cell 1
MDM2	Mouse homolog of double minute 2
MDR	Multidrug resistance
MEK	Mitogen-activated protein kinase
MKK	MAP kinase
MLST8	mTOR associated protein LST8 homolog
MiMP	Mitochondrial membrane permeability
MMP-9	Matrix metalloproteinase-9
mPGES-1	Microsomal prostaglandin E2 synthase-1
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NO	Nitric oxide
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
NRE	NF- κ B response elements
NRF2	Nuclear factor-erythroid 2 related factor 2
NSCLC	Non-small cell lung cancer
PCNA	Proliferating cell nuclear antigen
PD-1	Programmed death receptor
PD-L2	Programmed death receptor-ligand 2
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinase
PIKK	Phosphatidylinositol 3-kinase-related kinases

PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PUMA	P53 upregulated modulator of apoptosis
RA	Roburic acid
RAF	RAF proto-oncogene serine/threonine-protein kinase
RANKL	Receptor activator of nuclear factor kappa-B ligand
RAS	Rat sarcoma virus
ROS	Reactive oxygen species
RTKs	Receptor tyrosine kinases
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SLUG	Zinc finger protein SNAI2
SN38	7-ethyl-10-hydroxycamptothecin
SN38-G	SN38-glucuronide
SNAIL	Zinc finger protein SNAI1
SP1	Transcription factor Sp1
SP3	Transcription factor Sp3
SQTM	Sequestosome 1
STAT3	Signal transducer and activator of transcription 3
TGF- β	Transforming growth factor β
TGF- β R	Transforming growth factor β receptor
TLR4	Toll-like receptor 4
TME	Tumor microenvironment
TNF-R1	Tumor necrosis factor receptor 1
TNF- α	Tumor necrosis factor α
TP53	Tumor protein P53
TRAF6	TNF receptor-associated factor 6
UGT	Uridine 5'-diphosphate-glucuronosyltransferase
ULK1	Unc-51 like autophagy activating kinase
VEGF	Vascular endothelial growth factor
VPS34	Vesicular protein sorting kinase 34
XIAP	X-linked inhibitor of apoptosis protein
ZEB1/2	Zinc finger E-box binding homeobox 1/2
β G	β -glucuronidase

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Article

Anticancer Activity of Roburic Acid: In Vitro and In Silico Investigation

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Abstract

Natural compounds remain a valuable source of anticancer agents due to their structural diversity and multi-targeted mechanisms of action. Roburic acid (RA), a tetracyclic triterpenoid, has been identified as a substance capable of inhibiting key NF- κ B and MAPK signaling pathways through direct interaction with TNF- α , as well as preventing the production of inflammatory mediators and cancer progression. In this study, we evaluated the biological activity of RA against a panel of human cancer cell lines—DLD-1, HT-29, and HCT-116 (colorectal), PC-3 (prostate), and BxPC-3 (pancreatic)—as well as two non-malignant lines: WI-38 (fibroblasts) and CCD-841 CoN (colon epithelium). RA exhibited a concentration-dependent inhibitory effect on cancer cell metabolic activity, with colorectal cancer cells showing relatively higher sensitivity, particularly at shorter incubation times. To distinguish between cytotoxic and cytostatic effects, we performed trypan blue exclusion combined with a cell density assessment, clonogenic assay, and BrdU incorporation assay. The results from these complementary assays confirmed that RA acts primarily through an antiproliferative mechanism rather than by inducing cytotoxicity. In addition, NF- κ B reporter assays demonstrated that RA attenuates TNF- α -induced transcriptional activation at higher concentrations, supporting its proposed anti-inflammatory properties and potential to modulate pro-tumorigenic signaling. Finally, our in silico studies predicted that RA may interact with proteins such as CAII, CES1, EGFR, and PLA2G2A, implicating it in the modulation of pathways related to proliferation and cell survival. Collectively, these findings suggest that RA may serve as a promising scaffold for the development of future anticancer agents, particularly in the context of colorectal cancer.

Keywords: roburic acid; colorectal cancer; antiproliferative activity; inflammation; NF- κ B signaling



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1. Introduction

The clinical effectiveness of many currently used anticancer therapies is severely limited. The reason for this is the development of resistance of cells in response to the chemotherapy drugs used. Cancer cells can adapt and develop defense mechanisms, which cause a decrease in their sensitivity to drugs, especially in the case of long-term exposure.

Additionally, the phenomenon of tumor heterogeneity makes some cancers more resistant due to genetic differences between cancer cells. This creates another challenge in designing uniform and effective therapies, as molecular differences between tumors may require an individualized therapeutic approach. Ultimately, damage to healthy cells leads to side effects and the deterioration of patients' overall health. Due to the many challenges and dynamic nature of cancer diseases, there is a constant need to introduce new drugs to increase the effectiveness of and individualize treatment strategies [1].

In the search for new therapeutic agents, scientists take a variety of approaches, including computer-aided drug design (CADD) and de novo synthesis, or discovery of natural substances. New approaches based on natural substances include mainly research on phytochemicals, i.e., alkaloids, polyphenols, flavonoids, saponins, stilbene, lignans, and also compounds isolated from fungi or metabolites of marine organisms [2–4]. The wealth and diversity of chemical compounds present in the natural environment make them a valuable source of compounds with potential anticancer properties. Not only do they have unique chemical structures, but they also often exhibit specific, multidirectional mechanisms of action [5].

One such compound that has recently attracted the attention of researchers is roburic acid (RA). This tetracyclic triterpenoid, a terpene derivative composed of a combination of five-carbon isoprene units, has become the subject of intense research due to its diverse biological properties. Although it was probably used several centuries earlier in traditional Chinese medicine, its rediscovery occurred recently. This compound is isolated from oak galls, which are growths that result from damage to plant tissues by insects that feed on them [6,7]. Moreover, it also occurs in the plants of *Gentiana macrophylla* and *Gentiana dahurica*, growing in China, Kazakhstan, Mongolia, and Russia. It is known for its strong anti-inflammatory, antioxidant, and antimicrobial properties. Previous studies have demonstrated its ability to inhibit signaling pathways, including Nuclear factor NF- κ B (NF- κ B) and mitogen-activated protein kinases (MAPKs), through direct interaction with Tumor necrosis factor α (TNF- α) [7,8]. Furthermore, RA may influence the regulation of the nuclear factor erythroid 2-related factor 2 (NRF2)/Kelch-like ECH-Associating protein 1 (KEAP1)/antioxidant responsive element (ARE) pathway, which contributes to the reduction of reactive oxygen species levels [9]. RA has also been classified as an inhibitor of microsomal prostaglandin E2-1 synthase, which translates into its ability to inhibit the formation of prostaglandin E2, a key mediator in inflammatory processes and a significant promoter of cancer development [10]. The first in vitro studies of this compound using cancer cells showed its ability to regulate the expression of cyclins B, D, and E or c-MYC, which results in its antiproliferative potential and antiapoptotic proteins, leading to cell death [8]. In the present study, we tested the cytotoxic/cytostatic effects of RA in normal and cancerous human cell lines in vitro and investigated the molecular basis of its anticancer activity, including its impact on inflammatory signaling. We also assessed the pharmacokinetic and pharmacodynamic properties of RA using computational modeling.

2. Results

2.1. MTT Assay

A concentration-dependent decrease in absorbance was observed in all experimental series, indicating a reduction in cell viability following RA treatment. Dose-response curves obtained from three independent replicates are shown in Figure 1 (24 h incubation) and Figure 2 (72 h incubation). Among the tested cell lines, DLD-1 (colorectal adenocarcinoma) demonstrated the greatest sensitivity to RA after 24 h of incubation, followed by the HT-29 (colorectal adenocarcinoma), HCT-116 (colorectal carcinoma), PC-3 (prostatic adenocarcinoma), and BxPC-3 (pancreatic adenocarcinoma) cells. As expected, normal

human fibroblasts (WI-38) and human colon epithelial cells (CCD-841 CoN) showed the lowest sensitivity to the compound. After 72 h, the sensitivity profile remained generally consistent, with DLD-1 cells exhibiting the strongest response, followed by HCT-116 and HT-29 cells. However, the IC_{50} values for the non-malignant cell lines (CCD-841 CoN and WI-38) were closer to those of the less responsive cancer cell lines (PC-3 and BxPC-3), indicating a reduced distinction in sensitivity between malignant and non-malignant cells at this timepoint. The corresponding IC_{50} values are summarized in Table 1.

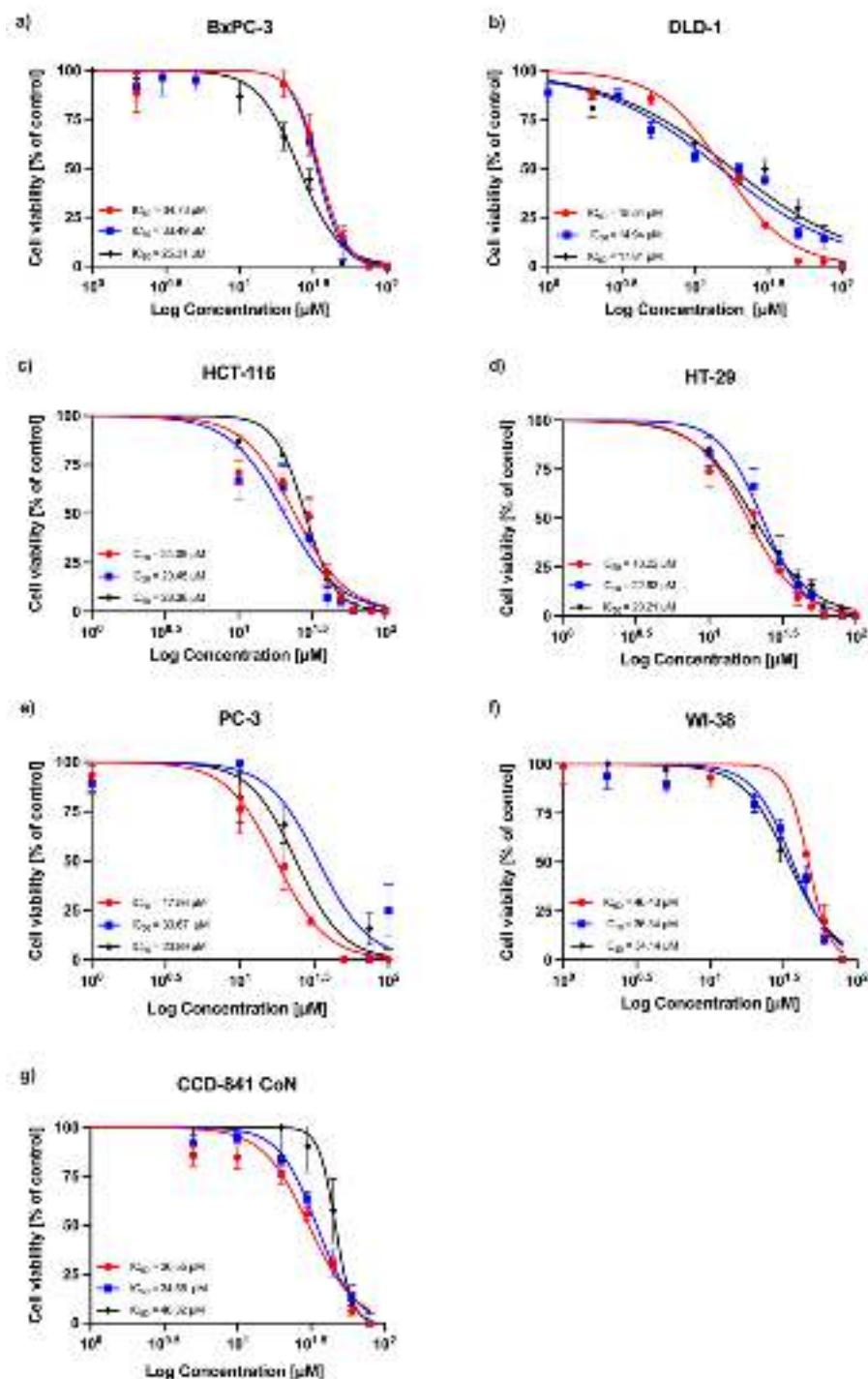


Figure 1. Relative cell viability (%) of cancer cell lines (a) BxPC-3, (b) DLD-1, (c) HCT-116, (d) HT-29, and (e) PC-3, and normal cells (f) WI-38 and (g) CCD-841 CoN after 24 h treatment with RA. The dose-response curve $\pm$ SD and the corresponding IC_{50} values are presented for each line.

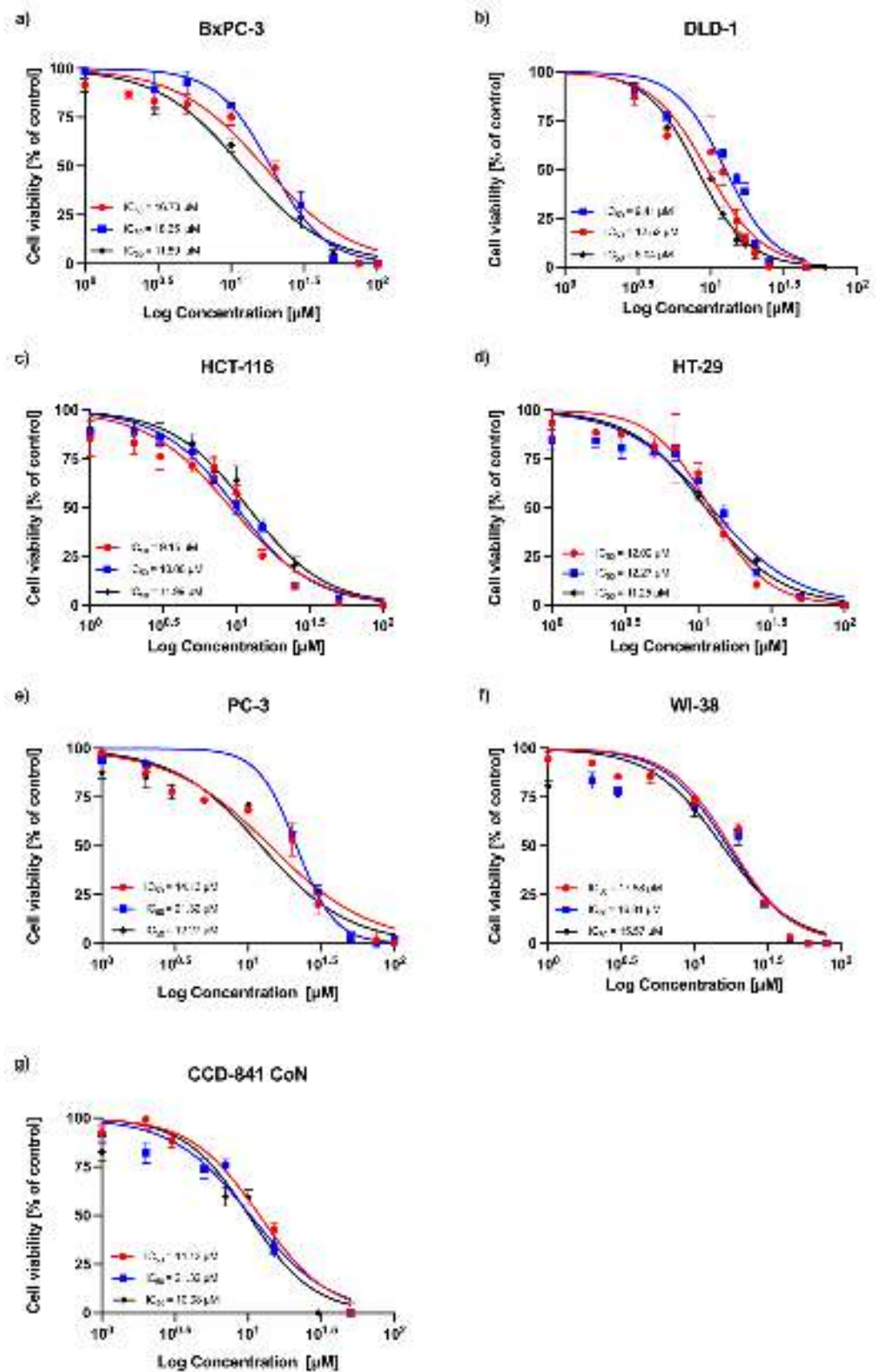


Figure 2. Relative cell viability (%) of cancer cell lines (a) BxPC-3, (b) DLD-1, (c) HCT-116, (d) HT-29, and (e) PC-3, and normal cells (f) WI-38 and (g) CCD-841 CoN after 72 h treatment with RA. The dose-response curve $\pm$ SD and the corresponding IC_{50} values are presented for each line.

Table 1. Mean IC₅₀ values ($\pm$ SD) obtained after 24 h and 72 h incubation of the tested cell lines with RA, as determined by the MTT assay.

Time	Cell Lines						
	Mean IC ₅₀ [μ M] of Roburic Acid $\pm$ SD						
	BxPC-3	DLD-1	HCT-116	HT-29	PC-3	WI-38	CCD-841 CoN
24 h	31.18 $\pm$ 5.12	16.1 $\pm$ 1.51	24.3 $\pm$ 4.0	20.45 $\pm$ 2.36	25.04 $\pm$ 8.12	38.97 $\pm$ 6.55	37.14 $\pm$ 8.2
72 h	15.76 $\pm$ 3.48	9.99 $\pm$ 2.3	10.38 $\pm$ 1.46	11.85 $\pm$ 0.53	15.91 $\pm$ 4.78	16.65 $\pm$ 1.01	15.18 $\pm$ 5.69

2.2. Neutral Red Uptake Assay

Based on the results of the MTT assay, the colon cancer cell lines DLD-1, HCT-116, and HT-29, along with the normal cell lines WI-38 and CCD-841 CoN, were selected for further evaluation using the neutral red uptake assay. This test measures the ability of viable cells to incorporate neutral red dye into lysosomes, serving as an indicator of lysosomal integrity and overall cell viability. A time-dependent decrease in dye accumulation was observed following 24 and 72 h exposure to RA, with the most pronounced effect noted in the DLD-1 cell line. In contrast, the CCD-841 CoN cells exhibited the lowest sensitivity to the tested compound at both time points. The IC₅₀ values calculated from two independent experiments are presented in Table 2.

Table 2. Mean IC₅₀ values ($\pm$ SD) obtained after 24 h and 72 h incubation of the tested cell lines with RA in the neutral red uptake assay.

Time	Cell Lines				
	Mean IC ₅₀ [μ M] of Roburic Acid $\pm$ SD				
	DLD-1	HCT-116	HT-29	WI-38	CCD-841 CoN
24 h	14.16 $\pm$ 1.37	17.83 $\pm$ 1.62	21.57 $\pm$ 5.25	33.53 $\pm$ 1.05	33.95 $\pm$ 0.76
72 h	7.43 $\pm$ 1.58	12.86 $\pm$ 3.06	11.69 $\pm$ 1.13	16.46 $\pm$ 0.62	19.34 $\pm$ 0.02

2.3. Crystal Violet Assay

The third and final test used to assess the cellular response to RA was the crystal violet assay. The experiments were conducted in a manner analogous to the neutral red assay. The IC₅₀ values obtained from two independent experiments are presented in Table 3. After 24 h, the greatest reduction in crystal violet uptake was observed in the DLD-1 cell line, followed by HCT-116, HT-29, and the normal cells WI-38 and CCD-841 CoN. However, after 72 h, the greatest reduction was observed in the HT-29 cell line, followed by DLD-1 and HCT-116. Notably, the IC₅₀ value for WI-38 cells after 72 h was comparable to that of the HCT-116 cancer cells. CCD-841 CoN cells consistently exhibited the lowest response to RA treatment.

Table 3. Mean IC₅₀ values ($\pm$ SD) obtained after 24 h and 72 h incubation of the tested cell lines with RA in the crystal violet assay.

Time	Cell Lines				
	Mean IC ₅₀ [μ M] of Roburic Acid $\pm$ SD				
	DLD-1	HCT-116	HT-29	WI-38	CCD-841 CoN
24 h	18.45 $\pm$ 0.42	18.88 $\pm$ 0.96	25.15 $\pm$ 1.27	33.14 $\pm$ 2.08	34.65 $\pm$ 0.28
72 h	10.2 $\pm$ 1.34	12.94 $\pm$ 0.37	8.25 $\pm$ 0.64	12.82 $\pm$ 1.25	17.99 $\pm$ 0.74

Conducting three different assays, each based on distinct mechanisms but all assessing cell viability, allowed for the determination of a unified value for each cell line, referred to as the consensus IC₅₀. These values were calculated as the arithmetic mean of the results obtained from the preceding assays and are summarized in Table 4.

Table 4. The consensus IC₅₀ values ($\pm$ SD), obtained after 24 h and 72 h incubation of the tested cell lines with RA, based on results from the MTT, neutral red uptake, and crystal violet assays.

Time	Cell Lines				
	Mean IC ₅₀ [μ M] of Roburic Acid $\pm$ SD				
	DLD-1	HCT-116	HT-29	WI-38	CCD-841 CoN
24 h	16.23 $\pm$ 1.1	20.33 $\pm$ 2.18	22.39 $\pm$ 2.96	35.21 $\pm$ 3.23	35.25 $\pm$ 3.08
72 h	9.21 $\pm$ 1.74	12.06 $\pm$ 1.63	10.6 $\pm$ 0.77	15.32 $\pm$ 0.96	17.5 $\pm$ 2.15

2.4. Cell Density and Viability Analysis Using Trypan Blue Exclusion

To further evaluate the impact of RA on cellular proliferation and survival, we focused on the three cancer cell lines—DLD-1, HCT-116, and HT-29—that exhibited the greatest response to RA in viability assays. The relative cell density and the proportion of viable cells were analyzed after 24 and 72 h incubation periods (Figure 3). RA was applied at concentrations corresponding to the previously established consensus IC₅₀ values for each cell line.

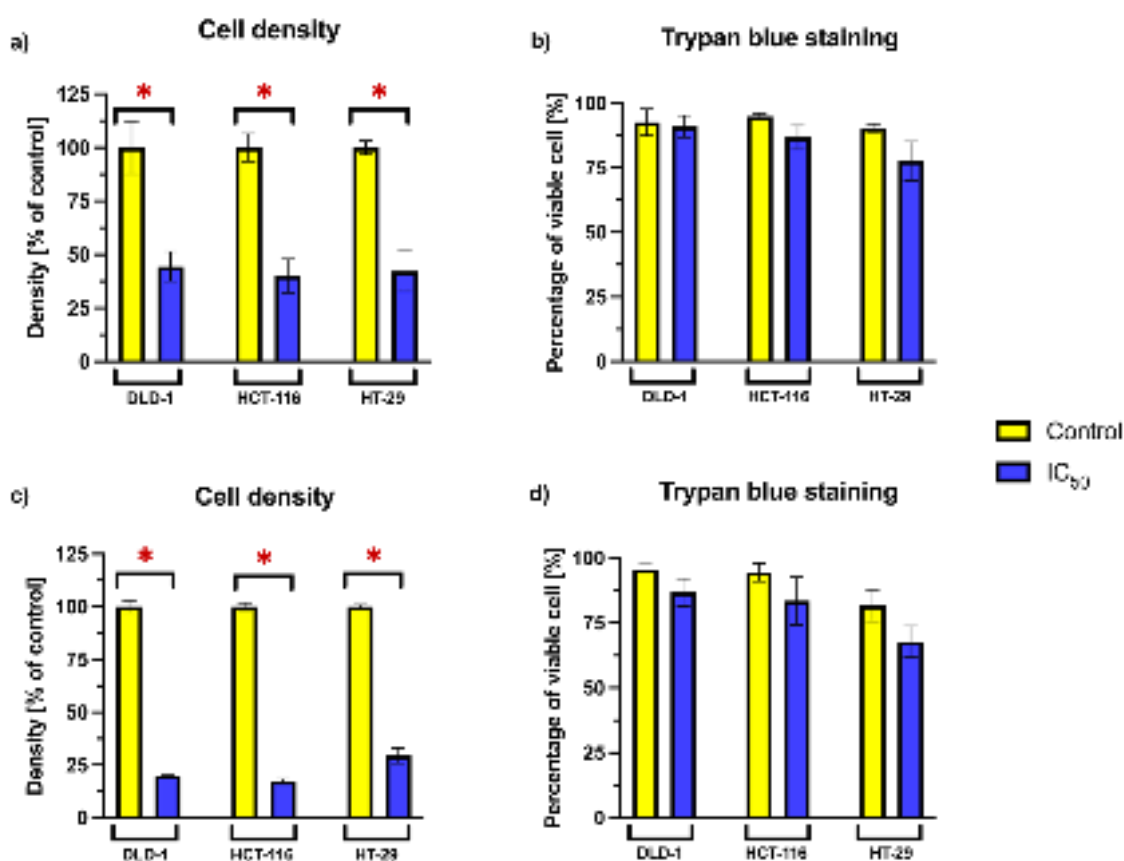


Figure 3. Relative cell density of colorectal cancer cell lines (DLD-1, HCT-116, and HT-29) treated with RA at consensus IC₅₀ concentrations for (a) 24 h and (c) 72 h, expressed as a percentage of the untreated control. Percentage of viable cells determined by trypan blue exclusion after (b) 24 h and (d) 72 h RA treatment. Data represent mean $\pm$ SD from two independent experiments. Asterisks (*) indicate statistically significant differences compared to the control ($p < 0.05$).

A statistically significant reduction in relative cell density was observed across all tested cell lines following RA treatment at both time points. This suggests a decrease in proliferative capacity or cell accumulation. However, the percentage of viable cells remained comparable to that of the untreated control. No statistically significant differences in viability were noted between RA-treated and untreated cells, suggesting that the reduced density was not associated with increased cell death.

2.5. Clonogenic Assay

To evaluate the long-term proliferative capacity of cancer cells following RA treatment, a clonogenic assay was performed. Based on preliminary optimization, a seeding of 500 cells per well was used, which ensured consistent colony formation. Cells were then treated with RA at concentrations corresponding to $0.5 \times IC_{50}$, IC_{50} , and $2 \times IC_{50}$ values. After a 10-day incubation period, colonies were stained and quantified (Figure 4). A statistically significant ($p < 0.05$) reduction in colony formation was observed in all tested cell lines at both IC_{50} and $2 \times IC_{50}$ concentrations (Table 5). The number of colonies formed decreased in a dose-dependent manner. These findings indicate that RA impairs the clonogenic potential of colorectal cancer cells.

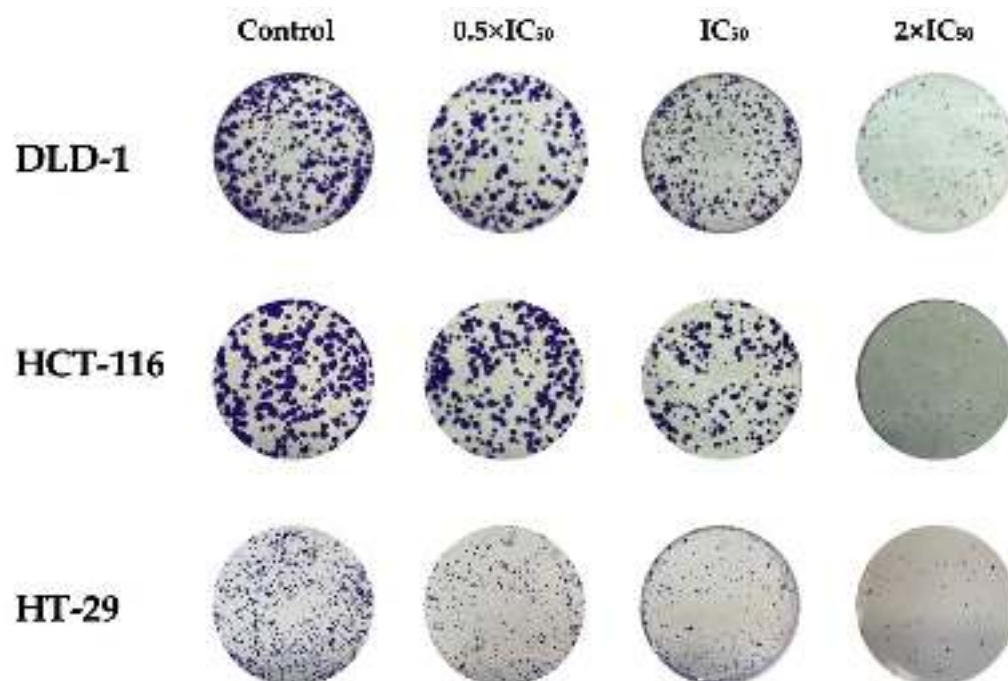


Figure 4. Representative images of colony formation following RA treatment. The effect of RA administered at $0.5 \times IC_{50}$, IC_{50} , and $2 \times IC_{50}$ on the clonogenic potential of colorectal cancer cell lines DLD-1, HCT-116, and HT-29 is compared to untreated controls.

Table 5. Mean number of colonies formed by cancer cells $\pm$ SD. Statistically significant differences compared to the negative control are indicated in bold.

RA Concentration	Cell Lines		
	Mean Number of Colonies $\pm$ SD		
	DLD-1	HCT-116	HT-29
Control	215.5 $\pm$ 21.92	244.0 $\pm$ 15.56	150.5 $\pm$ 7.78
$0.5 \times IC_{50}$	180.0 $\pm$ 15.56	168.5 $\pm$ 36.06	133.5 $\pm$ 6.36
IC_{50}	123.0 $\pm$ 7.07	127.0 $\pm$ 15.56	121.0 $\pm$ 4.24
$2 \times IC_{50}$	76.5 $\pm$ 6.36	76.0 $\pm$ 18.38	109.0 $\pm$ 1.41

2.6. Bromodeoxyuridine Incorporation Assay

To further investigate the antiproliferative potential of RA, the bromodeoxyuridine (BrdU) incorporation assay was employed. This method detects the integration of BrdU, a synthetic thymidine analog, into newly synthesized DNA during the S-phase of the cell cycle, thereby serving as a marker of active proliferation. Cells from the DLD-1, HCT-116, and HT-29 lines were incubated with RA at $0.5 \times IC_{50}$ and IC_{50} concentrations for 48 h. Then, 15 μ M oxaliplatin (OXA) was used as a positive control due to its established use in colorectal cancer therapy and well-documented antiproliferative properties. The concentration was selected based on previously reported IC_{50} values for colorectal cancer cell lines and applied uniformly across the tested models for consistency [11–15]. The results, expressed as the percentage of BrdU-positive cells, demonstrated a concentration-dependent decrease in proliferation following RA treatment (Figure 5 and Table 6). The most pronounced reduction was observed in the HT-29 cell line. In all tested lines, RA exhibited antiproliferative activity comparable to or exceeding that of the positive control. These findings, along with the clonogenic assay results, support the cytostatic effect of RA on colorectal cancer cells.

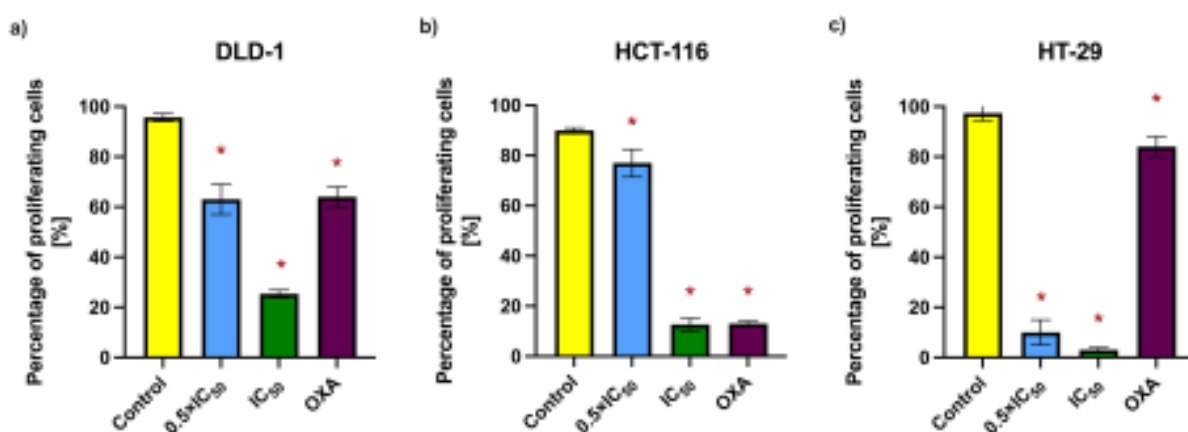


Figure 5. Percentage of proliferating cells in colorectal cancer cell lines (a) DLD-1, (b) HCT-116, and (c) HT-29 after 48 h exposure to RA at $0.5 \times IC_{50}$ and IC_{50} concentrations, assessed by a bromodeoxyuridine incorporation assay. Data are presented as the mean percentage of proliferating cells [%] $\pm$ SD. Statistically significant differences ($p < 0.05$) compared to the negative control are marked with an asterisk (*).

Table 6. Mean percentage [%] $\pm$ SD of proliferating cells in the populations of (a) DLD-1, (b) HCT-116, and (c) HT-29 cell lines after 48 h incubation with RA at $0.5 \times IC_{50}$ and IC_{50} concentrations.

Cell Lines	Mean of proliferating cells [%] $\pm$ SD			
	Control	$0.5 \times IC_{50}$	IC_{50}	OXA (15 μ M)
DLD-1	95.5 $\pm$ 2.12	66.0 $\pm$ 4.24	25.5 $\pm$ 2.12	66.0 $\pm$ 2.83
HCT-116	90.5 $\pm$ 0.71	79.5 $\pm$ 4.95	11.5 $\pm$ 2.12	12.5 $\pm$ 0.71
HT-29	97.33 $\pm$ 3.06	10.0 $\pm$ 5.0	3.0 $\pm$ 1.0	84.0 $\pm$ 4.0

Bold values indicate statistically significant differences ($p < 0.05$) compared to the negative control.

2.7. NF- κ B Reporter Cell Luciferase Assay

To investigate whether RA modulates NF- κ B transcriptional activity in response to inflammatory stimulation, we used the NF- κ B-TIME human endothelial reporter cell line, which contains a stably integrated luciferase construct under the control of NF- κ B response elements, allowing for sensitive and quantitative monitoring of pathway activation. NF- κ B-TIME cells were pre-treated with increasing concentrations of RA for 1 h, followed

by 6 h stimulation with TNF- α . TNF- α alone induced a robust activation of NF- κ B, as reflected by a significant increase in luminescence signal compared to the unstimulated control. RA at concentrations ranging from 0.5 to 5 μ M had no statistically significant effect on NF- κ B activity in TNF- α -stimulated cells. However, treatment with 10 μ M RA resulted in a modest but significant reduction in NF- κ B activation ($p = 0.0399$), which was further enhanced at 15 μ M ($p = 0.0036$) and 20 μ M ($p = 0.0016$) (Figure 6). These findings suggest that RA attenuates NF- κ B signaling in a concentration-dependent manner, with measurable inhibitory effects emerging at ≥ 10 μ M.

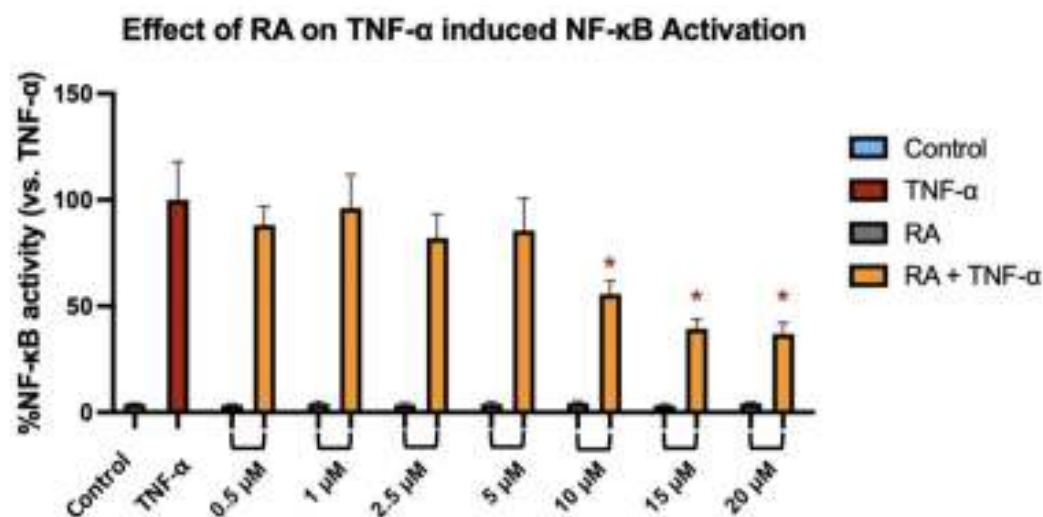


Figure 6. NF- κ B transcriptional activity in NF- κ B-TIME reporter cells following 6 h stimulation with TNF- α after 1 h pre-treatment with RA at concentrations of 0.5–20 μ M. Data are presented as the mean percentage of NF- κ B activity [%] relative to the TNF- α -stimulated control (set as 100%) $\pm$ SD. Statistically significant differences ($p < 0.05$) compared to the TNF- α control are marked with asterisks (*).

2.8. ADMET and Drug-Likeness

To assess the Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) characteristics and drug-likeness of RA, the Swiss-ADME and pkCSM platforms were used (<http://www.swissadme.ch>; <https://biosig.lab.uq.edu.au/pkcsm/prediction> accessed on 26 September 2023). The results are summarized in Table 7.

RA displays physicochemical and pharmacokinetic features characteristic of a lipophilic, moderately bioavailable compound. Its high lipophilicity supports its membrane permeability, but may compromise aqueous solubility and gastrointestinal absorption. Although predictive models provided mixed results, RA demonstrated good permeability in the Caco-2 model and is not a substrate for P-glycoprotein, which favors absorption [16].

The compound has a low steady state volume of distribution (VDss), suggesting high plasma protein binding and potentially reduced levels of pharmacologically active free drugs. RA is a substrate for CYP3A4, indicating possible first-pass metabolism and reduced oral bioavailability. However, it does not appear to inhibit key CYP enzymes, implying a low risk of drug–drug interactions.

The predicted total clearance is low, suggesting prolonged systemic retention, though RA is not a substrate for the renal Organic Cation Transporter 2 (OCT2). Toxicity models showed no mutagenic or cardiotoxic potential (negative AMES and hERG I/II), and the predicted maximum tolerated dose supports low acute toxicity. The long-term safety remains uncertain, particularly regarding liver effects.

Table 7. Predicted ADMET properties of RA obtained using the Swiss-ADME and pkCSM webserver.

Property	Prediction	Unit/Category
Absorption		
Water solubility	−4.485	log mol/L
CaCo-2 permeability	1.291	log Papp in 10 ^{−6} cm/s
Intestinal absorption (human)	96.428	% absorbed
Skin permeability	−2.55	log Kp
P-glycoprotein substrate	No	Yes/No
P-glycoprotein I inhibitor	No	Yes/No
P-glycoprotein II inhibitor	Yes	Yes/No
Distribution		
VDss (human)	−0.894	Log L/kg
Fraction unbound (human)	0	Fu
BBB permeability	−0.074	log BB
CNS permeability	−1.719	log PS
Metabolism		
CYP2D6 substrate	No	Yes/No
CYP3A4 substrate	Yes	Yes/No
CYP1A2 inhibitor	No	Yes/No
CYP2C19 inhibitor	No	Yes/No
CYP2C9 inhibitor	No/Yes (pkCSM/Swiss-ADME)	Yes/No
CYP2D6 inhibitor	No	Yes/No
CYP3A4 inhibitor	No	Yes/No
Excretion		
Total clearance	0.338	log mL/min/kg
Renal OCT2 substrate	No	Yes/No
Toxicity		
AMES toxicity	No	Yes/No
Max. tolerated dose (human)	0.079	log mg/kg/day
hERG I inhibitor	No	Yes/No
hERG II inhibitor	No	Yes/No
Oral rat acute toxicity (LD ₅₀)	2.188	mol/kg
Oral rat chronic toxicity	2.408	log mg/kg_bw/day
Hepatotoxicity	Yes	Yes/No
Druglikeness and Medicinal Chemistry		
Lipinski	Yes; 1 violation: MLOGP > 4.15	Yes/No
Ghose	No; 3 violation: WLOGP > 5.6, MR > 130, atoms > 70	Yes/No
Veber	Yes	Yes/No
Egan	No; 1 violation: WLOGP > 5.88	Yes/No
Muegge	No; 1 violation: XLOGP3 > 5	Yes/No
Bioavailability score	85	Percentage (%)
PAINS	0 alert	Number of alerts
Brenk	1 alert: isolated alkene	Number of alerts
Lead-likeness	No; 2 violations: MW > 350 XLOGP3 > 3.5	Yes/No
Synthetic accessibility	5.87	1–10 scale

Drug-likeness evaluation showed that RA violates some filters, mainly due to its high lipophilicity and structural complexity, but satisfies others, such as the Veber rule and bioavailability score. It triggered no PAINS alerts and had a moderate synthetic accessibility score.

In summary, RA appears to possess moderate toxicity and favorable bioavailability. Some challenges may arise from its complexity, potentially affecting synthesis and optimization. Nonetheless, more in-depth studies are warranted to evaluate RA's potential as a promising scaffold for drug development.

2.9. Prediction of Molecular Targets and Docking Studies

The crystal structures of protein–inhibitor complexes of tyrosine phosphatase 1B (PTP1 β) with 2-(oxalyl-amino)-4,7-dihydro-5H-thieno [2,3-*c*]pyran-3-carboxylic acid (PDB id: 1C87) [17], T-cell protein tyrosine phosphatase (PTPN2) with ABBV-CLS-484 (PDB id: 7UAD) [to be published], prostaglandin E synthase (PTGES) with N-[4-(4-chlorophenyl)-1H-imidazol-2-yl]-2-(difluoromethyl)-5-[(2-methylpropanoyl)amino]methyl]benzamide (PDB id: 5BQH) [18], carboxylesterase 1 (CES1) with tamoxifen (PDB id: 1YA4) [19], fatty acid-binding protein, adipocyte with 2-[(2-phenylphenyl)amino]benzoic acid (PDB id: 6LJS), DNA-(apurinic or apyrimidinic site) endonuclease, mitochondrial (APE1) with 5-nitro-1H-indole-2-carboxylic acid (PDB id: 7TC2) [20], Cathepsin D (CPSD) with N-(3,4-dimethoxybenzyl)-N-alpha-[N-[(3,4-dimethoxyphenyl)acetyl]carbamimidoyl]-D-phenylalaninamide (PDB id: 4OD9) [21], dual specificity protein kinase CLK4 (CLK4) with 5-[(3-chlorophenyl)amino]benzo[c][2,6]naphthyridine-8-carboxylic acid (PDB id: 6FYV) [22], glycogen phosphorylase (PYGL) with N-(2-chloro-4-fluorobenzoyl)-N'-(5-hydroxy-2-methoxyphenyl)urea (PDB id: 2ATI) [23], phospholipase A2 (PLA2G2A) with 6-phenyl-4(R)-(7-phenyl-heptanoylamino)-hexanoic acid (PDB id: 1KQU) [24], carbonic anhydrase 2 (CAII) with acetazolamide (PDB id: 3HS4) [25], and epidermal growth factor receptor (EGFR) with erlotinib (PDB id: 1M17) [26] were obtained from the PDB database (<https://www.rcsb.org/>), accessed on 25 September 2023. To validate the docking procedure used here, we have re-docked the co-crystallized ligands into the same binding site as described in the PDB files previously procured. Following the validation of the docking procedure, it was used to dock RA into the active site of the predicted targets (Table S1 of the Supplementary Information). It was shown that RA complexed with CAII, CES1, EGFR, and PLA2G2A showed the best binding score, with competitive binding affinity to reference inhibitors reported in the literature and deposited in the PDB database, and complexed with the protein target structures. The two-dimensional and three-dimensional representations of RA interactions within the active sites of these targets are shown in Figures S1–S13 of the Supplementary Information.

2.10. Molecular Dynamics Simulation

RA complexed with each of the CAII, CES1, EGFR, and PLA2G2A receptors were shortlisted for performing molecular dynamics (MD) simulation based on their docking score and observed drug–receptor chemical interactions.

A drug–receptor complex has to be sufficiently stable over a nano-scale time range to execute its therapeutic response. The complex of the CAII enzyme with RA was evaluated for thermodynamic stability by MD simulation for a specified timeframe of 100 ns by using Schrodinger's Desmond software version 2022.4. The CAII enzyme's monomeric chain has 257 amino acids consisting of 2049 heavy atoms out of an overall 4055 atoms. Structural alterations and root-mean-square deviation (RMSD) analysis of the macromolecular backbone were executed during the 100 ns simulation to evaluate their thermodynamic stability. The complexed ligand RA constitutes five flexible bonds comprising thirty-two

heavy atoms of eighty atoms in total. CAII conjugated with RA displayed that the bound ligand showed a couple of conformational changes at 20 ns and 75 ns, followed by attaining stabilized conformation. The RMSD value of the receptor's backbone was found to fluctuate between 0.8 and 1.6 Å, whereas the bound ligand RA exhibited minor fluctuations after achieving stabilized conformation at 75 ns and its RMSD value in the receptor cavity fluctuated between 7.0 and 9.0 Å.

The CES1 receptor's monomeric chain has 531 amino acids consisting of 4124 heavy atoms out of an overall 8257 atoms. The CES1 receptor conjugated with ligand RA displayed that the bound ligand showed some fluctuations until the initial 40 ns of simulation, followed by attaining stabilized conformation. The RMSD value of the receptor's backbone was found to fluctuate between 1.00 and 1.75 Å, whereas the bound ligand RA was found to fluctuate between 2.0 and 4.5 Å.

The EGFR receptor's monomeric chain has 312 amino acids consisting of 2497 heavy atoms out of an overall 5031 atoms. The EGFR conjugated with ligand RA was found to be stabilized throughout the simulation, with the RMSD value of the receptor's backbone found to fluctuate between 6.5 and 10.5 Å, whereas the bound ligand RA exhibited very minute fluctuations and maintained stabilized conformation throughout the simulation with an RMSD value in the receptor cavity between 4.5 and 6.5 Å.

The PLA2G2A receptor's monomeric chain has 124 amino acids consisting of 942 heavy atoms out of an overall 1834 atoms. The PLA2G2A receptor conjugated with ligand RA displayed that the bound ligand showed a high degree of stability during the simulation. The RMSD value of the receptor's backbone was found to fluctuate between 1.5 and 3.5 Å, whereas the bound ligand RA exhibited high stability in its RMSD value within the receptor cavity, ranging from 3.5 to 7.5 Å. The RMSD profiles of RA conjugated with CAII, CES1, EGFR, and PLA2G2A are presented in Figure S14 (Supplementary Information).

The atoms in a protein or ligand structure might deviate from their initial location, and this can be measured by using their root-mean-square fluctuation (RMSF) value. This is an important parameter for determining the flexibility and dynamic behavior of the macromolecular complex. Protein RMSF is important because it may be used to predict protein dynamics and evaluate stability by providing information about the relative flexibility of various regions. MD-based evaluation of ligand RA complexed with CAII has concluded that the RMSF for C α backbone was found to be within 0.1–1.6 Å, while for ligand RA it was found to range from 2.0 to 5.5 Å. The RMSF for ligand RA complexed with CES1 was found to be within 0.5–2.5 Å for the C α backbone, while for the complexed ligand RA, the average variation was found to be within 1.0–2.5 Å. The RMSF for ligand RA complexed with EGFR was found to be within 1.5–4.5 Å for the C α backbone, except for some terminal residues, while the complexed ligand RA's average variation was found to be within 2.0–4.5 Å. The RMSF for the PLA2G2A enzyme C α backbone complexed with ligand RA was revealed to be within 0.8–3.0 Å for the protein C α backbone, while the ligand RA's average variation was found to be within 2.0–3.0 Å. Figures S15–S18 (Supplementary Information) show the RMSF profiles of the C α backbones and bound RA ligands for CAII, CES1, EGFR, and PLA2G2A, respectively, as detected during MD simulations.

The development of hydrophobic contacts, ionic interactions, and hydrogen bonds during an MD simulation are responsible for the thermodynamic permanence of a receptor–ligand complex, and it is evaluated by the continuous monitoring of its strength throughout the simulation for all three macromolecular complexes. The simulation of the CAII receptor complexed with ligand RA was found to be interacting with the CAII protein via the formation of hydrophobic bonds with the amino acids Trp5, Leu60, Val121, Phe131, Val135, Leu141, Leu198, Pro202, Leu204, and Trp209, with Asn62, His96, Gln136, and Thr200 via hydrogen bonds, and the amino acids Trp5, Tyr7, His64, Asn67, Gln92, Gly132, Gln136,

Thr200, and Pro201 are found to be interacting via water bridges. In another simulation, the CES1 protein complexed with ligand RA was found to be interacting with the CES1 protein via the formation of hydrophobic bonds with the amino acids Ala93, Leu96, Leu97, Leu100, Phe101, Val146, Val254, Leu255, Leu304, Pro317, Leu318, Ile359, Leu363, Met364, Leu388, Met425, and Phe426, with Leu304 and Leu306 via hydrogen bonds, and the amino acids Leu304, Leu306, Pro317, Leu363, and Met364 are found to be interacting via water bridges. In another simulation, the EGFR receptor complexed with ligand RA was found to be interacting with the EGFR receptor via the formation of hydrophobic bonds with the amino acids Leu694, Phe699, Val702, Ala719, and Leu820, with Thr766, Gln767, and Met769 via hydrogen bonds, and the amino acids Thr766, Gln767, Met769, Phe771, and Thr830 were found to be interacting via water bridges. In another simulation, the PLA2G2A receptor complexed with ligand RA was found to be interacting with the PLA2G2A receptor via the formation of hydrophobic bonds with the amino acids Leu2, Phe5, Ile9, Ala17, Ala18, Tyr21, Tyr51, and Phe98, with His27, Gly29, Val30, and Gly31 via hydrogen bonds, and the amino acids His27, Gly29, Val30, Gly31, Gly32, Cys44, His47, and Asp48 were found to be interacting via water bridges. Figure S19 (Supplementary Information) illustrates the chemical interactions between RA and the binding site residues of the (a) CAII, (b) CES1, (c) EGFR, and (d) PLA2G2A receptors.

3. Discussion

Natural compounds derived from plants have long served as valuable sources of bioactive agents in cancer therapy. Among these, RA, a triterpenoid compound isolated from various *Gentiana* species, has recently gained attention due to its anti-inflammatory and potential anticancer properties. However, despite its promising biological profile, RA remains relatively understudied in the context of oncology, particularly regarding its activity in solid tumors and the mechanisms underlying its effects on cancer and normal cells.

Previous studies have indicated that RA may exert antiproliferative and proapoptotic effects in certain cancer models. These effects have been associated with cell cycle arrest at the G0/G1 phase, potentially mediated by downregulation of cyclins B1, D1, and E1, as well as with increased apoptotic signaling evidenced by poly(ADP-ribose) polymerase-1 (PARP1) cleavage and the activation of caspases 3, 7, and 9 [8]. Mechanistic studies suggest that RA may exert its activity through direct interaction with TNF- α , thereby inhibiting the NF- κ B pathway [8]. Additionally, RA has been reported to possess anti-inflammatory properties through inhibition of cyclooxygenase enzymes and by competitively binding to tumor necrosis factor receptor 1 (TNF-R1), further contributing to its proapoptotic potential [27]. In colorectal cancer cells, RA treatment has been shown to inhibit DNA synthesis, as demonstrated by 5-ethynyl-2'-deoxyuridine (EdU) incorporation assays, and to induce morphological changes including cell shrinkage, detachment from colonies, and cell death [8]. Apoptosis induction has been further confirmed by annexin V staining and flow cytometric analysis [8]. However, despite these promising findings, the current body of evidence remains fragmented, often limited to a single cell line or narrowly focused on individual pathways, and lacks systematic evaluation across different cancer types or normal cell counterparts. In particular, the distinction between the cytotoxic and cytostatic effects of RA has not been clearly established. Therefore, further studies are needed to comprehensively assess the biological activity of RA, to determine whether its observed effects are primarily due to cell death or proliferation arrest, and to evaluate its selectivity across both malignant and non-malignant human cell lines.

In the present study, we evaluated the biological activity of RA against a panel of human cancer cell lines derived from different tissue origins, including DLD-1 and HT-29 (colorectal adenocarcinoma), HCT-116 (colorectal carcinoma), PC-3 (prostate adenocarci-

noma), and BxPC-3 (pancreatic adenocarcinoma). To assess selectivity, we also included two non-malignant cell lines: WI-38 (normal human fibroblasts) and CCD-841 CoN (normal human colon epithelial cells). Cell viability and metabolic activity were assessed using MTT, neutral red uptake, and crystal violet assays following 24 and 72 h incubations with RA. Across all three assays, RA induced a concentration-dependent reduction in signal intensity, which initially suggested either decreased viability or inhibited proliferation. Cancer cell lines were more responsive, with IC_{50} values ranging from 14.16 to 31.18 μ M after 24 h and from 8.25 to 15.91 μ M after 72 h. In contrast, non-tumorigenic cells displayed higher IC_{50} values at 24 h (33.13–38.97 μ M), supporting a favorable selectivity profile at early timepoints. However, after 72 h of incubation, this distinction became less evident, as the IC_{50} values for normal cells (12.82–19.34 μ M) approached those of the less responsive cancer cell lines (e.g., BxPC-3 and PC-3). This observation suggests that the compound's selectivity decreases with prolonged exposure. Despite this overall pattern, colorectal cancer cell lines tended to respond more strongly to RA than the other tumor types. Based on these observations, DLD-1, HCT-116, and HT-29 cells were selected for subsequent mechanistic studies.

To further elucidate whether the observed decrease in metabolic activity was due to cytotoxicity or reduced proliferation, we employed three complementary approaches: a cell density analysis combined with trypan blue exclusion, clonogenic assay, and BrdU incorporation assay. These experiments were conducted using DLD-1, HCT-116, and HT-29 cells, selected for their pronounced response to RA in prior viability assessments. Initial evidence for a cytostatic effect came from cell density assessments, which revealed a marked reduction in cell confluence following RA treatment without a significant decrease in viability, as determined by trypan blue staining. Additional confirmation was provided by the clonogenic assay, which demonstrated a significant reduction in the number of colonies formed by DLD-1, HCT-116, and HT-29 cells treated with RA at both IC_{50} and $2 \times IC_{50}$ concentrations. Finally, the BrdU incorporation assay confirmed that RA inhibits DNA synthesis, thereby substantiating its antiproliferative mechanism of action. Notably, in the HT-29 cell line, RA was more effective at suppressing BrdU incorporation than OXA, a chemotherapeutic agent used as a positive control. Collectively, these findings suggest that RA exerts its anticancer effects primarily by suppressing cellular proliferation rather than inducing direct cytotoxicity. The diminished selectivity observed after prolonged exposure is likely a consequence of this antiproliferative mode of action, which, over time, also affects slower-dividing non-malignant cells. Unlike many classical chemotherapeutics, which rely on acute cytotoxic mechanisms, RA's ability to reduce proliferation without causing significant cell death may offer a more favorable safety profile. This characteristic positions RA as a potential candidate for combination therapies or for use in treatment regimens requiring lower systemic toxicity.

Beyond its effect on cancer cell proliferation, we investigated its potential to modulate NF- κ B signaling, a pathway critically involved in inflammation-driven tumor progression, resistance to apoptosis, and therapy failure in various cancers [28,29]. To this end, we used the NF- κ B-TIME reporter cell line, which harbors a stably integrated NF- κ B-responsive luciferase construct and provides a highly sensitive and reproducible model for monitoring transcriptional responses to inflammatory stimuli such as TNF- α . This system enables direct quantification of pathway activation without reliance on downstream target genes, making it particularly suited for pharmacological screening. Since this cell line is not tumor-derived and is optimized for transcriptional signaling rather than viability assays, it was not included in cytotoxicity or proliferation studies such as MTT or BrdU. We aimed to functionally verify previous observations suggesting the anti-inflammatory properties of RA by determining its ability to interfere with TNF- α -induced NF- κ B activity [7,8]. Our

results demonstrated that RA significantly reduced NF- κ B-mediated reporter expression at concentrations of 10 μ M, 15 μ M, and 20 μ M, even after short-term exposure (6 h total incubation and 1 h pre-treatment). Lower concentrations had no detectable effect. The compound was tested across a wide concentration range (0.5–20 μ M) to capture potential transcriptional effects. As the assay was not intended to assess cell viability, IC₅₀ values were not determined. Instead, fixed concentrations were selected based on prior results to avoid concentrations that significantly affect cell growth, ensuring a reliable assessment of NF- κ B modulation. These findings indicate that RA attenuates inflammatory signaling in a concentration-dependent manner, reinforcing the view that its biological effects may extend beyond proliferation control to include suppression of pro-tumorigenic signaling networks. This observation is particularly relevant given that NF- κ B is frequently hyperactivated in colorectal cancer and contributes to a microenvironment that supports tumor growth and immune evasion [30,31]. Therefore, the ability of RA to modulate this pathway may enhance its therapeutic profile and support its further evaluation as a candidate for inflammation-associated malignancies.

Additionally, we performed *in silico* screens to investigate RA as a scaffold for future drug development. Computational predictions indicated that RA may exhibit mild toxicity and satisfactory oral bioavailability, although its complex chemical structure could pose challenges for synthesis and optimization. Molecular docking and dynamics simulations suggested that RA may exert its anticancer effects through interactions with several proteins involved in cell proliferation and death, including CAII, CES1, EGFR, and PLA2G2A. While CAII, EGFR, and PLA2G2A are associated with various malignancies, CES1 showed the most stable interaction with RA in our simulations, with low RMSD and RMSF values and persistent binding within the active site. CES1 is a metabolic lipase regulated by NF- κ B and is known to support fatty acid oxidation and energy balance in aggressive colorectal cancers. Its overactivity contributes to cancer cell survival by preventing the accumulation of cytotoxic lipid species [32]. Previous studies have shown that pharmacological or genetic inhibition of CES1 alters lipid metabolism, compromises mitochondrial function, and sensitizes tumor cells to chemotherapeutic agents. Notably, targeting the CES1/PPAR α /stearoyl-CoA desaturase 1 pathway has been reported to enhance the efficacy of cisplatin in hepatocellular carcinoma models, with coadministration reducing tumor growth in xenografted mice [33]. Based on these computational findings and the biological relevance of CES1 in cancer, we propose that CES1 may represent a promising molecular target for RA and its derivatives. However, it should be emphasized that our docking and simulation results are predictive in nature and serve as a preliminary hypothesis-generating framework that awaits experimental confirmation.

4. Materials and Methods

4.1. Cell Culture

Human cancer cell lines BxPC-3 (pancreatic adenocarcinoma, ATCC[®] CRL-1687TM), DLD-1 (colorectal adenocarcinoma, ATCC[®] CCL-221TM), HCT-116 (colorectal carcinoma, ATCC[®] CCL-247TM), HT-29 (colorectal adenocarcinoma, ATCC[®] HTB-38TM), and PC-3 (prostate cancer, ATCC[®] CRL-1435TM), as well as normal cell lines WI-38 (human lung fibroblasts, ATCC[®] CCL-75TM) and CCD-841 CoN (human colon epithelial, ATCC[®] CRL-1790TM) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). In addition, the NF- κ B-TIME reporter cell line (human endothelial origin, ATCC[®] CRL-4049TM), which carries a luciferase reporter under the control of NF- κ B response elements, was kindly provided by the Institute of Vascular Biology and Thrombosis Research at the Medical University of Vienna, Vienna, Austria. BxPC-3, DLD-1, HCT-116, and HT-29 cells were cultured in RPMI-1640 medium; PC-3 cells in DMEM/F12; WI-38 and CCD-841

CoN in MEM Alpha medium; and NF- κ B-TIME cells in vascular cell basal medium supplemented with a bovine brain extract growth kit, 12.5 μ g/mL blasticidin, and 20 μ g/mL hygromycin B. All media were further supplemented with 10% (*v/v*) fetal bovine serum (FBS) and 1% (*v/v*) streptomycin–penicillin solution.

Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂. The culture medium was refreshed every 24–48 h. After the cells reached confluence, subculture was performed using 0.25% trypsin-EDTA. Mycoplasma contamination was routinely monitored using the MycoBlue™ Mycoplasma Detector kit (Vazyme BiotechCo., Ltd., Nanjing, China).

4.2. MTT Assay

To evaluate the effect of the test compound on cell viability, the MTT assay was employed. This colorimetric assay measures the metabolic activity of cells, based on their ability to reduce the yellow tetrazolium salt to insoluble violet-blue formazan crystals via mitochondrial succinate dehydrogenase. The amount of formazan produced is directly proportional to the number of metabolically active (viable) cells. After solubilization of the formazan crystals in an organic solvent, absorbance can be measured spectrophotometrically [34]. Cells ($8\text{--}10 \times 10^3$ /well) were seeded in 96-well plates and incubated for 24 h (37 °C, 5% CO₂), then treated with RA (0.5–100 μ M) for 24 or 72 h. After incubation, 20 μ L of MTT solution (5 mg/mL) was added per well and incubated for 3 h. Formazan crystals were dissolved in 100 μ L of dimethyl sulfoxide (DMSO), and absorbance was measured at 570 nm using a microplate reader (Power Wave XS, BioTek Instruments, Winooski, VT, USA). The IC₅₀ value—the concentration of RA required to reduce cell viability by 50%—was calculated based on absorbance values using non-linear regression analysis in GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA) [35].

4.3. Neutral Red Uptake Assay

Cells were seeded in 96-well plates following the same procedure as described for the MTT assay. After compound exposure, cells were incubated with 100 μ L of neutral red solution (40 μ g/mL) for 2 h. After washing with phosphate-buffered saline (PBS), 150 μ L of destaining solution (50% ethanol, 49% water, and 1% glacial acetic acid) was added, and plates were shaken for 15 min. Absorbance was measured at 540 nm using a microplate reader. The procedure was adapted from the protocol described by Kciuk et al. [36]. The GraphPad Prism 8.0 software was used to calculate the IC₅₀ values.

4.4. Crystal Violet Assay

Cells were seeded analogously to the previously described methods. After incubation, cells were washed with PBS and fixed with 75% methanol (100 μ L/well, 20 min, 4 °C), followed by 100% methanol (20 min, 4 °C). Fixed cells were stained with 0.2% crystal violet (100 μ L/well, 20 min, RT), washed with water, dried, and the dye was solubilized in DMSO. Absorbance was measured at 590 nm using a microplate reader. IC₅₀ values were determined using the GraphPad Prism 8.0 software.

4.5. Cell Density and Viability Analysis Using Trypan Blue Exclusion

To evaluate the mechanism of RA action, cell density and viability were assessed after 24 and 72 h incubations with RA at the consensus IC₅₀. Culture medium from each well was collected, and cells were washed with PBS and detached using trypsin-EDTA. After neutralization with complete medium, the total cell number was determined using a Thoma counting chamber and expressed as a percentage relative to untreated controls. In parallel, cell viability was assessed using the trypan blue exclusion method. A 1:1 mixture

of cell suspension and 0.4% trypan blue was prepared, and the viability was calculated as the percentage of unstained (viable) cells among a minimum of 300 counted.

4.6. Clonogenic Assay

To evaluate clonogenic potential, cells were first seeded at varying densities (100–2000 cells/well) in 6-well plates. The highest plating efficiency was observed at 500 cells/well, which was used for further experiments. After cell attachment (2–3 h), RA was added at $0.5 \times IC_{50}$, IC_{50} , and $2 \times IC_{50}$ concentrations. Cells were incubated for 10 days, with media replaced every 3 days. Colonies were then fixed with 6% formaldehyde (10 min), stained with crystal violet (10 min), washed, dried, and imaged. Colonies were counted using the ImageJ software, version 1.54f.

4.7. Bromodeoxyuridine Incorporation Assay

The BrdU incorporation assay was performed to assess RA's effect on DNA synthesis. Cells were seeded onto sterile round coverslips placed in 12-well plates at a density of 30,000 cells per well. After 24 h, cells were treated with RA at $0.5 \times IC_{50}$ and IC_{50} concentrations for 48 h. OXA (15 μ M) served as a positive control, with the concentration selected based on previously reported IC_{50} values for colorectal cancer cell lines to ensure standardized and pharmacologically relevant conditions [11–13,15]. BrdU was added to the culture medium at a final concentration of 10 μ M, 24 h prior to fixation. Following treatment, cells were fixed with 70% ethanol, and DNA was denatured using 4N HCl. After neutralization with 0.1 M borax, cells were incubated with a primary anti-BrdU antibody (1:100, Purified Mouse Anti-BrdU by BD™), followed by a secondary Alexa Fluor 488-conjugated anti-mouse antibody (1:650, Alexa Fluor 488 goat anti-mouse IgG (H + L) Invitrogen by Thermo Fisher Scientific). Nuclei were counterstained with DAPI. Coverslips were mounted using Fluoromount-G and examined under a fluorescence microscope (Olympus, Tokyo, Japan, CellSens V2.3 software). BrdU-positive cells were quantified based on green fluorescence, counting ~200 cells per sample.

4.8. NF- κ B Reporter Cell Luciferase Assay

To evaluate the effect of RA on TNF- α -induced NF- κ B transcriptional activity, we employed the NF- κ B-TIME luciferase reporter assay. Reporter cells were seeded into white-walled 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA) at a density of approximately 10,000 cells per well and incubated overnight under standard conditions. On the following day, cells were pre-treated with RA (0.5–20 μ M) for 1 h, followed by co-stimulation with TNF- α (10 ng/mL; PeproTech, London, UK) for an additional 6 h. NF- κ B activity was then quantified by luminescence measurement using the Nano-Glo® Luciferase Assay System (Promega, Madison, WI, USA) and recorded in relative light units with a TriStar² S LB 942 plate reader (Berthold Technologies, Bad Wildbad, Germany). The concentration range was chosen to avoid the antiproliferative effects observed at higher doses, enabling the interpretation of transcriptional responses independent of growth inhibition. IC_{50} values were not determined, as the assay was not designed to assess cytotoxicity.

4.9. ADMET and Drug-Likeness Analyses

ADMET profiling and drug-likeness assessments are crucial for evaluating the suitability of chemical compounds as potential drug candidates. ADMET analysis provides insight into the absorption, distribution, metabolism, excretion, and potential toxicity of a compound, helping to predict its behavior in biological systems. In parallel, drug-likeness evaluation determines whether a compound exhibits physicochemical and structural features typical of known drugs, thereby aiding in the selection of candidates with promising

pharmacokinetic and pharmacodynamic properties. In this study, the ADMET characteristics and drug-likeness of RA were evaluated using two publicly available *in silico* tools: Swiss-ADME (<http://www.swissadme.ch>) (accessed on 26 September 2023) and pkCSM (<https://biosig.lab.uq.edu.au/pkcsm/prediction>) (accessed on 26 September 2023).

4.10. Prediction of Molecular Targets and Docking Studies

The PDB databank (<https://www.rcsb.org/>), accessed on 25 September 2023, was checked for possible molecular targets of RA previously predicted based on the isomeric SMILES code obtained from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/compound/Roburic-acid> (accessed on 25 September 2023)) with the use of SwissTargetPrediction (<http://www.swisstargetprediction.ch/> (accessed on 25 September 2023)), SuperPred3 (<https://prediction.charite.de/> (accessed on 25 September 2023)), and BindingDB (<https://www.bindingdb.org/> (accessed on 25 September 2023)) for ligand similarity of at least 0.8) webserver. Their involvement in cancer pathogenesis was confirmed through the mining of the PubMed database (<https://pubmed.ncbi.nlm.nih.gov/> (accessed on 25 September 2023)) using the following queries: “cancer” AND “protein name”.

The structure of RA was created using the ChemDraw 8.0 software, and its three-dimensional arrangement was obtained by performing energy minimization using an MM2 force field. Before conducting the molecular docking simulation, we utilized AutoDock 4, a software developed by The Scripps Research Institute in La Jolla, CA, USA. This software was used to detect aromatic carbons and rotatable bonds, establish automatic torsion parameters, consider non-polar hydrogens, and add Gasteiger charges. The AutoDock 4 tools were employed to assign autodock atom types (AD4) to the macromolecules, and Gasteiger charges were integrated and distributed throughout the macromolecule. Subsequently, the structures were stored in the PDBQT file format. To validate the process of molecular docking simulation, the structure representations of all the produced macromolecular targets were first docked with their respective reference ligands, which were already in complex structural models.

4.11. Molecular Dynamics Simulations

RA complexed with the CAII, CES1, EGFR, and PLA2G2A proteins were shortlisted for performing MD simulation based on their docking score and observed drug–receptor chemical interactions. MD simulation was executed for each of the above-mentioned macromolecular complexes for a time of 100 ns by using the Desmond module of Schrodinger’s Maestro software version 2022.1 [37–39]. The addition of explicit solvent molecules was followed by their neutralization by adding the respective ions. The steepest-descent algorithm was used to relax the system and eliminate any steric clashes or poor contacts within atoms to minimize the system’s energy. Using a short series having a low temperature with constant pressure simulations, the system was brought to equilibrium. Positional constraints are applied to the system in addition to a progressive increase in temperature [40–42]. This makes it more likely that the system will be in a stable, balanced state before the simulation; to obtain the appropriate outcomes, the simulation is performed for 100 ns while taking into account the system’s energies, atom positions, and RMSD values. This aids in comprehending the system’s dynamic behavior and provides long-term intuitions on the complex’s structure and functional stability [43,44].

4.12. Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 10.0 software (GraphPad Prism Software Inc., San Diego, CA, USA). The results obtained in the MTT test came from 3 independent experiments and were presented as mean values with standard deviations (SDs). The outcomes of the neutral red uptake assay and the crystal violet assay, as

complementary methods, were performed in duplicate. All results were transformed by using the logarithmic function $\log(X)$, where X was the concentration used. The next step was to normalize the results, and finally, plot a response–inhibition curve using a non-linear regression model. The use of these tools made it possible to determine the IC_{50} parameter values. For cell density and viability data obtained via the trypan blue exclusion assay, statistical significance between the treated and control groups was evaluated using the unpaired two-tailed Student's t -test. Prior to analysis, the assumption of normal distribution was verified using the Shapiro–Wilk test. For the clonogenic assay and BrdU incorporation assay, differences among multiple treatment groups were assessed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for pairwise comparisons. In the NF- κ B reporter assay, luminescence values were normalized to the TNF- α -stimulated control group, and statistical comparisons were performed using one-way ANOVA with Dunnett's post hoc test. A p -value of less than 0.05 was considered statistically significant in all analyses.

5. Conclusions

RA is a plant-derived compound primarily known for its anti-inflammatory properties, but recent studies have highlighted its potential as an anticancer agent. In this study, we demonstrated that RA reduces metabolic activity, inhibits colony formation, and decreases DNA synthesis in various cancer cell lines, with colorectal models (DLD-1, HCT-116, and HT-29) showing relatively greater sensitivity, particularly at shorter incubation times. These findings support an antiproliferative rather than cytotoxic mode of action. In addition, we showed that RA significantly attenuates TNF- α -induced NF- κ B activation in a reporter assay, indicating its ability to modulate inflammatory signaling pathways commonly involved in tumor progression. Furthermore, computational studies suggest that RA may exert its therapeutic potential through interaction with the CES1 receptor, as the macromolecular complex of CES1-RA was found to be highly stabilized throughout the simulation time, with a very low RMSD and RMSF value for the C α chain as well as complexed RA, with strong interactions with the macromolecular residues throughout the simulation. In particular, CES1 may represent an interesting molecular target for RA or its derivatives in the context of anticancer therapy for aggressive colorectal cancers.

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Abbreviations

ADMET	Absorption, Distribution, Metabolism, Excretion, and Toxicity
APE1	(Apurinic or apyrimidinic site) endonuclease 1
ARE	Antioxidant responsive element
BrdU	Bromodeoxyuridine
CAII	Carbonic anhydrase 2
CADD	Computer-aided drug design
CES1	Carboxylesterase 1
CLK4	CDC like kinase 4
CPSD	Cathepsin D
DMSO	Dimethyl sulfoxide
EdU	5-Ethynyl-2'-deoxyuridine
EGFR	Epidermal growth factor receptor
FBS	Fetal bovine serum
KEAP1	Kelch-like ECH-Associating protein 1
MAPKs	Mitogen-activated protein kinases
MD	Molecular dynamics
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NF- κ B	Nuclear factor NF-kappa-B
NRF2	Nuclear factor erythroid 2-related factor 2
OCT2	Organic Cation Transporter 2
OXA	Oxaliplatin
PARP1	Poly(ADP-ribose) polymerase-1
PBS	Phosphate-buffered saline
PLA2G2A	Phospholipase A2 Group IIA
PTGES	Prostaglandin E synthase
PTP1 β	Protein tyrosine phosphatase 1 β
PTPN2	T-cell protein tyrosine phosphatase
PYGL	Glycogen phosphorylase
RA	Roburic acid
RMSD	Root-mean-square deviation
RMSF	Root-mean-square fluctuation
TNF- α	Tumor necrosis factor α
TNF-R1	Tumor necrosis factor receptor 1
VDss	Volume of distribution at steady state

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Supplementary Information

Anticancer activity of roburic acid: in vitro and in silico investigation

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Table S1. Molecular docking results for RA with the predicted molecular targets, including binding affinities and identified interacting residues. The binding properties were compared to those of reference inhibitors co-crystallized with the target protein structure obtained from the PDB database (<https://www.rcsb.org/>).

Predicted target with symbol	PDB id	Reference ligand	Binding affinity reported for experimental study/Key interacting residues reported from crystallographic studies	Binding score (affinity)	Interacting residues from docking validation study	Binding score (affinity) for RA and predicted target	Interacting residues
tyrosine phosphatase 1B (PTP1 β)	1C87	2-(oxalyl-amino)-4,7-dihydro-5H-thieno[2,3-c]pyran-3-carboxylic acid	Ki = 6.30e+4 nM Gly-22, Arg-22, Tyr-46, Val-49, Lys-120, Asp-181, Ala-217, Ile-219.	-9.2	Tyr46, Val49, Lys120, Asp181, Ser216, Ala217, Gly220, Arg221.	-6.98	Tyr46, Asp48, Val49, Lys116, Asp181, Phe182, Ser216, Ala217, Ile219.
T-cell protein tyrosine phosphatase (PTPN2)	7UAD	ABBV-CLS-484	Not available	-11.88	Tyr48, Asp50, Lys122, Asp182, Phe183, Cys216, Ser217, Ala218, Gly219, Gly221, Gln260, Gln264, Arg292.	-5.96	Tyr22, Arg26, Asp50, Phe183, Ile220, Met256, Gln260.
prostaglandin synthase (PTGES)	5BQH	N-[4-(4-chlorophenyl)-1H-imidazol-2-yl]-2-(difluoromethyl)-5-[(2-methylpropanoyl)amino]methylbenzamide	IC ₅₀ = 3 nM Ile-32, Gln-36, Arg-52, His-53, Ser-127.	-7.03	Arg70, Tyr117, Arg126, Ser127, Tyr130, Thr131.	-8.52	Arg70, Glu77, Tyr117, Ser127, Tyr130.
carboxylesterase 1 (CES1)	1YA4	tamoxifen	Ki = 1.52e+4 nM Phe-101, Ser-221, Leu-304, Leu-318, Leu-363, Met-364, Leu-388, His-468.	-8.42	Leu97, Val254, Leu255, Phe303, Leu304, Ser305, Leu318, Ile359, Leu363,	-10.3	Ala93, Leu97, Val146, Leu255, Leu304, Leu363, Met364, Ile359, His468.

					Met364, His468.		
fatty acid-binding protein, adipocyte (FABP4)	6LJS	2-[(2-phenylphenyl)amino]benzoic acid	Kd = 110.6 nM Phe-16, Ser-53, Ser-55, Phe-57, Lys-58, Thr-60, Ala-75, Arg-126, Tyr-128.	-8.59	Phe16, Met20, Ala33, Ala36, Pro38, Phe57, Ala75, Ile104, Val115, Cys117, Arg126, Tyr128.	-9.04	Phe16, Tyr19, Met20, Val23, Val25, Ala33, Pro38, Met40, Ile51, Phe57, Ala75, Arg106, Tyr128.
NA-(apurinic or apyrimidinic site) endonuclease, mitochondrial (APE1)	7TC2	5-nitro-1H-indole-2-carboxylic acid	Leu-62, Ile-64, Ile-91, Ser-135, Arg-136, Gln-137, Phe-162, Ser-164, Phe-165.	-5.81	Leu62, Ser135, Arg136, Gln137, Ser164.	-6.26	Thr61, Gln137, Phe162, Phe165, Leu318.
Cathepsin D (CPSD)	4OD9	N-(3,4-dimethoxybenzyl)-N-alpha-{N-[(3,4-dimethoxyphenyl)acetyl]carbamimidoyl}-D-phenylalaninamide	IC ₅₀ = 58 nM Asp-33, Tyr-78, Gly-79, Phe-126, Phe-131, Ile-134, Tyr-205, Asp-231, Thr-234, Met-309, Ile-311.	-9.94	Val31, Asp33, Tyr78, Gly79, Phe126, Ala129, Phe131, Ile134, Tyr205, Asp234, Met309, Ile311, Ile320.	-7.61	Val31, Tyr78, Phe131, Ile311, Ile320.
dual specificity protein kinase CLK4 (CLK4)	6FYV	5-[(3-chlorophenyl)amino]benzo[c][2,6]naphthyridine-8-carboxylic acid	IC ₅₀ = 11 nM Leu-248, Ser-249, Ser-301, Val-326.	-9.82	Leu167, Gly168, Phe172, Val175, Ala189, Lys191, Val225, Phe241, Leu244, Leu295, Val324, Asp325.	-9.19	Glu169, Val175, Ala189, Lys191, Phe241, Leu244, Leu295, Val324.

glycogen phosphorylase (PYGL)	2ATI	N-(2-chloro-4-fluorobenzoyl)-N'-(5-hydroxy-2-methoxyphenyl)urea	IC ₅₀ = 23 nM Val-40, Lys-41, Asp-42, Asn-44 Trp-67, Tyr-75, Arg-193.	-3.6	His36, Val40 , Lys41 , Asp42, Val45 .	-4.72	Val40 , Lys41 , Val45 .
phospholipase A2 (PLA2G2A)	1KQU	6-phenyl-4(R)-(7-phenyl-heptanoylamino)-hexanoic acid	Gly-30, Gly-32, His-48, Asp-49.	-9.33	Leu2, Phe5 , His6 Ile9, Ala17, Gly29, Val30, Gly31, Tyr51, Lys62, Phe98 .	-11.83	Phe5 , Cys44, His47, Asp48, Tyr51, Lys52, Ala94, Phe98 .
carbonic anhydrase 2 (CAII)	3HS4	acetazolamide	IC ₅₀ : min: 0.49 nM, max: 4500 nM His-94, His-96, His-119, Val121, Phe131, Leu198, Thr199, Thr200, Pro201.	-5.91	His94 , His96 , His119 , Val121 , Leu198 , Thr200 , Trp209 .	-8.43	Asn62, His94 , His96 , His119 , Val121 , Phe131, Leu141, Val143, Leu198 , Thr200 , Trp209 .
epidermal growth factor receptor (EGFR)	1M17	erlotinib	IC ₅₀ : min: 0.03 nM, max: 260 nM Ser-671, Gly-672, Asn-676, Leu-680, Glu-685, Tyr-740, Ser-744.	-6.74	Leu694 , Val702 , Ala719, Lys721 , Met742, Leu764, Met769, Leu820.	-8.58	Leu694 , Phe699, Val702 , Lys721 , Leu768, Thr830, Asp831.

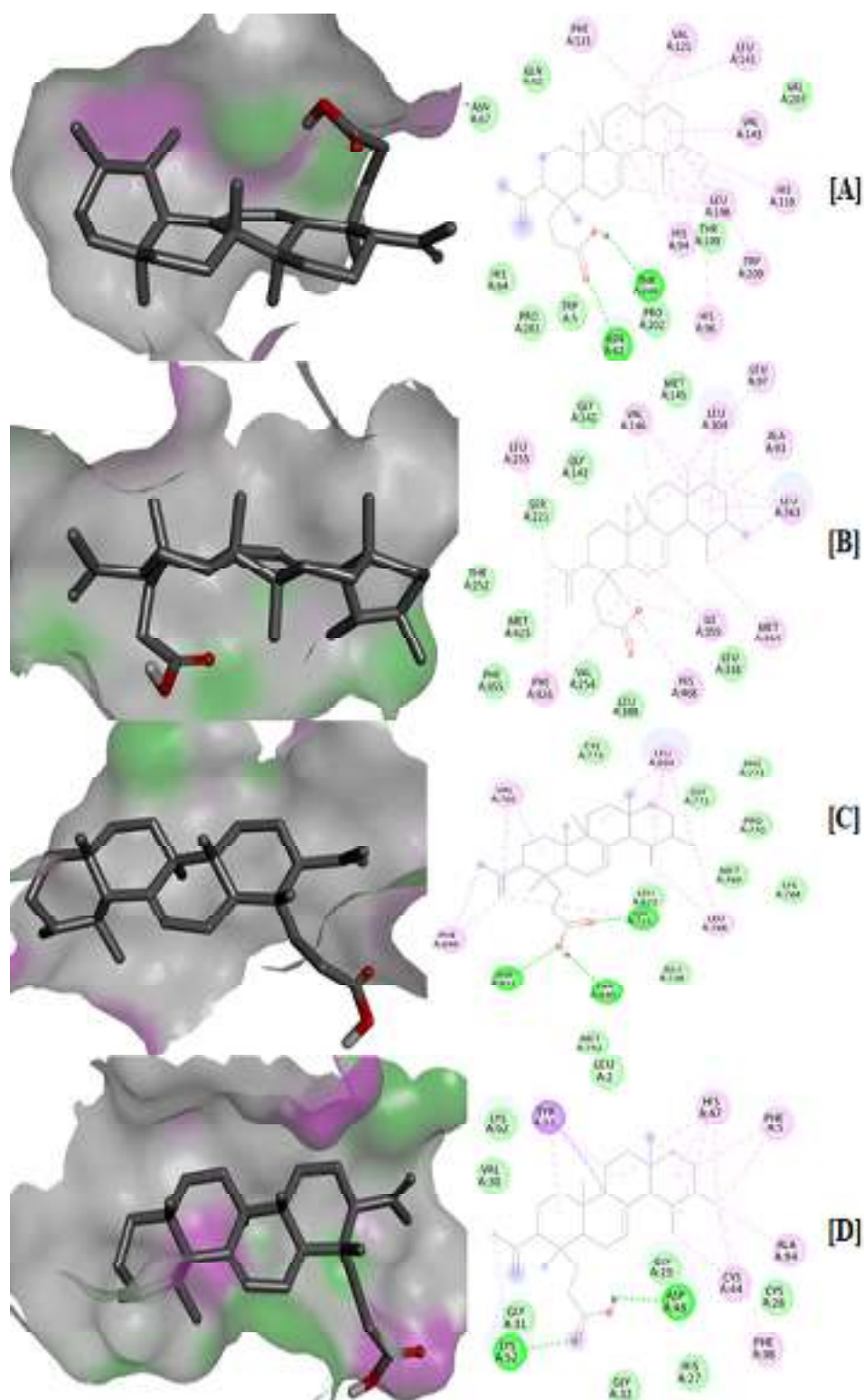


Figure S1. Two-dimensional and three-dimensional binding modes of RA with [A] CAII, [B] CES1, [C] EGFR, and [D] PLA2G2A.

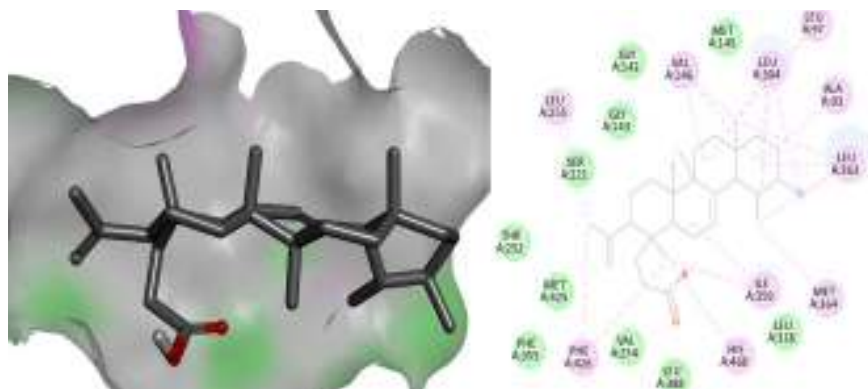


Figure S5. The two-dimensional and three-dimensional binding interactions of RA within the active site of 1ya4 as predicted by molecular docking.

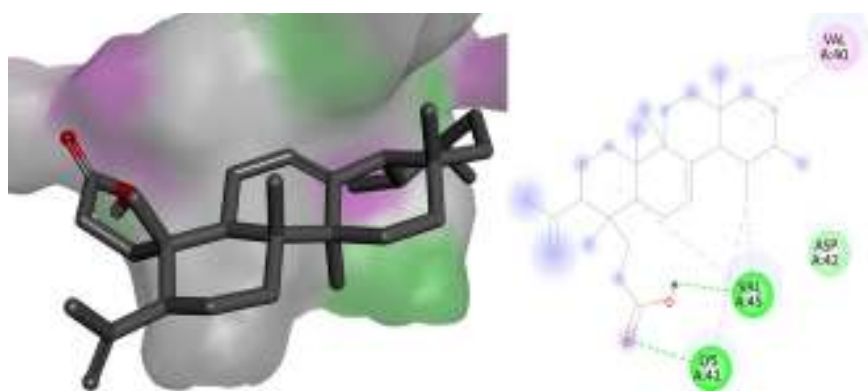


Figure S6. The two-dimensional and three-dimensional binding interactions of RA within the active site of 2ati as predicted by molecular docking.

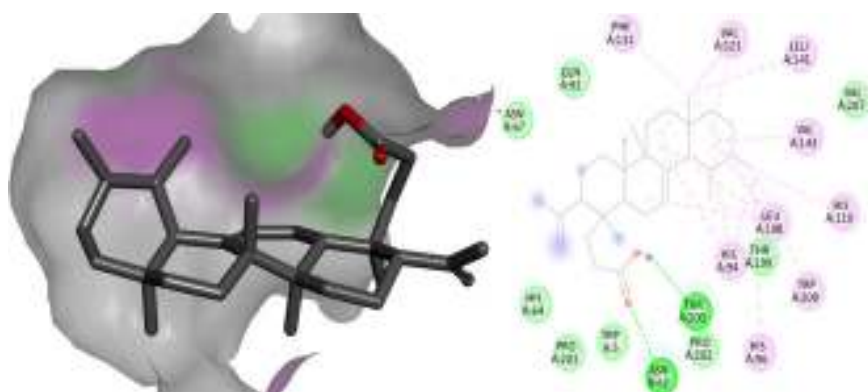


Figure S7. The two-dimensional and three-dimensional binding interactions of RA within the active site of 3hs4 as predicted by molecular docking.

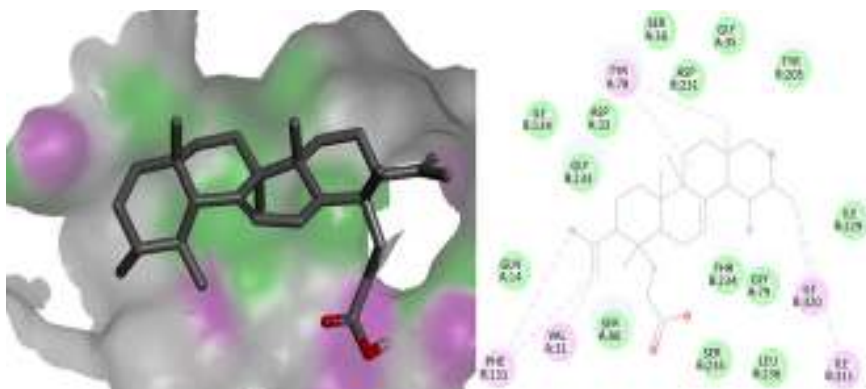


Figure S8. The two-dimensional and three-dimensional binding interactions of RA within the active site of 4od9 as predicted by molecular docking.

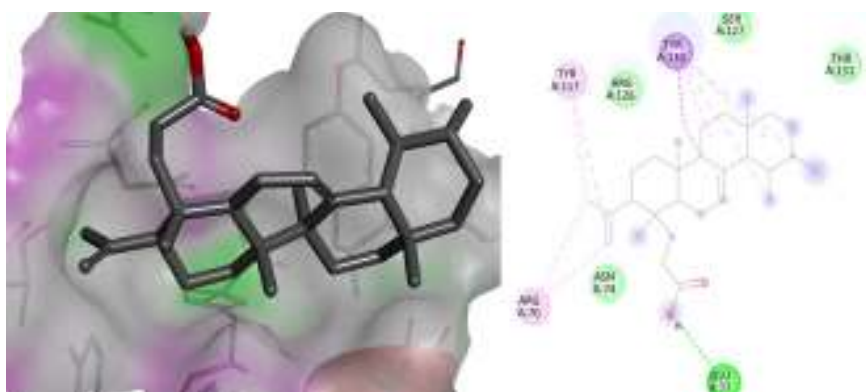


Figure S9. The two-dimensional and three-dimensional binding interactions of RA within the active site of 5bqh as predicted by molecular docking.

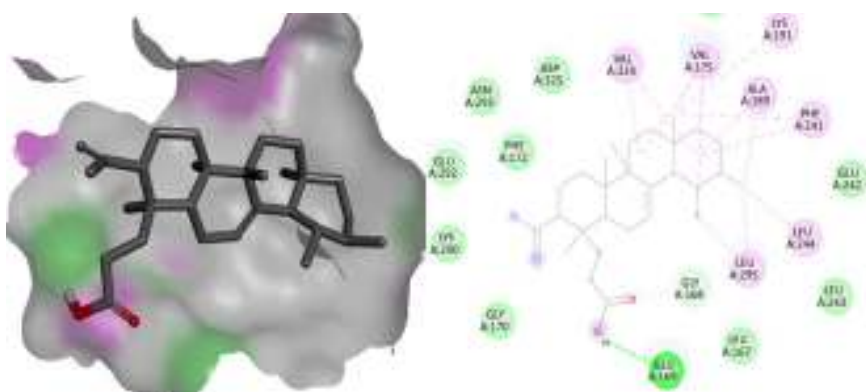


Figure S10. The two-dimensional and three-dimensional binding interactions of RA within the active site of 6fyv as predicted by molecular docking.

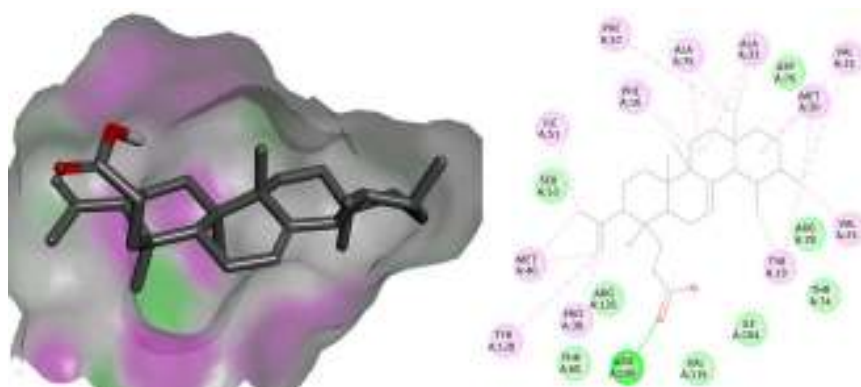


Figure S11. The two-dimensional and three-dimensional binding interactions of RA within the active site of 6ljs as predicted by molecular docking.

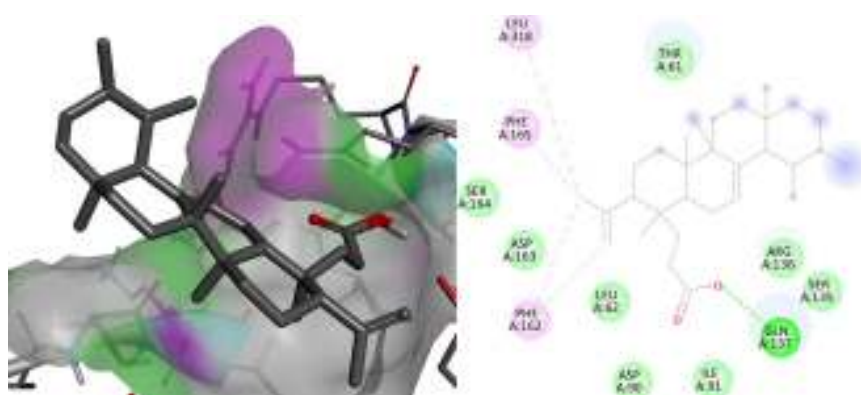


Figure S12. The two-dimensional and three-dimensional binding interactions of RA within the active site of 7tc2 as predicted by molecular docking.

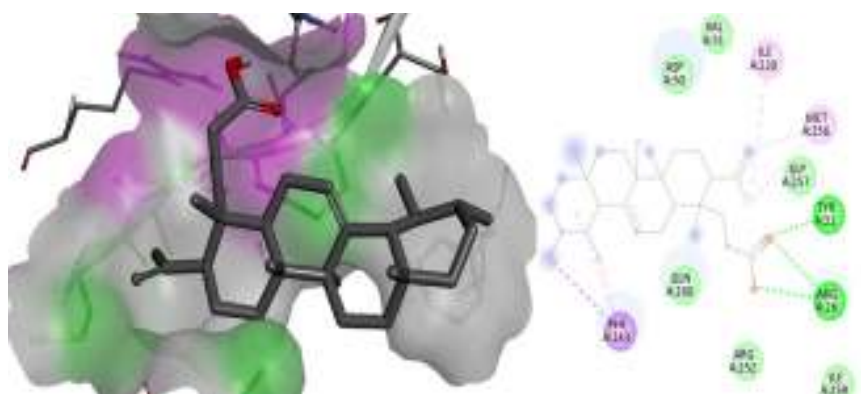


Figure S13. The two-dimensional and three-dimensional binding interactions of RA within the active site of 7uad as predicted by molecular docking.

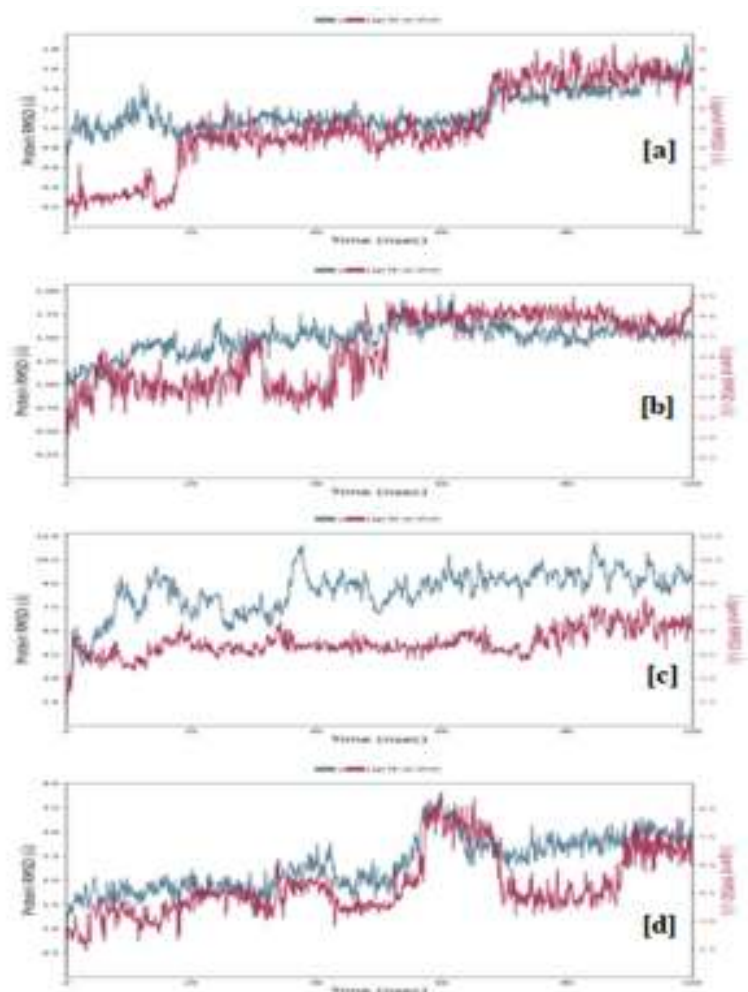


Figure S14. The RMSD for the ligand RA complexed with (a) CAII, (b) CES1, (c) EGFR, and (d) PLA2G2A detected while executing a 100 ns MD simulation.

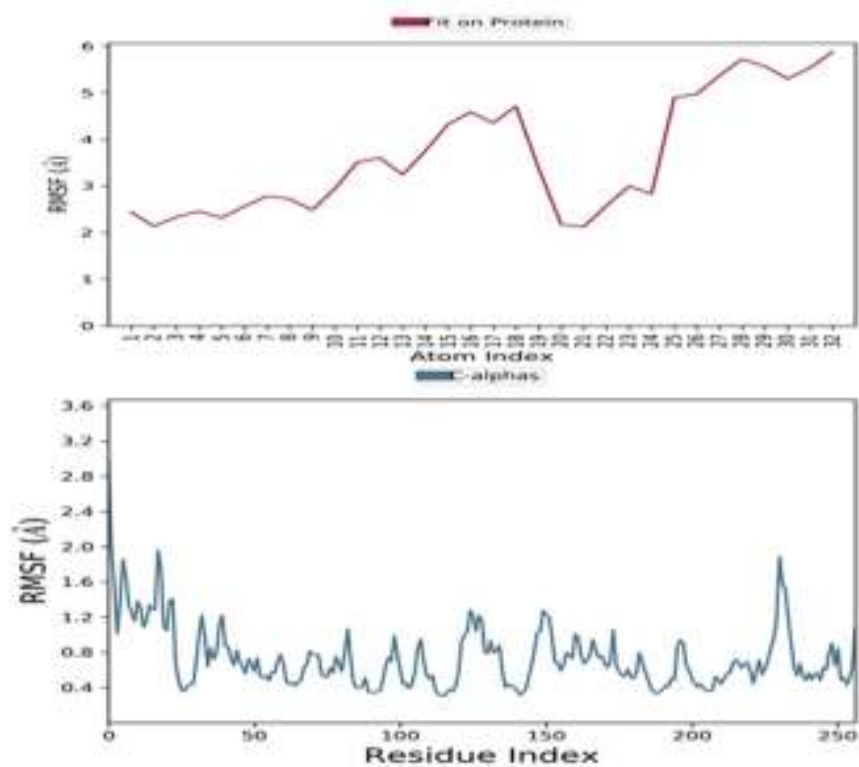


Figure S15. RMSF for the CAIL complexed with ligand RA detected while executing MD simulation.

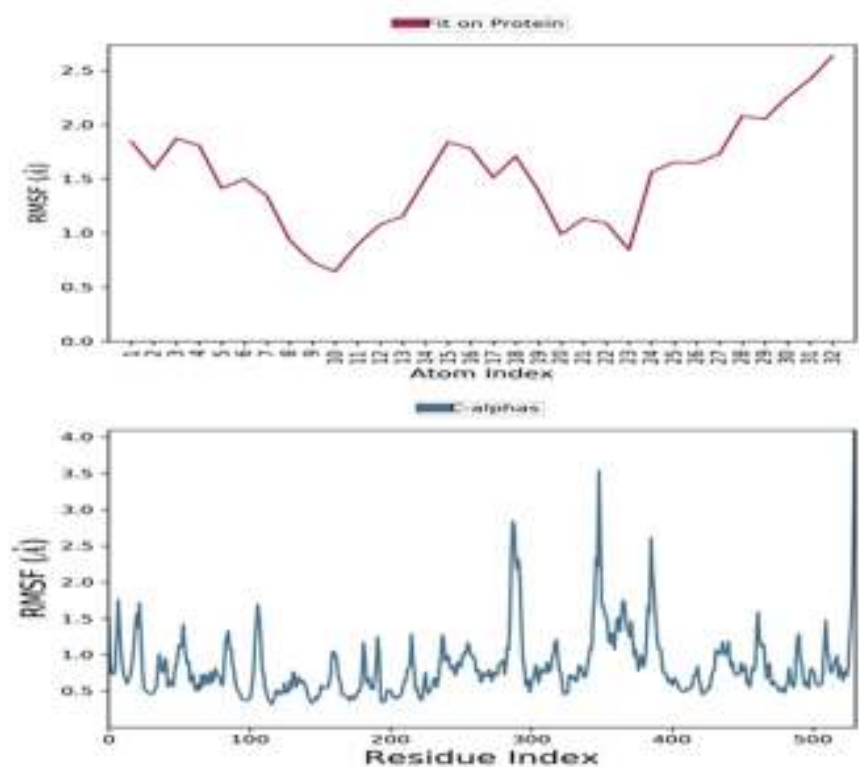


Figure S16. RMSF for the CES1 complexed with ligand RA detected while executing MD simulation.

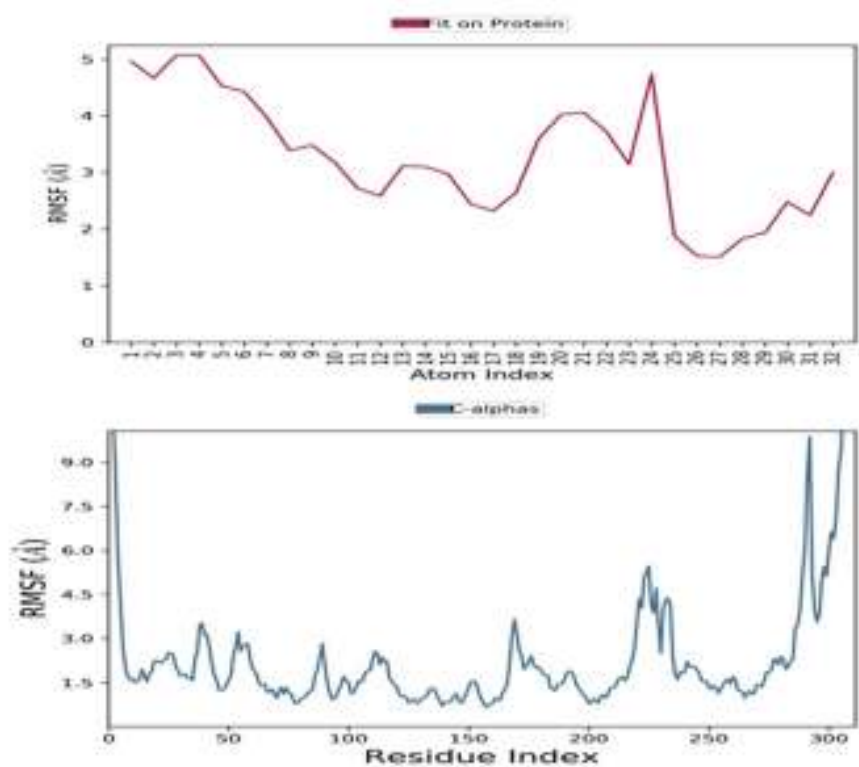


Figure S17. RMSF for the EGFR complexed with ligand RA detected while executing MD simulation.

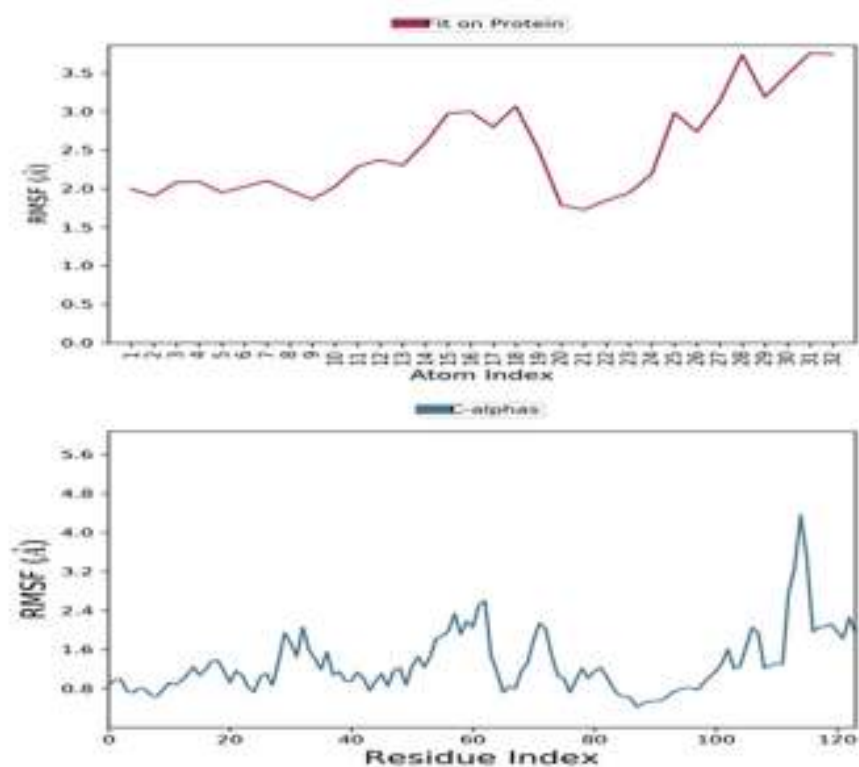


Figure S18. RMSF for the PLA2G2A complexed with ligand RA detected while executing the MD simulation.



Article

Roburic Acid as a Therapeutic Candidate: Antiproliferative Activity and Secondary Cell Death Response in Colorectal Cancer Cells

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Abstract

Natural compounds are increasingly recognized as valuable sources of pharmacologically active agents for cancer therapy. Among them, plant-derived triterpenoids attract attention due to their structural diversity and broad biological activity. Roburic acid (RA), a tetracyclic triterpenoid, has previously been shown to exert antiproliferative effects in colorectal cancer (CRC) cells with limited cytotoxicity. In the present study, we investigated the cellular mechanisms underlying RA activity in CRC cells, focusing on cell cycle regulation, mitochondrial function, apoptosis, oxidative stress, and DNA integrity. RA treatment markedly suppressed CRC cell proliferation, resulting in G₀/G₁ cell cycle arrest and downregulation of key proliferation markers. Mitochondrial analysis revealed an early reduction in mitochondrial membrane potential (MMP) following RA exposure, indicating mitochondrial dysfunction. Importantly, these effects occurred in the absence of intracellular reactive oxygen species (ROS) generation and without induction of DNA strand breaks, demonstrating a non-pro-oxidant and non-genotoxic profile of RA. Apoptotic features were observed mainly at higher concentrations and after prolonged exposure and were strongly dependent on cell line and assay type. Overall, RA limits CRC cell growth predominantly through cytostatic mechanisms, including cell cycle arrest and mitochondrial modulation, while apoptosis is a secondary, context-dependent response. The lack of oxidative stress and genotoxicity distinguishes RA from many conventional cytotoxic agents and supports its further investigation as a non-genotoxic anticancer compound.

Keywords: roburic acid; colorectal cancer; cytostatic activity; cell cycle arrest; mitochondrial dysfunction; anti-proliferative activity



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1. Introduction

Colorectal cancer (CRC) remains one of the most frequently diagnosed malignancies worldwide and represents a major global health burden. According to the most recent GLOBOCAN 2022 estimates, CRC accounts for approximately 1.9 million new cases and over 930,000 deaths annually, ranking third in global cancer incidence and second in cancer-related mortality [1]. Although incidence rates are stabilizing or declining in some high-income countries due to screening programs, CRC incidence continues to rise in

many low- and middle-income regions. Notably, an alarming increase in early-onset CRC has been observed in individuals under 50 years of age, particularly in developed countries, suggesting shifts in environmental, lifestyle, and metabolic risk factors [2]. Despite advances in surgical techniques, chemotherapy, targeted therapies, and immunotherapy, treatment efficacy remains limited by the frequent development of drug resistance and the occurrence of severe adverse effects, often compromising patient outcomes and quality of life. In this context, increasing attention has been directed toward therapeutic approaches that suppress tumor progression while minimizing cytotoxicity. In particular, cytostatic agents that inhibit cancer cell proliferation without inducing extensive cell death are gaining interest, as they may reduce systemic toxicity and limit selective pressure favoring resistant tumor clones [3]. Natural compounds have emerged as a promising source of such agents due to their remarkable chemical diversity, multitargeted mechanisms of action, and generally favorable safety profiles. Consequently, they have attracted growing interest as potential anticancer therapeutics capable of modulating multiple pathways involved in cancer progression and therapy resistance.

Roburic acid (RA) is a tetracyclic triterpenoid isolated from the roots of *Gentiana* species and from oak galls (Figure 1). It has been reported to exhibit strong anti-inflammatory, antibacterial, anti-viral, and anti-atherogenic activities [3–6]. These effects are thought to stem from mechanisms that include the inhibition of cyclooxygenase enzymes and direct binding to tumor necrosis factor- α (TNF- α), thereby modulating pathways such as nuclear factor kappa B (NF- κ B), which is associated with inflammatory responses, regulation of apoptotic proteins, and oncogenic transformation [4,7,8]. In our previous study, we demonstrated that RA significantly reduces metabolic activity, inhibits colony formation, and suppresses DNA synthesis across multiple cancer cell lines, with CRC models (DLD-1, HCT-116, and HT-29) showing relatively greater sensitivity [9]. These findings support an antiproliferative (cytostatic), rather than cytotoxic mode of action of this natural compound. However, the cellular and molecular mechanisms underlying this growth-inhibitory effect have not yet been fully elucidated.

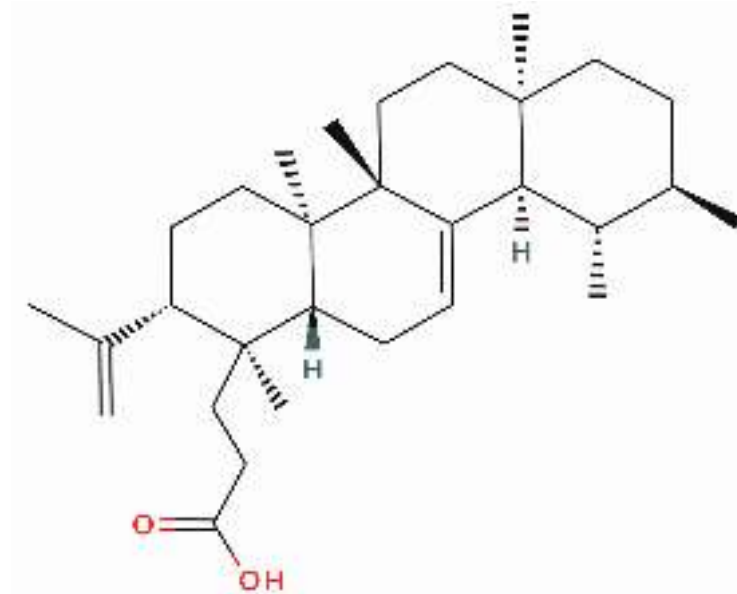


Figure 1. Chemical structure of roburic acid (RA). Image adapted from PubChem (CID: 5317800).

Cytostatic responses in cancer cells may arise from diverse mechanisms, including alterations in cell cycle progression, activation of checkpoint pathways, mitochondrial dysfunction, or DNA damage signaling, even in the absence of pronounced cytotoxic effects. Therefore, a comprehensive analysis integrating functional, morphological, and

molecular approaches is required to clarify the basis of RA-mediated growth inhibition. In the present study, we aimed to further elucidate the mechanisms of action of RA in CRC cells by evaluating its effects on cell proliferation, cell cycle regulation, DNA integrity, oxidative stress levels, mitochondrial function, and apoptotic responses. To this end, we employed a combination of fluorescence-based assays, cytometric analyses, and gene expression profiling to characterize RA activity across CRC cell lines representing distinct mutational backgrounds and chemoresistance profiles. Interestingly, our findings differ from some previously reported observations [7,10], potentially reflecting differences in experimental models, treatment conditions, or cellular contexts.

Based on these considerations, we hypothesized that RA exerts its antiproliferative effects in CRC cells through modulation of cell cycle progression and regulation of key molecular components involved in cell cycle control. To test this hypothesis, we performed a comprehensive mechanistic analysis evaluating RA-induced changes in cell cycle distribution, proliferation markers, mitochondrial function, oxidative stress levels, DNA integrity, and apoptotic responses in CRC cell lines representing distinct molecular backgrounds. Elucidating the mechanisms underlying RA-mediated growth inhibition may provide important insights into its potential as a novel anticancer candidate and contribute to a broader understanding of regulatory mechanisms controlling tumor cell proliferation in CRC.

2. Results

2.1. Ki-67 Immunofluorescence Assay

Building on the antiproliferative effects of RA previously reported by our group [9], the impact of RA on the expression of the proliferation marker Ki-67 was evaluated in CRC cell lines. Following 48 h of exposure to RA at $0.5 \times IC_{50}$ and IC_{50} concentrations, Ki-67 immunofluorescence was significantly altered in all analyzed models (Figure 2). In DLD-1, treatment with RA at $0.5 \times IC_{50}$ resulted in a modest but statistically significant increase in Ki-67 fluorescence compared with untreated controls ($p < 0.0001$), whereas exposure at IC_{50} led to a pronounced reduction in the Ki-67 signal ($p < 0.0001$). In HCT-116 and HT-29 cells, RA treatment resulted in a significant reduction in Ki-67 fluorescence at both $0.5 \times IC_{50}$ and IC_{50} concentrations, indicating a robust antiproliferative response across CRC models. Quantitative analysis confirmed a statistically significant reduction in Ki-67 signal in RA-treated cells ($p < 0.05$), demonstrating a decreased proportion of actively cycling cells. These findings corroborate earlier observations obtained using bromodeoxyuridine incorporation assays and support the notion that RA limits CRC cell growth predominantly by inhibiting proliferative activity [7].

2.2. Cell Cycle Analysis

Cell cycle profiling revealed that RA treatment induced marked alterations in cell cycle distribution in all examined CRC cell lines. After 24 h of exposure, an increased proportion of cells in the G_0/G_1 phase was observed in DLD-1, HCT-116, and HT-29 cells compared with untreated controls (Figure 3A). This increase reached statistical significance at the IC_{50} concentration in DLD-1 ($p = 0.0029$) and HCT-116 cells ($p = 0.0305$), whereas in HT-29 cells, a significant accumulation in the G_0/G_1 phase was already detected at $0.5 \times IC_{50}$ ($p = 0.0002$) and remained significant at IC_{50} ($p < 0.0001$). These shifts were accompanied by a reduction in the S-phase population, which was statistically significant in selected conditions (DLD-1: $p = 0.0155$; HCT-116: $p = 0.0315$; HT-29: $p < 0.0001$ for both concentrations), indicating impaired cell cycle progression. Following prolonged exposure to RA (48 h), alterations in cell cycle distribution became more pronounced (Figure 3B). All three cell lines exhibited a significant increase in the G_0/G_1 fraction (DLD-

1: $p < 0.0001$ for both concentrations; HCT-116: $p = 0.001$ for $0.5 \times \text{IC}_{50}$ and $p = 0.05$ for IC_{50} ; HT-29: $p < 0.0001$ for IC_{50}), particularly at the IC_{50} concentration, along with a concomitant decline in the S-phase population (HCT-116: $p = 0.0002$; HT29: $p < 0.0001$). In addition, a sub-G1 fraction became detectable after 48 h of treatment, most notably in HT-29 cells at the higher RA concentration ($p = 0.0015$), suggesting the emergence of DNA fragmentation in a subset of cells. Overall, these findings indicate that RA disrupts cell cycle progression in CRC cells, with a predominant accumulation in the G_0/G_1 phase. The magnitude and statistical significance of these effects varied between cell lines and treatment conditions, consistent with a primarily cytostatic response that becomes more pronounced with prolonged exposure.

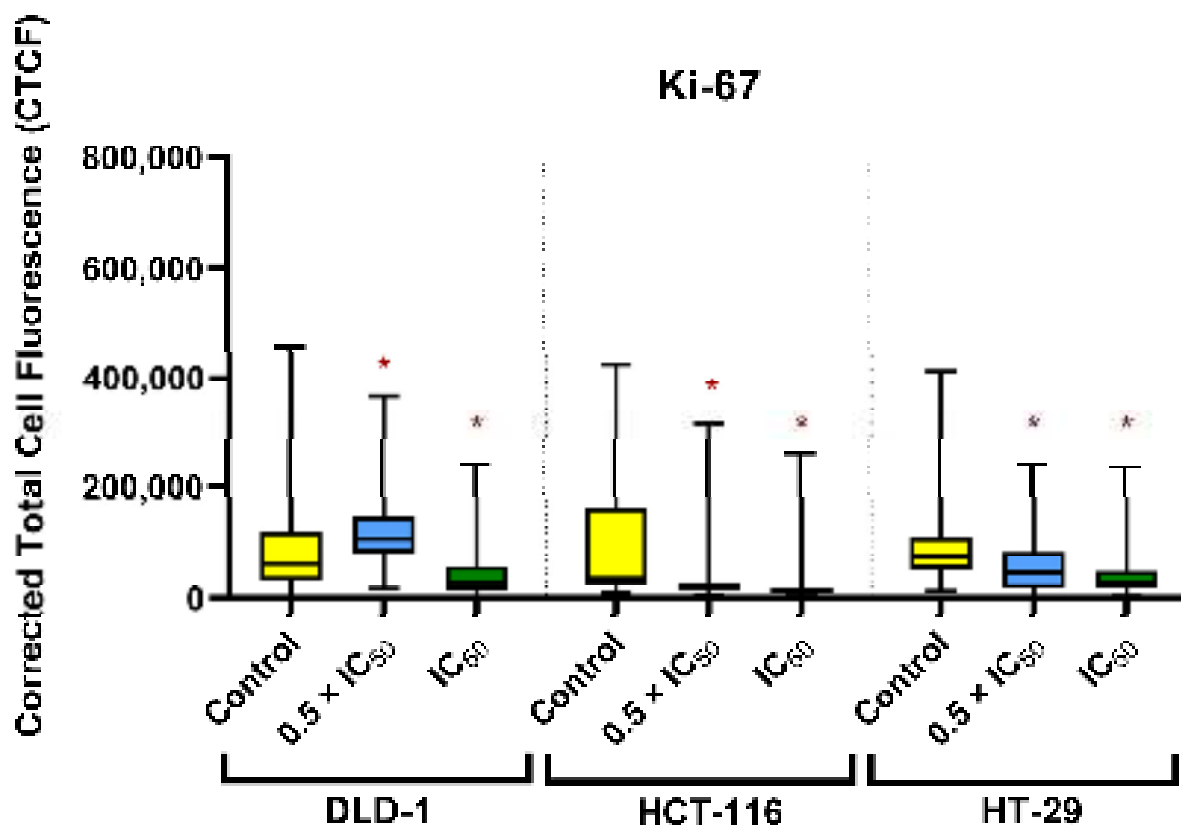


Figure 2. Effect of roburic acid (RA) on Ki-67 expression in colorectal cancer (CRC) cell lines. Ki-67 immunofluorescence staining in DLD-1, HCT-116, and HT-29 following 48 h of exposure to RA at concentrations corresponding to $0.5 \times \text{IC}_{50}$ and IC_{50} . Quantitative data are presented as medians with interquartile ranges, minimums, and maximums. Statistical analysis was performed using the Kruskal–Wallis test, followed by Dunn’s post hoc test; an asterisk indicates significant differences relative to untreated controls (*) ($p < 0.05$).

2.3. Annexin V-FITC Binding Assay

Given the appearance of a sub-G1 population following prolonged RA exposure, apoptotic responses were further evaluated using annexin V conjugated with fluorescein isothiocyanate (FITC) binding. After 24 h of treatment, a statistically significant increase in the proportion of annexin V-positive cells were detected only at the highest RA concentration ($2 \times \text{IC}_{50}$) in DLD-1 ($32.13 \pm 5.10\%$; $p < 0.0001$) and HCT-116 cells ($28.07 \pm 3.80\%$; $p < 0.0001$), whereas no significant changes were observed in HT-29 cells at this time point (Figure 4A). Prolonged exposure to RA markedly enhanced apoptotic responses (Figure 4B). After 72 h of treatment at $2 \times \text{IC}_{50}$, annexin V-positive cells accounted for more than half of the cell population in all examined CRC cell lines ($57.9 \pm 8.0\%$ in DLD-1, $51.6 \pm 1.42\%$

in HCT-116, and $51.8 \pm 4.07\%$ in HT-29; $p < 0.05$). In addition, treatment with RA at IC_{50} resulted in statistically significant increases in apoptotic cell fractions in HCT-116 ($31.1 \pm 3.15\%$; $p < 0.0001$) and HT-29 ($20.73 \pm 0.68\%$; $p = 0.0019$). Importantly, the fraction of necrotic cells remained low and did not reach statistical significance ($p < 0.05$) under any of the tested conditions (Supplementary Figures S1 and S2), indicating that RA-induced cell death was predominantly apoptotic. Together, these results indicate that apoptosis is not an immediate response to RA exposure but rather emerges following prolonged treatment and/or higher exposure conditions, consistent with a predominantly cytostatic effect at earlier time points.

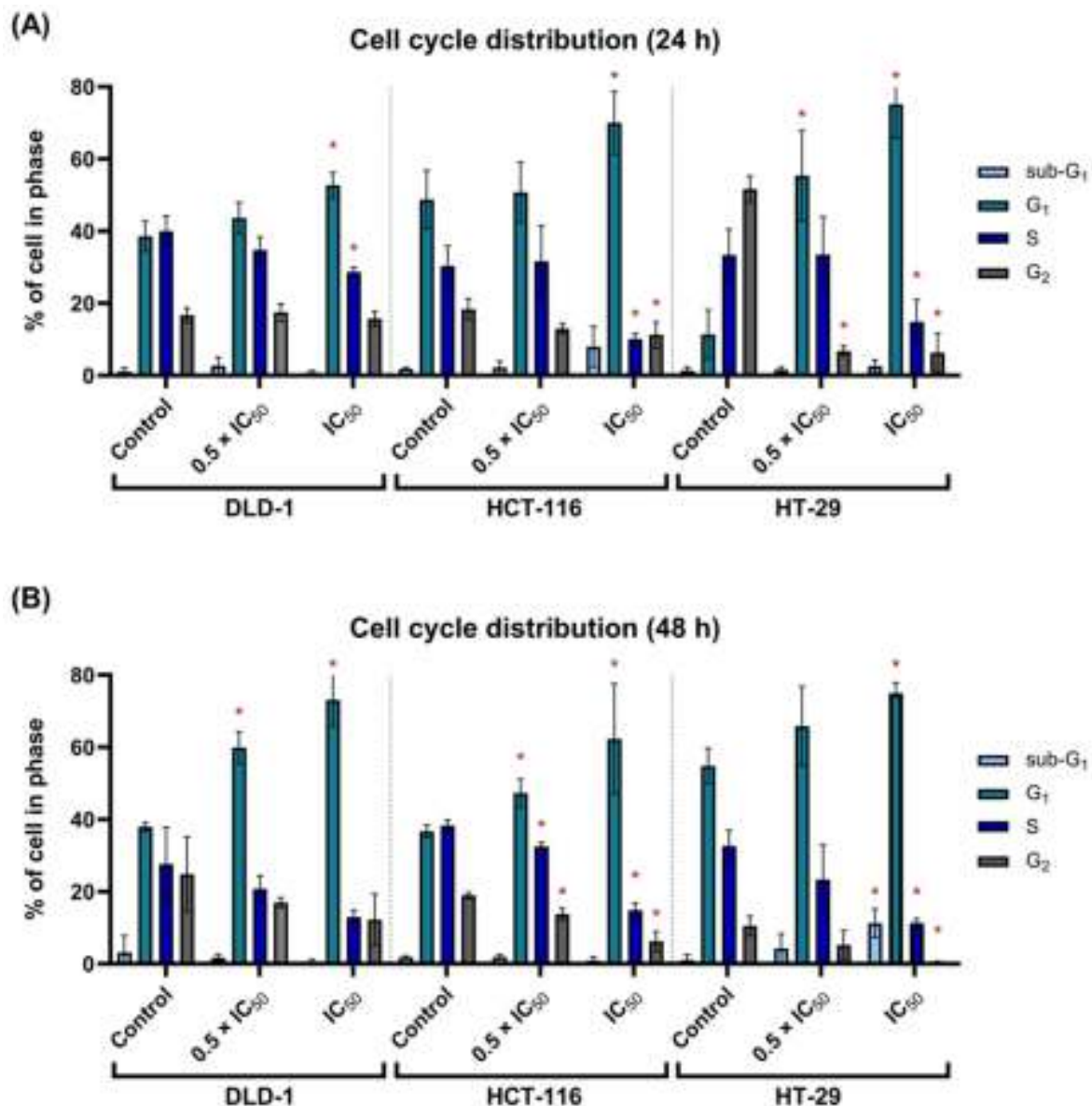


Figure 3. Cell cycle distribution of DLD-1, HCT-116, and HT-29 colorectal cancer (CRC) cells following (A) 24 h and (B) 48 h of exposure to roburic acid (RA) at concentrations corresponding to $0.5 \times IC_{50}$ and IC_{50} . The data are presented as the mean percentages of cells [%] $\pm$ SD in G₀/G₁, S, and G₂ phases. Statistical analysis was performed using a two-way ANOVA, followed by Dunnett's multiple comparisons test; an asterisk indicates significant differences relative to untreated controls (*) ($p < 0.05$).

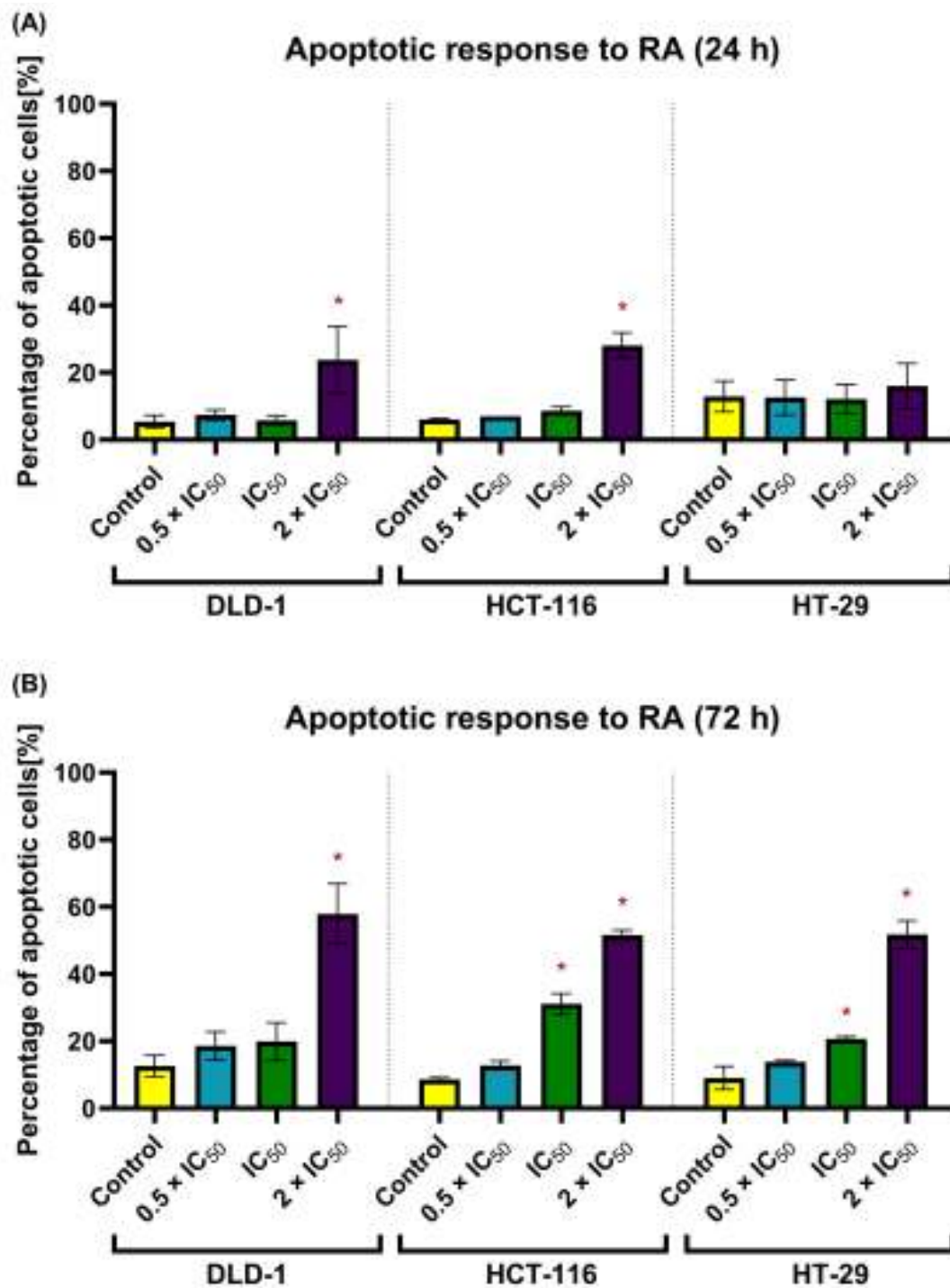


Figure 4. Apoptotic cell fraction in DLD-1, HCT-116, and HT-29 colorectal cancer (CRC) cell lines following (A) 24 h and (B) 72 h of exposure to roburic acid (RA) at concentrations corresponding to $0.5 \times \text{IC}_{50}$, IC_{50} , and $2 \times \text{IC}_{50}$. Apoptosis was assessed by annexin V-FITC binding. The data are presented as the mean percentages [%] $\pm$ SD of annexin V-positive cells. Statistical analysis was performed using one-way ANOVA, followed by Dunnett's post hoc test; an asterisk indicates significant differences relative to untreated controls (*) ($p < 0.05$), $N > 1 \times 10^4$.

2.4. Dual Acridine Orange/Ethidium Bromide Fluorescent Staining

Apoptotic morphology was evaluated in CRC cell lines using dual acridine orange/ethidium bromide (AO/EB) staining. After 24 h of RA exposure, a statistically significant increase ($p < 0.05$) in the proportion of apoptotic cells was observed only in DLD-1 cells treated with the highest RA concentration ($2 \times \text{IC}_{50}$), reaching $16.75 \pm 3.0\%$

($p = 0.001$) compared with the untreated control ($3.5 \pm 2.65\%$) (Figure 5A). No significant changes were observed in HCT-116 or HT-29 cells at this time point. Prolonged exposure to RA (72 h) resulted in a more pronounced apoptotic response (Figure 5B). A statistically significant increase in apoptotic cells was observed in HCT-116 and HT-29 cell lines treated with RA at $2 \times \text{IC}_{50}$ ($14.75 \pm 2.5\%$; $p = 0.0387$; and $22.75 \pm 5.19\%$; $p = 0.0003$, respectively) compared with the untreated control ($5.75 \pm 4.57\%$ and $8.13 \pm 3.4\%$, respectively). In addition, HT-29 cells also exhibited a significant increase in apoptotic morphology at the IC_{50} concentration ($18.00 \pm 1.41\%$; $p = 0.002$). Overall, AO/EB staining confirmed that morphologically evident apoptosis occurs primarily following prolonged exposure and at higher RA concentrations, consistent with a delayed and secondary apoptotic response.

2.5. Mitochondrial Membrane Potential Assay

Alterations in mitochondrial membrane potential (MMP; $\Delta\Psi\text{m}$) were examined in CRC cell lines following RA exposure. After 24 h of treatment, RA induced a reduction in MitoTracker Red CMXRos fluorescence intensity in DLD-1, HCT-116, and HT-29 cells, indicating mitochondrial membrane depolarization (Table 1, Figure 6). This effect was observed across most tested conditions and cell lines, except for HCT-116 cells treated with $0.5 \times \text{IC}_{50}$ RA, where the decrease in $\Delta\Psi\text{m}$ did not reach statistical significance ($p = 0.0501$), although a clear downward trend was evident. The magnitude of $\Delta\Psi\text{m}$ reduction varied among the cell lines and concentrations. The most pronounced decrease in mitochondrial membrane potential was observed in HT-29 cells treated with RA at $2 \times \text{IC}_{50}$, reaching $75.09 \pm 2.67\%$ ($p < 0.0001$) of the control value. Overall, the observed changes indicate that RA affects mitochondrial function within 24 h of exposure.

Table 1. Changes in the mitochondrial membrane potential (MMP; $\Delta\Psi\text{m}$) in DLD-1, HCT-116, and HT-29 cells following 24 h of exposure to roburic acid (RA). Results are expressed as mean relative fluorescence $\pm$ SD compared to untreated controls (normalized to 100%). Statistical analysis was performed using one-way ANOVA, followed by Dunnett's post hoc test ($p < 0.05$; significant values indicated in bold).

Cell Line	Results of 24 h Incubation of Cancer Cells with RA		
	[%] Fluorescence Intensity Compared to Negative Control $\pm$ SD *		
	$0.5 \times \text{IC}_{50}$	IC_{50}	$2 \times \text{IC}_{50}$
DLD-1	84.67 ± 6.00 ($p = 0.005$)	86.00 ± 6.33 ($p = 0.005$)	82.00 ± 6.33 ($p = 0.0012$)
HCT-116	91.61 ± 6.67 ($p = 0.0501$)	91.23 ± 2.33 ($p = 0.0404$)	84.87 ± 3.33 ($p = 0.0013$)
HT-29	88.46 ± 2.00 ($p = 0.0022$)	81.90 ± 4.67 ($p < 0.0001$)	75.09 ± 2.67 ($p < 0.0001$)

(*) The asterisk indicates significant differences relative to untreated controls ($p < 0.05$).

2.6. Determination of Intracellular Reactive Oxygen Species Level

Intracellular reactive oxygen species (ROS) levels were evaluated in CRC cell lines following RA exposure. Treatment with RA did not result in statistically significant changes ($p > 0.05$) in ROS production in DLD-1, HCT-116, or HT-29 cells at any of the tested concentrations compared with untreated controls (Figure 7). This lack of ROS induction was consistently observed throughout the monitoring period. In contrast, exposure to hydrogen peroxide, used as a positive control, led to a marked increase in fluorescence signal, confirming the sensitivity and responsiveness of the assay. Overall, these results

indicate that RA does not promote intracellular ROS accumulation under the experimental conditions employed.

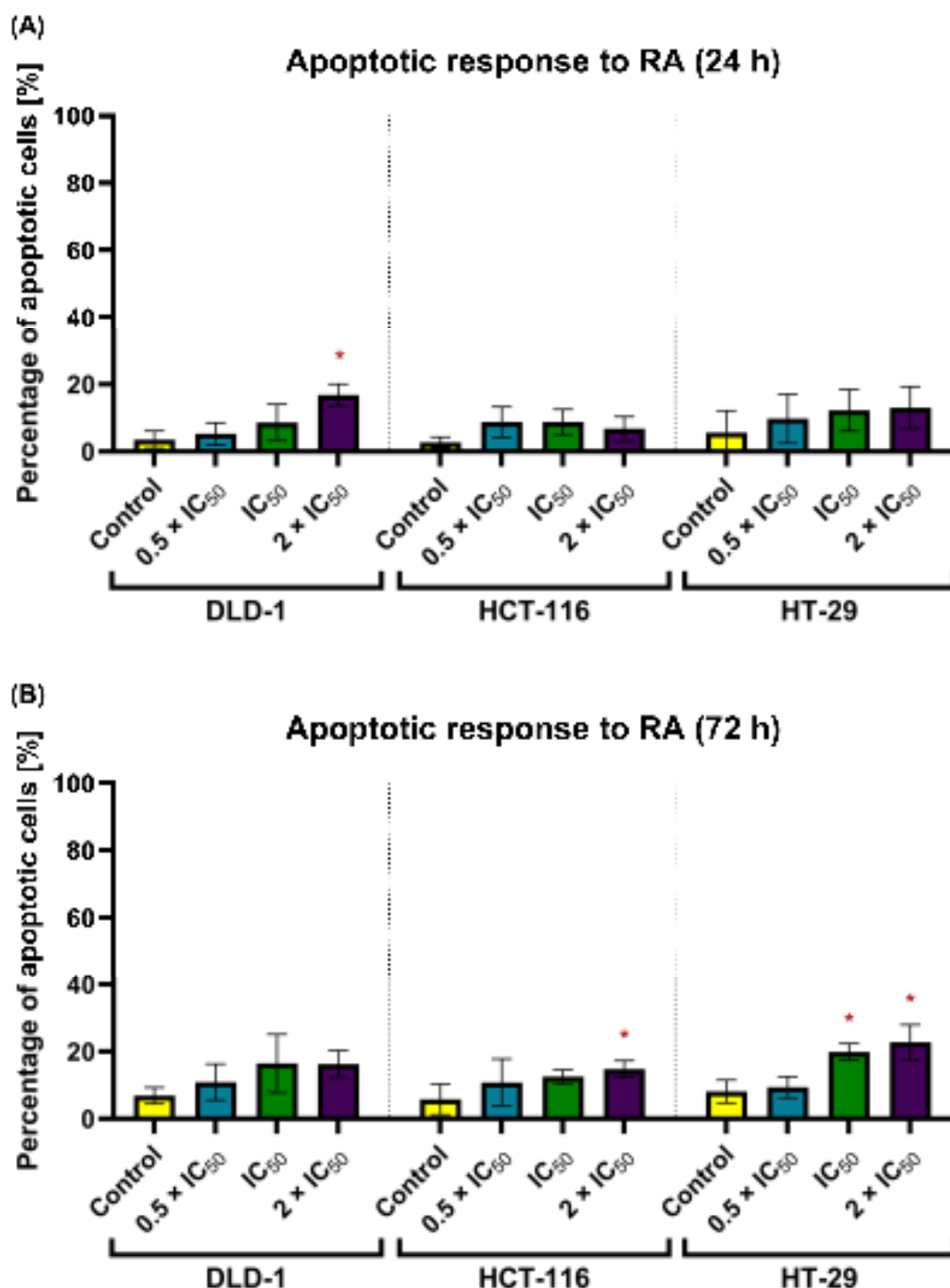


Figure 5. Percentage of apoptotic cells in colorectal cancer (CRC) cell lines DLD-1, HCT-116, and HT-29 following (A) 24 h and (B) 72 h of exposure to roburic acid (RA) at concentrations corresponding to $0.5 \times \text{IC}_{50}$, IC_{50} , and $2 \times \text{IC}_{50}$, assessed by dual acridine orange/ethidium bromide (AO/EB) staining. The data are shown as mean percentages [%] $\pm$ SD of AO/EB-identified apoptotic cells. Statistical analysis was performed using one-way ANOVA, followed by Dunnett's post hoc test; an asterisk indicates significant differences relative to untreated controls (*) ($p < 0.05$), $N > 1 \times 10^4$.

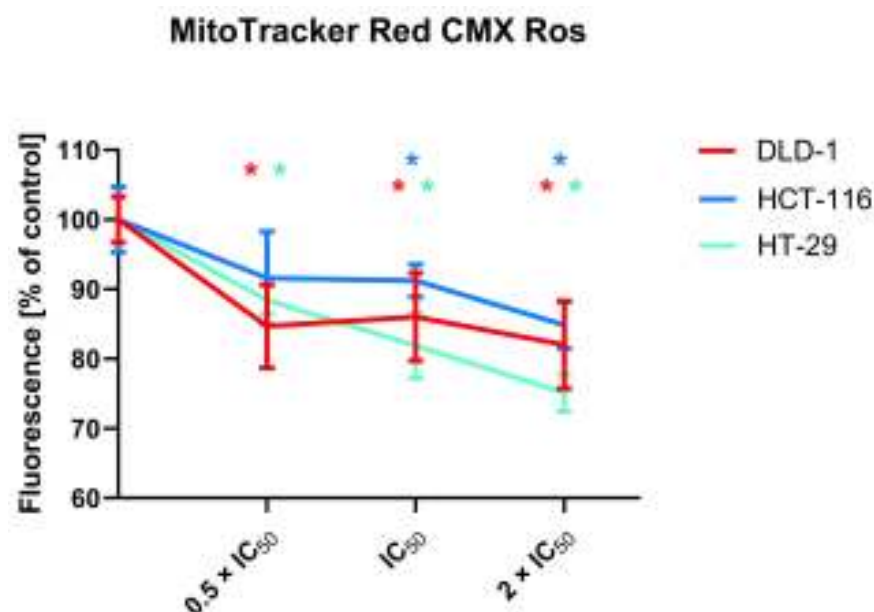


Figure 6. Changes in mitochondrial membrane potential (MMP; $\Delta\Psi_m$) in DLD-1, HCT-116, and HT-29 cells following 24 h of exposure to roboric acid (RA) at concentrations corresponding to $0.5 \times IC_{50}$, IC_{50} , and $2 \times IC_{50}$, assessed using MitoTracker Red CMX Ros. The data are presented as mean fluorescence intensity $\pm$ SD relative to untreated control (normalized to 100%). Statistical analysis was performed using one-way ANOVA, followed by Dunnett's post hoc test; an asterisk indicates significant differences relative to untreated controls (*) ($p < 0.05$).

2.7. Genotoxicity—Alkaline Comet Assay

The genotoxic potential of RA was examined using the alkaline comet assay in CRC cell lines. After 24 h of exposure, RA did not induce a significant increase in DNA damage in DLD-1, HCT-116, or HT-29 cells at any of the tested concentrations compared with untreated controls (Figure 8). At the IC_{50} concentration, median DNA content in comet tails reached 3.82% in DLD-1 cells, 2.86% in HCT-116 cells, and 2.68% in HT-29 cells, values comparable to those observed in control samples. No statistically significant differences were detected between RA-treated cells and the negative control across all concentrations tested, indicating the absence of detectable DNA strand breaks under the experimental conditions employed. Representative comet images illustrating the lack of RA-induced DNA damage are shown in Figure 9, whereas cells treated with bleomycin, used as a positive control, exhibited pronounced DNA fragmentation. Importantly, no genotoxic effects of RA were detected in non-cancerous human cells (CCD-841 CoN and WI-38), further supporting the compound's non-genotoxic profile (Supplementary Figure S3). Overall, these results demonstrate that RA does not exhibit genotoxic activity in CRC cells after 24 h of exposure.

2.8. Gene Expression Analysis by Quantitative Real-Time PCR

The expression of genes associated with cell proliferation and cell cycle regulation was analyzed by quantitative real-time PCR (RT-qPCR) following RA treatment at the IC_{50} concentration. Gene expression was normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and evaluated after 24 and 72 h of exposure. Across all examined CRC cell lines, RA treatment resulted in a statistically significant ($p < 0.05$) decrease in the proliferation marker Kiel 67 (MKI67) expression at both time points, consistent with reduced proliferative activity (Figure 10). A similar pattern was observed for proliferating cell nuclear antigen (PCNA), with significant reductions in transcript levels in DLD-1 and HCT-116 cells after 24 and 72 h of treatment. In HT-29 cells, a significant

decrease in *PCNA* expression was observed only after prolonged exposure (72 h). Analysis of cyclin-dependent kinase inhibitor 1A (*CDKN1A*) expression revealed a time- and cell line-dependent response. A significant increase in *CDKN1A* transcript levels was detected in DLD-1 and HT-29 cells after 72 h of RA treatment, whereas in HCT-116 cells, elevated expression was observed at both 24 and 72 h. Expression of cellular tumor antigen p53 (*TP53*) exhibited divergent patterns among the analyzed cell lines. In DLD-1 cells, RA treatment led to a significant increase in *TP53* expression at both time points analyzed. In contrast, a significant decrease in *TP53* transcript levels was observed in HCT-116 cells after 24 h of exposure, while in HT-29 cells, a reduction in *TP53* expression was detected only after 72 h. Collectively, these gene expression changes are consistent with the observed inhibition of proliferation and alterations in cell cycle progression induced by RA.

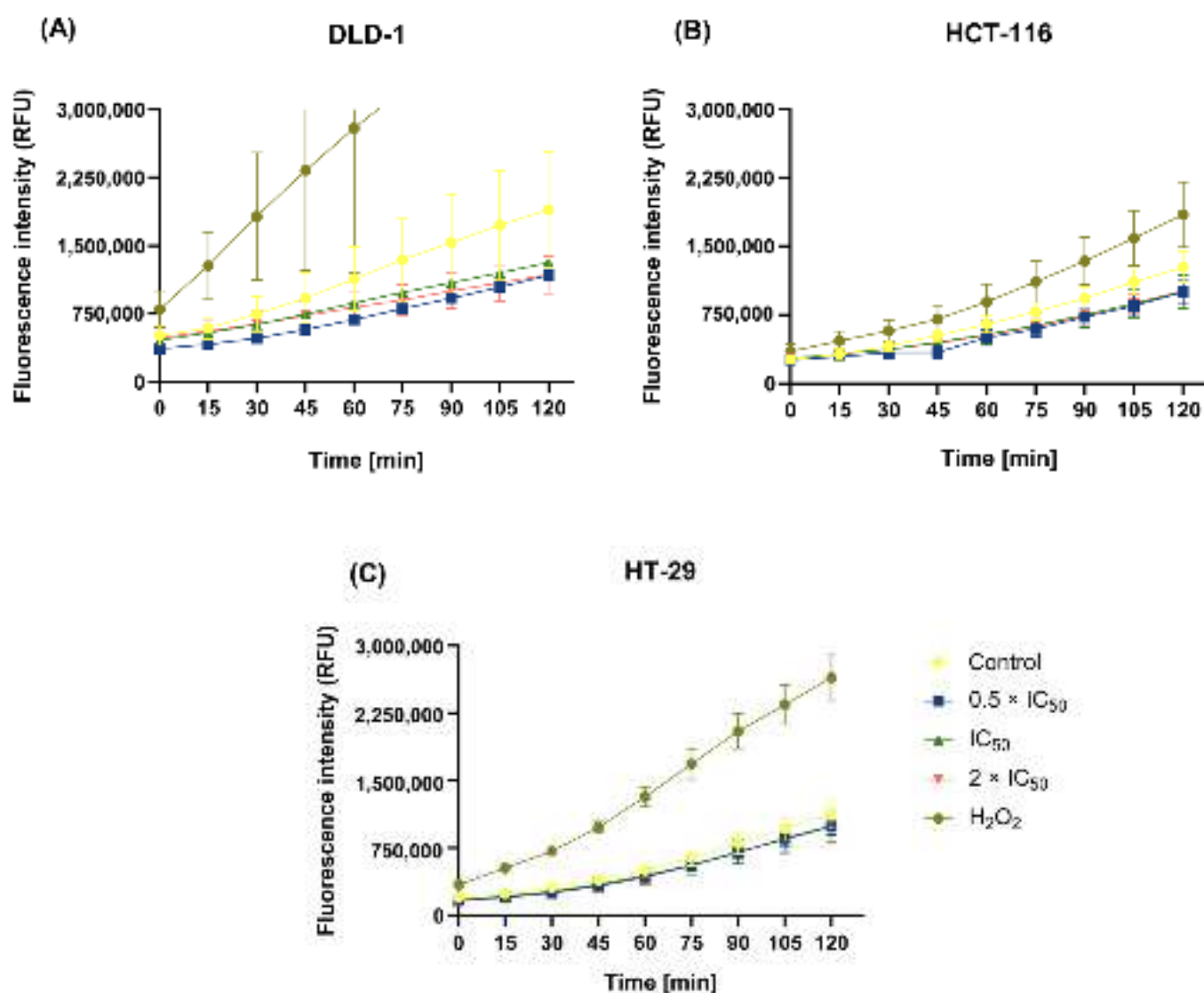


Figure 7. Intracellular reactive oxygen species (ROS) levels in colorectal cancer (CRC) cell lines (A) DLD-1, (B) HCT 116, and (C) HT-29 following exposure to RA at concentrations corresponding to $0.5 \times \text{IC}_{50}$, IC_{50} , and $2 \times \text{IC}_{50}$, assessed using the 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) probe. The data are presented as means relative fluorescence intensity $\pm$ SD over 2 h. Hydrogen peroxide (H_2O_2) was used as a positive control. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test ($p < 0.05$).

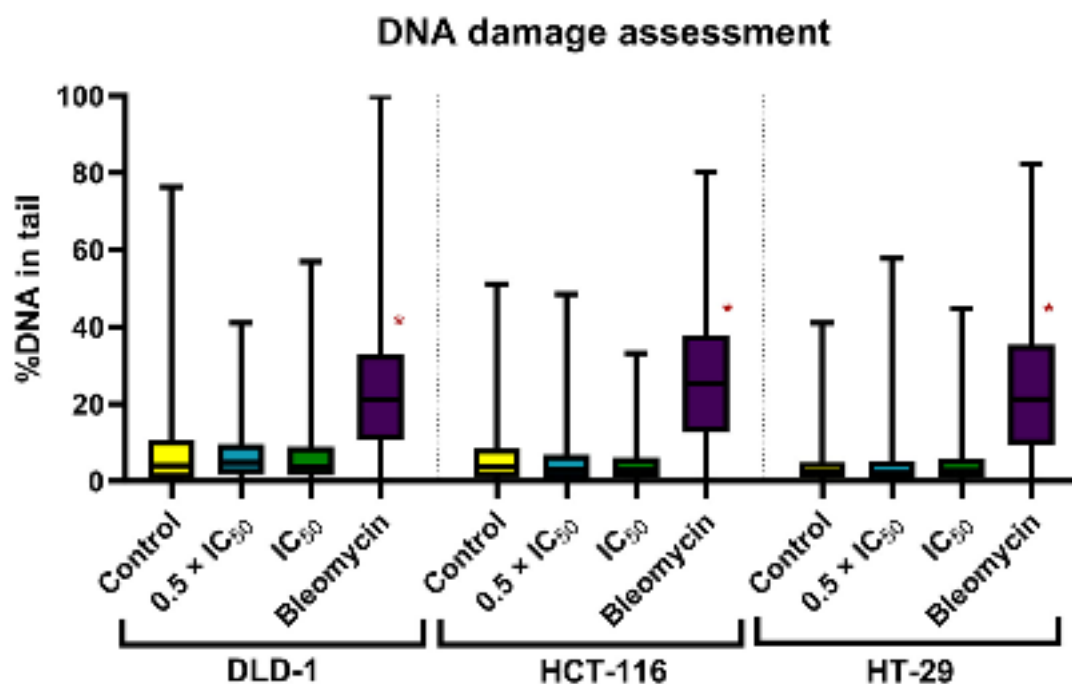


Figure 8. DNA damage in DLD-1, HCT-116, and HT-29 colorectal cancer (CRC) cells was assessed by the alkaline comet assay after 24 h of exposure to roburic acid (RA) at concentrations corresponding to $0.5 \times \text{IC}_{50}$ and IC_{50} . The data are presented as mean percentage of DNA in comet tails with interquartile range, minimum, and maximum. Statistical analysis was performed using the Kruskal–Wallis test, followed by Dunn’s post hoc test; an asterisk indicates significant differences relative to untreated controls (*) ($p < 0.05$), $N > 200$.

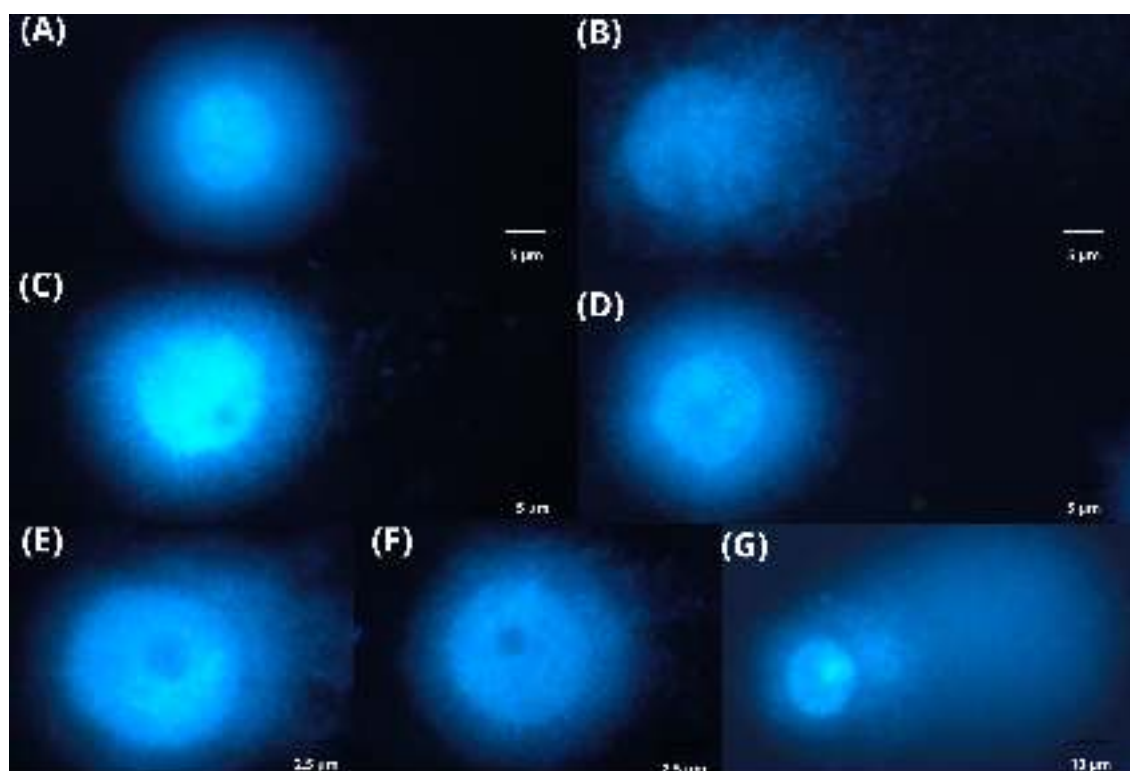


Figure 9. Representative images from the alkaline comet assay. DLD-1: (A) control, (B) RA IC_{50} ; HCT-116: (C) control, (D) RA IC_{50} ; HT-29: (E) control, (F) RA IC_{50} ; and (G) bleomycin (20 μM)-treated cells used as a positive control. The images are shown at identical magnification (40×).

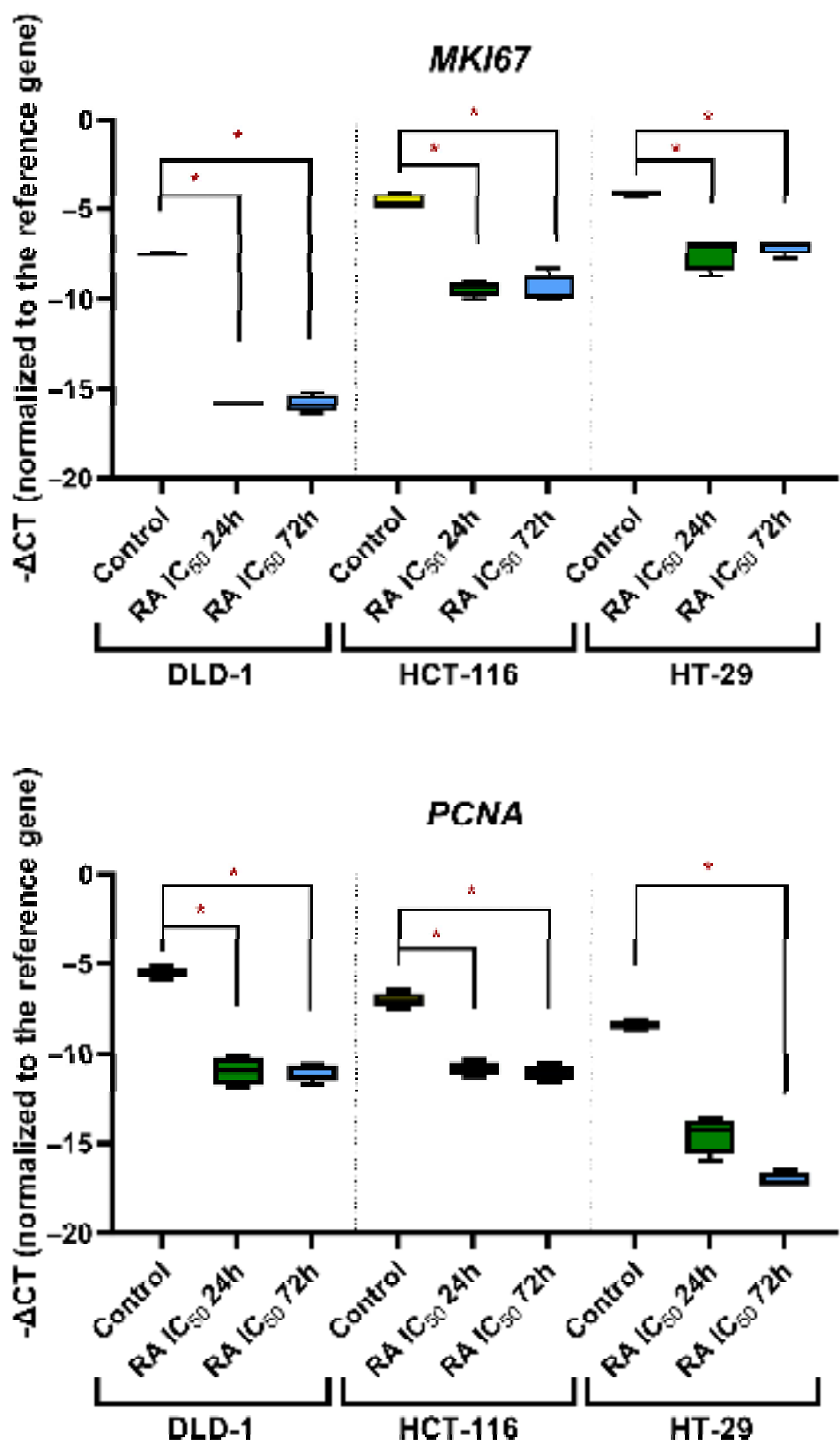


Figure 10. Cont.

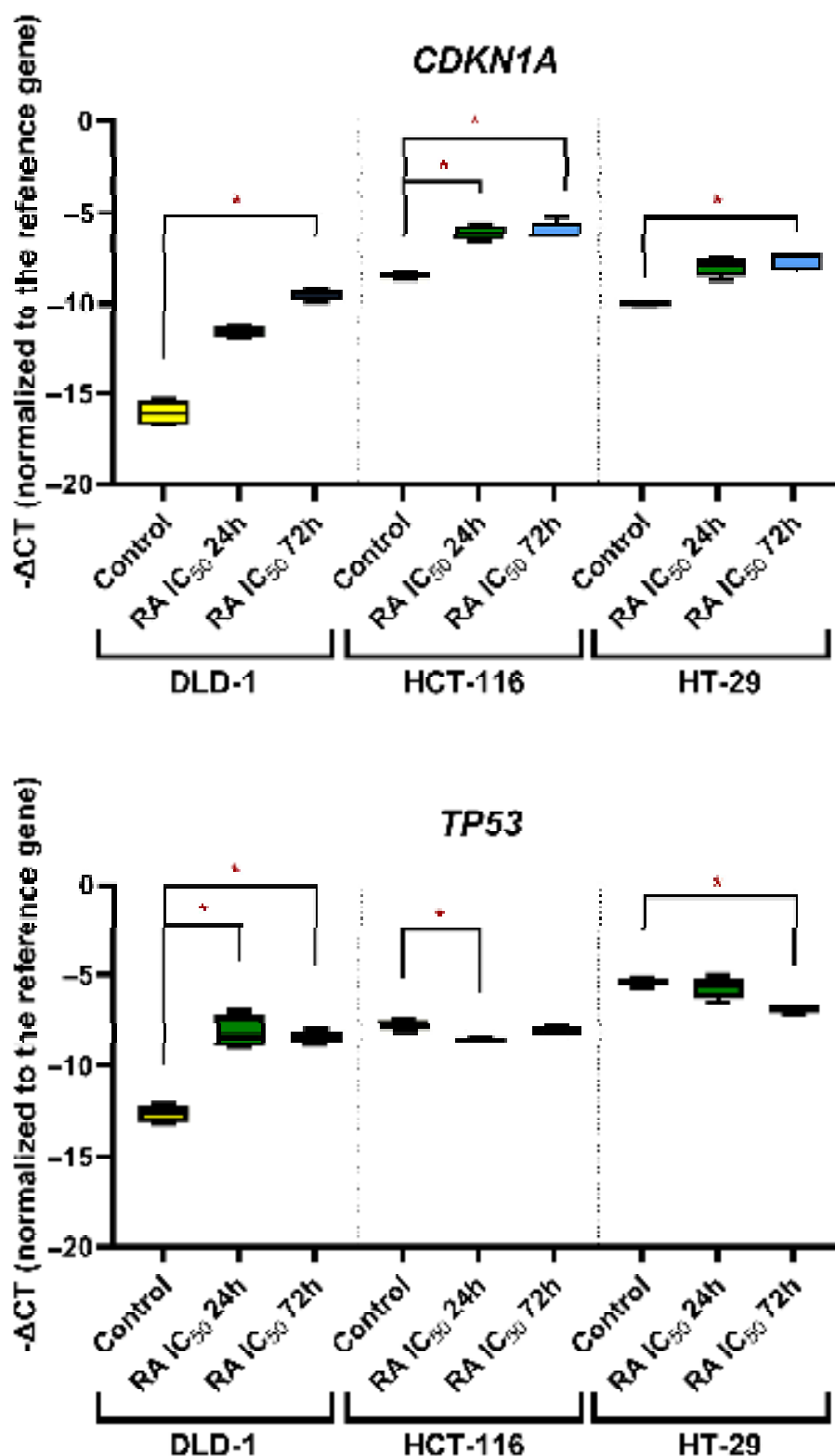


Figure 10. Transcript levels of *MKI67*, *PCNA*, *CDKN1A*, and *TP53* in colorectal cancer (CRC) cell lines DLD-1, HCT-116, and HT-29 following treatment with roburic acid (RA) at the concentrations corresponding to IC₅₀ for 24 and 72 h. Gene expression was analyzed by quantitative real-time PCR (RT-qPCR) and normalized to the housekeeping gene *GAPDH*. The data are presented as $-\Delta C_t$ values (medians with interquartile ranges). Statistical analysis was performed using the Kruskal–Wallis test, followed by Dunn’s post hoc test; an asterisk indicates significant differences relative to untreated controls (*) ($p < 0.05$).

3. Discussion

In our previous study, RA was shown to exert pronounced cytostatic and antiproliferative effects in cancer and normal cells, with selective activity toward CRC models [9]. While these findings established the growth-inhibitory potential of RA, the cellular mechanisms underlying this effect remained unresolved. The present study addressed this gap by providing a comprehensive mechanistic evaluation of RA activity in CRC cells. Importantly, our data demonstrated that RA-mediated growth inhibition occurs independently of genotoxic stress, oxidative damage, and extensive cytotoxicity at concentrations corresponding to IC_{50} values. Instead, RA induced coordinated alterations in cell cycle progression and mitochondrial function, with apoptotic features emerging only under conditions of prolonged exposure or elevated concentrations. These findings support the concept that RA limits CRC cell growth predominantly through cytostatic mechanisms, acting as a regulator of proliferation rather than a conventional cytotoxic or cell-killing agent.

The marked inhibition of DNA synthesis and clonogenic potential observed in our previous study prompted a more detailed investigation into the mechanisms underlying RA-mediated growth inhibition, with a particular focus on cell cycle regulation. Since both bromodeoxyuridine incorporation and colony formation assays reflect the ability of cells to proliferate and sustain long-term growth actively, these findings suggest that RA interferes with fundamental proliferative processes. Flow cytometric evaluation revealed that RA treatment led to a clear accumulation of CRC cells in the G_0/G_1 phase, accompanied by a reduction in the S-phase population and, in some conditions, a decrease in the G_2/M fraction. This pattern indicated that RA primarily disrupts early cell cycle checkpoints, enforcing a G_0/G_1 arrest that restricts the G_1/S transition and limits entry into DNA synthesis. In selected cell lines and following prolonged exposure, the appearance of a sub- G_1 population was also observed, which may reflect early apoptotic features emerging as a secondary and time-dependent consequence of sustained cell cycle perturbation. A similar concentration- and time-dependent shift from cytostatic to cytotoxic effects has been reported for several bioactive natural compounds [3,11–14]. Consistent with our findings, Xu et al. demonstrated that RA induces a pronounced G_0/G_1 accumulation in CRC cells (HCT-116 and HCT-115) after 24 h of exposure, accompanied by a reduction in the S-phase population [7]. However, based primarily on metabolic viability assays, they interpreted RA as predominantly cytotoxic and reported lower IC_{50} values. Differences in potency estimates across studies may arise from methodological and experimental factors, including the use of metabolic viability assays that are sensitive to cytostatic growth inhibition, as well as differences in exposure conditions, such as serum deprivation, which can increase cellular susceptibility to stress and cell death [15,16].

Given the observed alterations in cell cycle distribution, we next focused on the analysis of established molecular markers for cellular proliferation [17,18]. *MKI67* and *PCNA* are widely used indicators of proliferative status, as they reflect the proportion of actively cycling cells and the cellular capacity for DNA replication, respectively [19,20]. Ki-67 is expressed exclusively during the active phases of the cell cycle (G_1 , S, G_2 , and M) and is absent in quiescent (G_0) cells, making it a sensitive marker of proliferative activity rather than cell viability [20–23]. Consistent with the observed G_0/G_1 arrest, RA treatment resulted in a significant reduction in *MKI67* transcript levels across all analyzed CRC cell lines, as determined by RT-qPCR. This transcriptional downregulation was further corroborated at the protein level by immunofluorescence staining using anti-Ki-67 antibodies, which revealed a marked decrease in Ki-67 expression. Notably, in DLD-1 cells exposed to a sub-inhibitory concentration of RA ($0.5 \times IC_{50}$), a modest but statistically significant increase in Ki-67 signal was observed. This apparent biphasic response is unlikely to reflect a genuine pro-proliferative effect of RA, but rather suggests adaptive or selective processes occurring

under partial growth-inhibitory pressure. At low concentrations, antiproliferative agents may fail to suppress all cycling cells uniformly, thereby favoring the transient expansion or enrichment of more proliferative or resistant subpopulations [24,25]. Importantly, at the IC_{50} concentration, RA induced a pronounced and statistically significant reduction in Ki-67 expression in DLD-1 cells, consistent with effective suppression of proliferative activity. In contrast, HCT-116 and HT-29 cells exhibited a significant decrease in Ki-67 expression already at $0.5 \times IC_{50}$, indicating a more uniform antiproliferative response in these models. Taken together, these changes provide molecular evidence for a shift in RA-treated cells toward a non-proliferative, quiescent-like state. While *MKI67* reflects the fraction of actively cycling cells, *PCNA* represents a complementary marker associated with DNA replication competence. At the protein level, PCNA functions as a central coordinator of DNA replication and S-phase progression, acting as a sliding clamp for DNA polymerases and participating in DNA repair processes [19,20,23,26]. The observed reduction in *PCNA* expression, particularly in DLD-1 and HCT-116 cells and following prolonged exposure in HT-29 cells, is consistent with impaired DNA replication competence and further complements the changes observed in proliferation markers. Given PCNA's established role in coordinating DNA synthesis and repair, its modulation under RA treatment is compatible with an adaptive cytostatic response, in which replication-associated processes are progressively restrained rather than abruptly disabled.

To further explore the molecular basis of RA-induced G_0/G_1 arrest, we analyzed transcripts involved in checkpoint regulation. A consistent feature across the models was the induction of *CDKN1A* (P21), particularly after prolonged exposure. P21 is a canonical cyclin-dependent kinase inhibitor that restrains G_1/S progression by inhibiting cyclin-dependent kinase 2 (CDK2)–cyclin E/A complexes and thereby limits commitment to DNA synthesis, functioning as a key effector of cytostatic checkpoint enforcement [27–30]. Importantly, although P21 is classically regulated by P53, its expression can also be induced through *TP53*-independent pathways, enabling effective checkpoint control even in tumor cells harboring *TP53* mutations [29,30]. This regulatory flexibility is particularly relevant in the context of the heterogeneous *TP53* status of the analyzed CRC cell lines and provides a mechanistic framework for interpreting the divergent *TP53* transcriptional responses observed in this study. *CDKN1A* upregulation occurred rapidly and was sustained in HCT-116 cells, which retain wild-type *TP53*, whereas in DLD-1 and HT-29 cells, both characterized by mutant *TP53* backgrounds, *CDKN1A* induction became apparent predominantly after prolonged exposure [31–33]. The lack of uniform correlation between *TP53* transcript and *CDKN1A* induction likely reflects the predominantly post-translational regulation of P53 and cell-line specific feedback mechanisms [34,35]. Functionally, P21-mediated checkpoint activation links these transcriptional changes to the observed antiproliferative phenotype. By enforcing G_0/G_1 arrest, P21 reduces the proportion of actively cycling cells, as reflected by reduced *MKI67* expression and decreased Ki-67 protein levels [36]. In parallel, P21 can directly interact with PCNA and restrain DNA replication processes, thereby contributing to the reduced replication competence observed in RA-treated cells. Modulation of *PCNA* expression, particularly in DLD-1 and HCT-116 cells and following prolonged exposure in HT-29 cells, is consistent with progressive limitation of DNA replication capacity characteristic of an adaptive cytostatic state. In this context, the previously reported ability of RA to suppress NF- κ B signaling may be mechanistically relevant [9]. RA has been shown to inhibit TNF-induced phosphorylation of I κ B kinase (IKK) α/β , inhibitor of κ B α (I κ B α), and the P65 (RelA) subunit of NF- κ B, prevent I κ B α degradation, and block nuclear translocation of P65, leading to reduced NF- κ B–dependent gene expression [7,9]. Given the well-established antagonistic crosstalk between NF- κ B and the P53/P21 pathways, suppression of NF- κ B activity by RA could alleviate constraints on pro-survival and

pro-proliferative signaling, thereby facilitating P21-driven G₀/G₁ arrest independently of TP53 transcriptional dynamics [37–39]. Together with our transcriptional and functional data, these observations indicate that RA interferes with cell cycle progression at multiple regulatory levels, reinforcing its role as a modulator of proliferative capacity.

Importantly, the magnitude and nature of these molecular effects appeared to be strongly dependent on RA concentration and exposure time. At concentrations corresponding to IC₅₀ values, RA predominantly induced transcriptional modulation of proliferation-associated genes, including downregulation of *MKI67* and *PCNA* and upregulation of *CDKN1A*, consistent with checkpoint activation and G₀/G₁ arrest. These alterations were detectable prior to the emergence of substantial apoptotic fractions, indicating that RA initially exerts a growth-regulatory effect rather than immediate cytotoxicity. In contrast, at higher concentrations (2 × IC₅₀) and following prolonged exposure, apoptotic features became more pronounced, suggesting that increased RA burden may exceed the adaptive capacity of CRC cells and shift the cellular response toward activation of cell death pathways. Thus, the balance between cytostatic and cytotoxic outcomes appears to be determined by RA concentration and treatment duration [40,41].

Mitochondrial function is tightly linked to cellular energy homeostasis, proliferation, and survival, and its modulation may underlie both cytostatic and cytotoxic responses [42,43]. In the present study, RA induced a significant reduction in MMP, as assessed using the mitochondria-selective fluorescent probe MitoTracker Red CMXRos, within 24 h of exposure across CRC cell lines, indicating an early disturbance of mitochondrial function. Importantly, these depolarizations occurred under conditions in which extensive apoptotic or necrotic cell death was not predominant, suggesting that mitochondrial perturbation represents an early, mechanistically distinct event rather than a direct trigger of acute cell death. A decrease in $\Delta\Psi_m$ does not inevitably signify activation of the intrinsic apoptotic pathway but may instead reflect impaired mitochondrial bioenergetics [44]. Because progression through the cell cycle and DNA synthesis are highly energy-dependent processes, even moderate disruption of mitochondrial polarization may restrict ATP availability and thereby limit the proliferative capacity of cancer cells [45–47]. In this context, the observed mitochondrial depolarization is fully consistent with the G₀/G₁ arrest, reduced S-phase entry, and downregulation of proliferation markers induced by RA, supporting a model in which mitochondrial dysfunction contributes to a predominantly cytostatic phenotype. Notably, RA-induced changes in $\Delta\Psi_m$ were not accompanied by increased intracellular ROS generation. Given that mitochondrial dysfunction is frequently associated with oxidative stress and ROS-driven cytotoxicity, the absence of ROS accumulation indicates that RA-mediated mitochondrial alterations occur independently of overt oxidative damage. Only at higher concentrations and prolonged exposure did mitochondrial perturbation coincide with increased apoptotic fractions, suggesting that sustained mitochondrial stress may eventually exceed the adaptive capacity of CRC cells and trigger activation of cell death pathways.

Consistent with the absence of ROS generation, RA did not induce DNA strand breaks, as assessed by the alkaline comet assay, further supporting the lack of genotoxic stress. Importantly, to our knowledge, this is the first study (as of 27 January 2026) to systematically evaluate the genotoxic potential of RA in both CRC cells and non-cancerous human cell models (CCD-841 CoN and WI-38). Under the applied experimental conditions, RA did not increase DNA damage levels relative to untreated controls, while the assay's responsiveness was confirmed using bleomycin as a positive control. The combined absence of ROS induction and DNA damage clearly distinguishes RA from many conventional anticancer agents, whose mitochondrial effects are tightly coupled to oxidative injury and genome destabilization [48–50]. In contrast, RA appears to modulate mitochondrial

function, limiting proliferative capacity without eliciting acute genomic damage. Such a mechanistic profile distinguishes RA from classical chemotherapeutic agents that suppress tumor growth primarily through DNA damage or oxidative stress. This non-genotoxic mode of action is particularly relevant in the context of long-term or combination-based therapeutic strategies, as it implies a reduced risk of therapy-induced mutagenesis.

The emergence of a sub-G₁ population in cell cycle analysis, together with the early loss of MMP, prompted further investigation into RA's pro-apoptotic activity in CRC cells. These observations suggested that, under certain conditions, sustained cell cycle and mitochondrial perturbations might progress toward activation of cell death pathways. Assessment of apoptosis by annexin V-FITC flow cytometry revealed an increase in phosphatidylserine-positive cells, predominantly at the highest tested concentration ($2 \times \text{IC}_{50}$) and following prolonged exposure. In contrast, at concentrations corresponding to IC_{50} values, annexin V positivity was limited or absent at earlier time points. Further evaluation using dual AO/EB staining, which identifies apoptotic cells based on morphological criteria associated with later stages of cell death, revealed a markedly lower fraction of apoptotic cells compared with annexin V/FITC analysis [51]. This discrepancy reflects the different biological processes captured by these methods. Annexin V/FITC binding detects early externalization of phosphatidylserine, which may represent an initial and potentially reversible stage of apoptosis, whereas AO/EB staining identifies cells that have progressed to advanced, morphologically defined apoptotic stages [52,53]. Consequently, annexin V positivity does not necessarily indicate irreversible commitment to apoptotic execution [54–56]. Notably, even under prolonged exposure conditions, the necrotic fraction remained low and non-dominant across all tested cell lines, indicating that RA does not induce nonspecific membrane damage or acute cytotoxicity. Instead, these data suggest that RA primarily imposes sustained cytostatic stress, with apoptotic features emerging only when mitochondrial and cell-cycle perturbations exceed the adaptive capacity of cancer cells. In contrast to our findings, Xu et al. reported pronounced apoptosis at lower RA concentrations [7]. However, their experiments were conducted under serum-deprived conditions, which may lower the apoptotic threshold. Differences in baseline stress levels and biological characteristics of the analyzed cell lines further complicate direct comparison. These discrepancies likely reflect context-dependent variability in RA responsiveness rather than fundamental mechanistic divergence.

Taken together, the present study extends our previous observations by providing an integrated mechanistic framework linking mitochondrial perturbation, transcriptional modulation, and growth inhibition in RA-treated CRC cells. At concentrations corresponding to IC_{50} values, RA induced early mitochondrial depolarization accompanied by coordinated downregulation of proliferation markers and induction of *CDKN1A*, resulting in G₀/G₁ arrest and a sustained cytostatic phenotype. Importantly, these effects occurred in the absence of detectable ROS accumulation or DNA damage, indicating that RA does not act as a classical oxidative or genotoxic cytotoxic agent. A clear concentration-dependent relationship emerged: moderate RA exposure predominantly enforced checkpoint-mediated growth restraint, whereas higher concentrations and prolonged exposure were required for substantial apoptotic engagement. Thus, mitochondrial perturbation and transcriptional reprogramming appear mechanistically linked to the observed antiproliferative effect, while apoptotic cell death represents a secondary consequence of sustained proliferative and metabolic stress rather than the primary driver of growth inhibition. This distinction is particularly relevant when interpreting viability-based readouts and underscores the importance of integrating complementary functional and molecular assays when characterizing natural compounds. Collectively, these findings position RA as a non-genotoxic, non-pro-oxidative modulator of cancer cell proliferation that restricts tumor growth through

regulatory interference with bioenergetic and cell cycle networks rather than through acute destructive cytotoxicity. Future studies should further delineate the upstream signaling pathways connecting mitochondrial dysfunction to checkpoint activation and explore how this growth-restrictive profile may be leveraged in rational combination-based therapeutic strategies.

4. Materials and Methods

4.1. Chemicals

Cell culture reagents: Fetal bovine serum (FBS), phosphate-buffered saline (PBS), MEM, and RPMI-1640 culture medium were purchased from Biowest (CytoGen, Zgierz, Poland). Stabilized penicillin-streptomycin solution and trypsin-ethylenediamine tetraacetic acid (EDTA) were obtained from Merck/Sigma Aldrich Chemical Co. (Burlington, MA, USA). Black and clear-bottom 96-well plates, as well as 12-well and 6-well plates, were supplied by Thermo Fisher Scientific (Waltham, MA, USA). Comet assay reagents: normal melting point agarose, low-melting point agarose, bleomycin sulfate, sodium hydroxide solution, EDTA, disodium salt dihydrate, tris(hydroxymethyl)aminomethane (TRIS), Triton X-100, sodium chloride solution, and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Merck/Sigma Aldrich Chemical Co. (Burlington, MA, USA). Apoptosis, mitochondrial function, and oxidative stress assays: MitoTracker CMX Ros was obtained from Invitrogen (Waltham, MA, USA). The Annexin V/FITC Apoptosis Detection Kit I was supplied by B.D. Biosciences (Franklin Lakes, NJ, USA). AO/BE, as well as the ROS probe H₂DCFDA, were purchased from Merck/Sigma Aldrich Chemical Co. (Burlington, MA, USA). RNA isolation and gene expression analysis: Total RNA was isolated using FenoZol reagent (A&A Biotechnology, Gdańsk, Poland). Reverse transcription was performed using the TranScriba noGenome Kit (A&A Biotechnology, Gdańsk, Poland). Quantitative PCR was carried out using qPCR-HS SYBR Mix (A&A Biotechnology, Gdańsk, Poland).

4.2. Cell Culture

Human CRC cell lines DLD-1 (adenocarcinoma; ATCC[®] CCL-221TM), HCT-116 (adenocarcinoma; ATCC[®] CCL-247TM), and HT-29 (carcinoma; ATCC[®] HTB-38TM), as well as normal cell lines WI-38 (human lung fibroblasts, ATCC[®] CCL-75TM) and CCD-841 CoN (human colon epithelial, ATCC[®] CRL-1790TM), were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). CRC cell lines were cultured in RPMI-1640 medium, whereas non-cancerous WI-38 and CCD-841 CoN cells were maintained in MEM medium, both supplemented with 10% (*v/v*) fetal bovine serum and 1% (*v/v*) penicillin-streptomycin solution. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. The culture medium was replaced every 24–48 h, and cells were subcultured using 0.25% trypsin-EDTA upon reaching approximately 80–90% confluence. Mycoplasma contamination was routinely monitored monthly using the MycoBlueTM Mycoplasma Detector kit (Vazyme Biotech, Nanjing, China).

4.3. Cell Treatment

RA concentrations applied in the present study were selected based on previously published cytotoxicity and antiproliferative data obtained using MTT, crystal violet, and neutral red assays. Consensus IC₅₀ values derived from these assays were used to establish the working concentrations for subsequent experiments. Depending on the experimental design, cells were treated with RA at 0.5 × IC₅₀, IC₅₀, or 2 × IC₅₀. The exact RA concentrations used in individual assays are summarized in Table 2.

Table 2. Roburic acid (RA) concentrations were applied in the individual experimental assays.

Time	Concentration	Cell Line				
		DLD-1	HCT-116	HT-29	CCD-841 CoN	WI-38
24 h	$0.5 \times \text{IC}_{50}$	8.12	10.17	11.2	17.63	17.61
	IC_{50}	16.23	20.33	22.39	35.25	35.21
	$2 \times \text{IC}_{50}$	32.47	40.66	44.78	70.5	70.42
72 h	$0.5 \times \text{IC}_{50}$	4.61	6.03	5.3	8.75	7.66
	IC_{50}	9.21	12.06	10.6	17.5	15.32
	$2 \times \text{IC}_{50}$	18.42	24.12	21.19	35.0	30.64

4.4. Ki-67 Immunofluorescence Assay

Ki-67 immunofluorescence staining was performed using standard procedures for the detection of nuclear proliferation markers, with modifications [54]. Cell proliferation was assessed by immunofluorescence staining of the nuclear proliferation marker Ki-67. DLD-1, HCT-116, and HT-29 cells were seeded onto glass coverslips placed in 12-well plates at a density of 3×10^4 cells/well and allowed to adhere for 24 h. Cells were subsequently treated with RA at concentrations corresponding to $0.5 \times \text{IC}_{50}$ and IC_{50} for 48 h. Following treatment, cells were washed with PBS and fixed in 70% ethanol at -20°C . After permeabilization with PBS containing 0.5% Triton X-100, DNA was denatured using 4 N HCl and neutralized with 0.1 M borax. Samples were then incubated for 1 h with a rabbit anti-Ki-67 primary antibody (Thermo Fisher, MA5-14520; 1:250 dilution in PBS supplemented with 1% bovine serum albumin and 0.5% Tween-20), followed by a 1 h incubation with an Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (Thermo Fisher, A-11008; 1:500 dilution). Cell nuclei were counterstained with DAPI (1 $\mu\text{g}/\text{mL}$), and coverslips were mounted using Fluoromount-G. Fluorescence images were acquired using an Olympus BX60 F5 fluorescence microscope equipped with 20 $\times$ and 40 $\times$ objectives. For each sample, images of at least 200 cells were captured using CellSens imaging software v1.7 (Olympus Corporation, Tokyo, Japan). Quantitative analysis of Ki-67 fluorescence intensity was performed using ImageJ version 1.54f (ImageJ software, National Institutes of Health, Bethesda, MD, USA). Nuclear regions of interest were defined, and fluorescence intensity was quantified as corrected total cell fluorescence (CTCF), calculated as integrated density minus background fluorescence. The data are presented as median values and expressed relative to untreated controls.

4.5. Cell Cycle Analysis

Cell cycle distribution was analyzed by flow cytometry following propidium iodide (PI) staining. DLD-1, HCT-116, and HT-29 cells were seeded in 6-well plates at a density of 0.5×10^6 cells/mL and allowed to attach for 24 h. The assay was performed according to the previously described protocols [55,56]. Cells were subsequently treated with RA at concentrations corresponding to $0.5 \times \text{IC}_{50}$ and IC_{50} for 24 or 48 h. After treatment, cells were harvested and centrifuged at 1400 rpm for 10 min at 4°C . Cell pellets were washed with PBS and fixed dropwise in ice-cold 70% ethanol with continuous vortexing. Prior to flow cytometry analysis, cells were washed with PBS and incubated for 30 min at 37°C in the dark in a staining solution containing 5 $\mu\text{g}/\text{mL}$ PI and 10 U/mL RNase A in PBS. DNA content was measured using an LSR II flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 488 nm excitation laser and a 530 nm emission filter. A minimum

of 10,000 events were collected per sample. The data were analyzed using FlowJo version 10.8 (FlowJo software, Becton, Dickinson, and Company, Ashland, OR, USA), and cell populations were quantified in the G₀/G₁, S, G₂/M, and sub-G₁ phases.

4.6. Annexin V-FITC Binding Assay

Apoptotic cell death was evaluated in DLD-1, HCT-116, and HT-29 cells using the FITC Annexin V Apoptosis Detection Kit I, based on the detection of phosphatidylserine externalization on the outer leaflet of the plasma membrane [52,53]. The combined use of annexin V-FITC and PI enabled discrimination between viable, early apoptotic, late apoptotic, and necrotic cells. The assay was performed according to the previously described protocols [55,56]. Cells were cultured in 6-well plates at densities of 7×10^5 cells/mL or 3.5×10^5 cells/mL and allowed to attach for 24 h. Subsequently, cells were treated with RA at concentrations corresponding to $0.5 \times \text{IC}_{50}$, IC_{50} , and $2 \times \text{IC}_{50}$ for 24 or 72 h. After treatment, cells were harvested, washed with PBS, and centrifuged at 1400 rpm for 10 min at 4 °C. Cell pellets were resuspended in Annexin V binding buffer and stained with Annexin V-FITC and PI for 15 min in the dark at room temperature. Samples were analyzed using an LSR II flow cytometer with fluorescence detection at 530 nm. A minimum of 10,000 events were acquired per sample. Data were analyzed using FlowJo version 10.8.

4.7. Dual Acridine Orange/Ethidium Bromide Fluorescent Staining

Dual staining with AO and EB, combined with morphological assessment, was used to distinguish viable, necrotic, and apoptotic cells [57,58]. AO penetrates intact cell membranes and intercalates into nucleic acids, emitting green fluorescence in viable cells, whereas EB is membrane-impermeable and stains only cells with compromised membrane integrity, emitting red fluorescence. The assay was performed according to the previously described protocols [56,59]. DLD-1, HCT-116, and HT-29 cells were seeded in 12-well plates at densities of 1.5×10^5 cells/mL (for 24 h treatment) or 7.5×10^4 cells/mL (for 72 h treatment) and allowed to attach for 24 h. Cells were then treated with RA at concentrations corresponding to $0.5 \times \text{IC}_{50}$, IC_{50} , and $2 \times \text{IC}_{50}$ for 24 and 72 h. After treatment, cells were harvested, centrifuged at 1400 rpm for 10 min at 4 °C, and resuspended in 25 µL of PBS. AO/EB staining solution (1 µL; 100 µg/mL of each dye) was added to the cell suspension, and 10 µL of the mixture was placed on glass microscope slides. Samples were examined immediately under a fluorescence microscope (Olympus BX60 F5, Olympus Optical Co., Ltd., Nagano, Japan). At least 200 cells per condition were scored and classified as viable, necrotic, or apoptotic based on differential dye uptake and characteristic morphological features.

4.8. Mitochondrial Membrane Potential Assay

MMP was assessed using the fluorescent probe MitoTracker Red CMXRos, which selectively accumulates in active mitochondria. The assay was performed according to the previously described protocols [56,59]. DLD-1, HCT-116, and HT-29 cells were seeded in black 96-well plates at a density of 1.2×10^4 cells/mL and allowed to attach for 24 h. Cells were then treated with RA at concentrations corresponding to $0.5 \times \text{IC}_{50}$, IC_{50} , and $2 \times \text{IC}_{50}$ for 24 h. Following treatment, cultures were washed with PBS and incubated with MitoTracker Red CMXRos (0.1 µM) for 40 min in the dark. After staining, fluorescence intensity was measured using a SpectraMax[®] i3x Multi-Mode Detection Platform (Molecular Devices, San Jose, CA, USA) at excitation and emission wavelengths of 579 and 599 nm, respectively. Results were expressed as mean fluorescence intensity compared with untreated control cells (normalized to 100%).

4.9. Determination of Intracellular Reactive Oxygen Species Level

Intracellular ROS levels were determined using the fluorescent probe H₂DCFDA according to the protocol described by Ruiz-Leal and George [60]. DLD-1, HCT-116, and HT-29 cells were seeded in black 96-well plates at a density of 1.2×10^4 cells/mL and allowed to attach for 24 h. Cells were then loaded with H₂DCFDA (20 μ M) for 20 min in the dark, washed with PBS, and treated with RA at concentrations corresponding to $0.5 \times \text{IC}_{50}$, IC_{50} , and $2 \times \text{IC}_{50}$. H₂O₂ (50 μ M) was used as a positive control. Fluorescence intensity was recorded at excitation and emission wavelengths of 485 and 535 nm, respectively, using a SpectraMax® i3x Multi-Mode Detection Platform over 2 h at 15 min intervals.

4.10. Alkaline Comet Assay

The alkaline comet assay was performed to evaluate DNA strand breaks in CRC cell lines according to the protocol described by Kciuk et al. [61]. DLD-1, HCT-116, and HT-29 cells, as well as non-cancerous WI-38 and CCD-841 CoN cells, were seeded in 12-well plates at a density of 1.2×10^5 cells/mL and allowed to attach for 24 h. Cells were then treated with RA at concentrations corresponding to $0.5 \times \text{IC}_{50}$ and IC_{50} for an additional 24 h. The 20 μ M bleomycin was chosen as a positive control. Following treatment, cells were processed for the comet assay as previously described [61]. Briefly, cells were embedded in agarose on microscope slides, lysed, and subjected to electrophoresis under alkaline conditions. After electrophoresis, DNA was stained with DAPI (1 μ g/mL) and visualized using a fluorescence microscope (Olympus) equipped with CellSens software at an excitation wavelength of 360 nm. For each experimental condition, at least 200 cells were analyzed. DNA damage was quantified as the mean percentage of DNA content in comet tails with the interquartile range, minimum, and maximum.

4.11. Gene Expression

4.11.1. RNA Isolation and cDNA Synthesis

DLD-1, HCT-116, and HT-29 cells were seeded in 6-well plates at densities of 5×10^5 cells/well (24 h incubation) or 2×10^5 cells/well (72 h incubation) and treated with RA at the IC_{50} concentrations or left untreated as controls. After 24 or 72 h, total RNA was isolated using the phenol–chloroform extraction method with FenoZol Plus reagent (cat. no. 203-100P; A&A Biotechnology, Gdańsk, Poland) according to the manufacturer's instructions. RNA concentration and purity were determined spectrophotometrically using a BioDrop μ Lite + system (Biochrom Ltd., Cambridge, UK), based on absorbance ratios at 260/280 nm and 260/230 nm. Complementary DNA was synthesized from 250 ng of total RNA using the TranScriba noGenome Kit (cat. no. 4000NG-100; A&A Biotechnology) according to the manufacturer's protocol.

4.11.2. Quantitative Real-Time PCR (RT-qPCR)

Quantitative real-time PCR was performed with SYBR Green chemistry with qPCR-HS Mix SYBR (cat. no. 2008HS-100; A&A Biotechnology) on a real-time PCR thermocycler (RotorGene Q, QIAGEN, Hilden, Germany). Primer sequences used for RT-qPCR analysis are provided in Supplementary Table S1. Thermal cycling conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 54–56 °C (primer-dependent) for 30 s, and extension at 72 °C for 30 s.

Relative gene expression was calculated using the ΔCt method. Threshold cycle (Ct) values were obtained using Rotor-Gene Q Series software v2.3.5. For each sample, Ct values of target genes were normalized to the reference gene *GAPDH* according to the following formula:

$$\Delta\text{Ct} = \text{Ct}_{\text{target gene}} - \text{Ct}_{\text{ref gene}}$$

Due to the non-normal distribution of RT-qPCR data, statistical analyses were performed on ΔCt values. For graphical presentation, gene expression data are shown as $-\Delta\text{Ct}$ values to facilitate visualization of relative changes in transcript levels. All reactions were performed in duplicate, and mean Ct values were used for further analyses.

4.12. Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA) and Statistica version 13.3 (TIBCO Software Inc., Palo Alto, CA, USA). Data normality was assessed using the Shapiro–Wilk test. Data obtained from annexin V binding assay, MMP measurements, intracellular ROS determination, AO/EB double staining assays, and cell cycle analysis are presented as mean $\pm$ SD. Results from Ki-67 immunofluorescence analysis, the alkaline comet assay, and RT-qPCR are presented as medians with interquartile ranges because of non-normal distributions. For comparisons involving more than two groups, one-way analysis of variance (ANOVA), followed by Dunnett's or Tukey's post hoc tests, was applied for normally distributed data. Cell cycle distribution and intracellular ROS measurements were additionally analyzed using two-way ANOVA, followed by Dunnett's or Tukey's multiple comparisons tests as appropriate. For non-normally distributed data, the Kruskal–Wallis test followed by Dunn's post hoc test was used. Statistical significance was defined as $p < 0.05$. For the alkaline comet assay, at least 200 cells were analyzed per experimental condition. The comet assay, MMP assay, annexin V binding assay, and intracellular ROS determination were performed in triplicate. The AO/EB double staining assay was conducted in two independent experiments. RT-qPCR reactions were performed in duplicate for each sample.

5. Conclusions

RA does not exhibit genotoxic activity in CRC cells, indicating that its antiproliferative effects are not mediated by DNA damage. RA induces an early reduction in mitochondrial membrane potential without concomitant ROS generation or oxidative stress, distinguishing its mode of action from that of many conventional cytotoxic anticancer agents. Under conditions corresponding to IC_{50} values, RA primarily exerts cytostatic effects, manifested by cell cycle arrest, suppression of proliferation markers, and limited induction of cell death. Apoptotic features become apparent mainly at the highest concentrations ($2 \times \text{IC}_{50}$) or following prolonged exposure and are highly dependent on cell line-specific and experimental conditions, suggesting that apoptosis represents a secondary consequence of sustained cellular stress rather than a primary mechanism of action. Collectively, these findings indicate that RA limits CRC cell growth predominantly through cytostatic mechanisms of the cell cycle and mitochondrial function, rather than through direct cytotoxicity, oxidative stress, or genotoxic injury. This non-genotoxic, non-oxidant profile highlights RA as a biologically active compound with a distinct mechanism of action and potential relevance for long-term or combination-based anticancer strategies. In particular, its growth-modulatory rather than DNA-damaging activity suggests possible utility as an adjuvant agent aimed at restraining tumor proliferation while minimizing therapy-associated mutagenic risk. Future investigations should therefore include *in vivo* validation, pharmacokinetic assessment, and evaluation of RA in rational combination regimens with standard chemotherapeutics or targeted agents. Furthermore, additional studies are warranted to elucidate the precise signaling pathways involved, including those related to cell cycle control, mitochondrial regulation, and adaptive stress responses such as autophagy or senescence.

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Abbreviations

AO/EB	Acridine orange/ethidium bromide
CAII	Carbonic anhydrase II
<i>CDKN1A</i>	Cyclin-dependent kinase inhibitor 1A
CES1	Carboxyl esterase 1
CRC	Colorectal cancer
Ct	Threshold cycle
CTCF	Corrected total cell fluorescence
DAPI	4,6-diamidino-2-phenyl-indole
EDTA	Ethylenediamine tetraacetic acid
EGFR	Epidermal growth factor receptor
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase
H ₂ DCFDA	2',7'-dichlorodihydrofluoresceindiacetate
H ₂ O ₂	Hydrogen peroxide
IKK	IκB kinase
IκBα	Inhibitor of κB α
<i>MKI67</i>	Marker of proliferation Kiel 67
MMP	Mitochondrial membrane potential
NF-κB	Nuclear factor kappa B
PBS	Phosphate-buffered saline
<i>PCNA</i>	Proliferating cell nuclear antigen
PI	Propidium iodide
PLA2G2A	Phospholipase A2
RA	Roburic acid
ROS	Reactive oxygen species
RT-qPCR	Quantitative real-time PCR
TNF-α	Tumor necrosis factor-alpha
<i>TP53</i>	Tumor antigen p53
TRIS	Tris(hydroxymethyl)aminomethane

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Supplementary Information

Roburic Acid as a Therapeutic Candidate: Antiproliferative Activity and Secondary Cell Death Response in Colorectal Cancer Cells

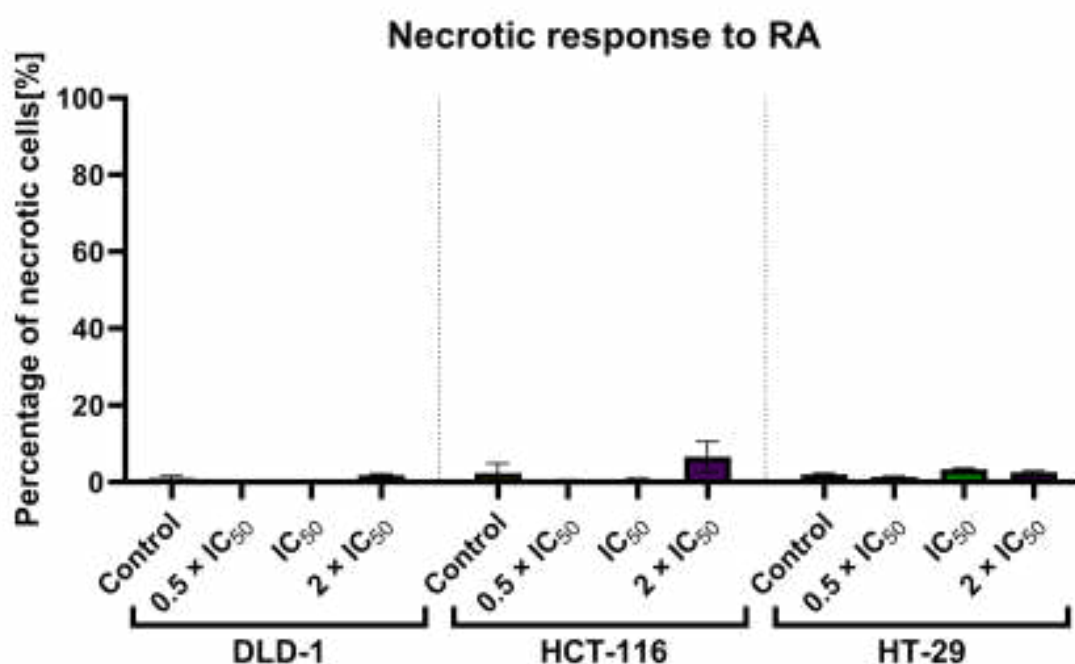
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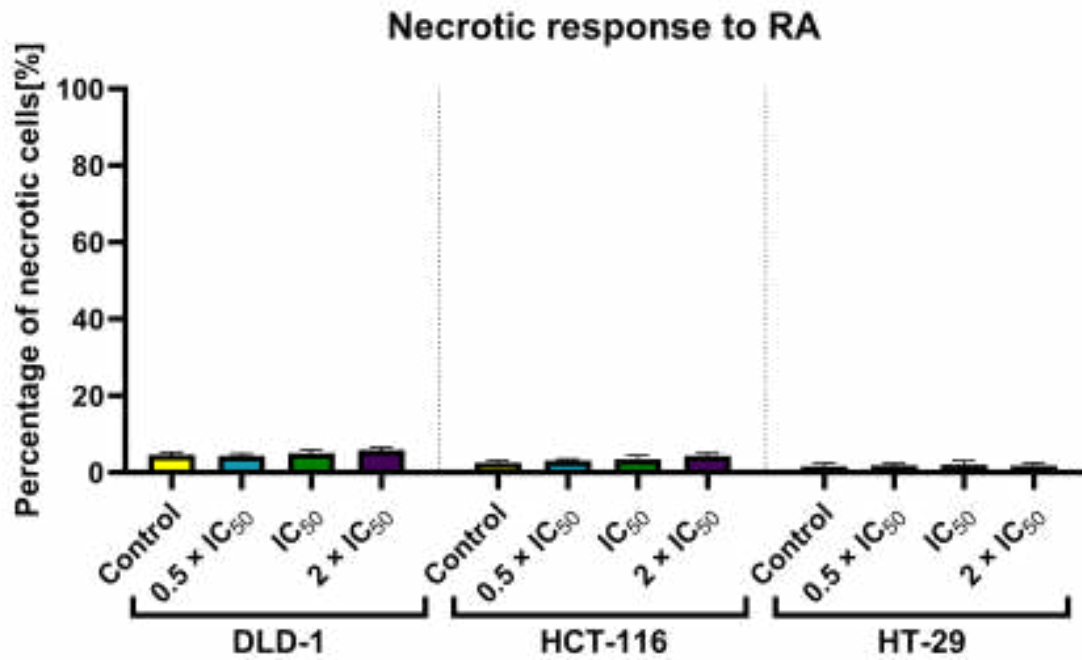
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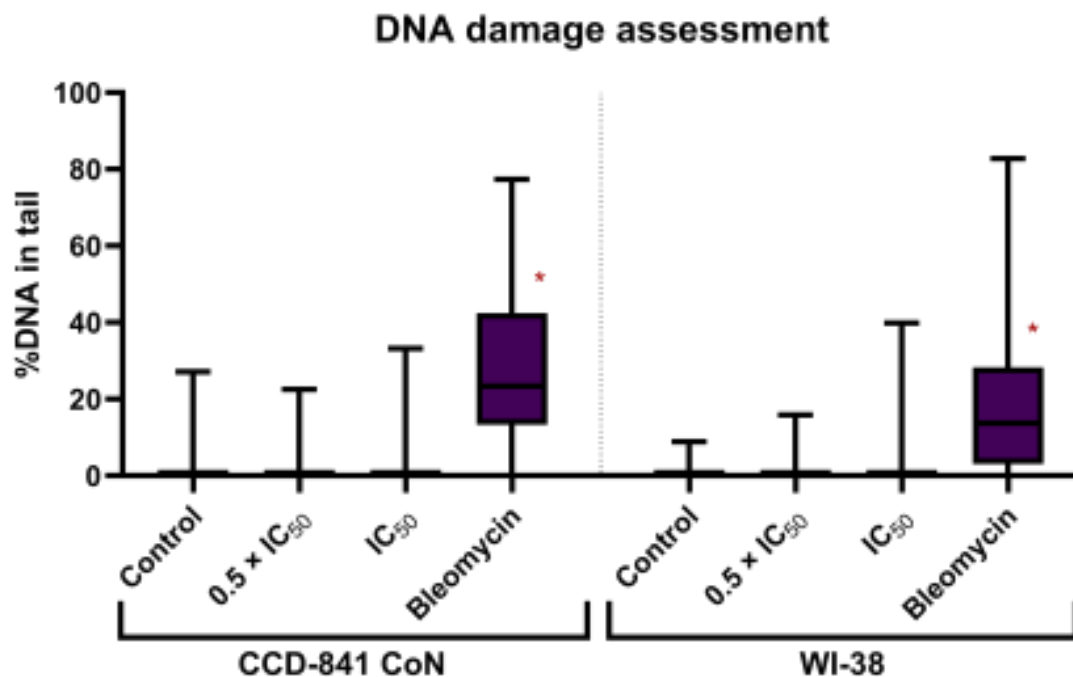
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Supplementary Figure S1. Necrotic response of DLD-1, HCT-116, and HT-29 colorectal cancer (CRC) cells to RA treatment at concentrations of 0.5 × IC₅₀, IC₅₀, and 2 × IC₅₀ for 24 hours, determined by annexin V–fluorescein isothiocyanate (FITC)/propidium iodide (PI). Data are presented as mean percentage [%] ± SD of PI-positive cells. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test; an asterisk indicates significant differences relative to untreated controls (*) ($p < 0.05$), $N > 1 \times 10^4$.



Supplementary Figure S2. Necrotic response of DLD-1, HCT-116, and HT-29 colorectal cancer (CRC) cells to RA treatment at concentrations of $0.5 \times \text{IC}_{50}$, IC_{50} , and $2 \times \text{IC}_{50}$ for 72 hours, determined by annexin V–fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining. Data are presented as mean percentage [%] $\pm$ SD of PI-positive cells. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test; an asterisk indicates significant differences relative to untreated controls (*) ($p < 0.05$), $N > 1 \times 10^4$.



Supplementary Figure S3. DNA damage in CCD-841 CoN and WI-38 normal human cell lines assessed by the alkaline comet assay after 24 hours of exposure to RA at concentrations of $0.5 \times \text{IC}_{50}$ and IC_{50} . Data are presented as mean percentage of DNA content in comet tails with interquartile ranges, minimum, and maximum values. Statistical analysis was performed using the Kruskal–Wallis

test followed by Dunn's post hoc test; an asterisk indicates significant differences relative to untreated controls (*) ($p < 0.05$), $N > 200$.

Supplementary Table S1. Primer sequences used for RT-qPCR.

Gene	Forward primer sequence (5' → 3')	Reverse primer sequence (5' → 3')
<i>GAPDH</i>	CCGCATCTTCITTTGCGTCG	GCCCAATACGACCAAATCCGT
<i>MKI-67</i>	TGACCCTGATGAGAAAGCTCAA	CCCTGAGCAACACTGTCTTTT
<i>PCNA</i>	GCAGAGTGGTCGTTGTCTTT	TCTAGCTGGTTTCGGCTTCA
<i>CDKN1A</i>	CAGTACCCTCTCAGCTCCAG	AGCTTCCCTGGTTAGTACGG
<i>TP53</i>	ACTGGTGTTTTGTTGTGGGG	AGCTTCCCTGGTTAGTACGG

Revisiting Apoptosis: What Lies Behind the Antiproliferative Activity of Roburic Acid in Colorectal Cancer Cells?

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Abstract

Roburic acid (RA) exhibits antiproliferative activity in colorectal cancer (CRC) cells, but its mechanism of action remains unclear. Here, we investigated whether RA induces regulated cell death or alternative cellular responses in CRC models with different tumor antigen P53 (*TP53*) status. Pharmacological inhibition of apoptosis, necroptosis, and ferroptosis did not restore cell viability, indicating that these pathways are not major contributors to RA-induced growth inhibition. RA also failed to induce cellular senescence. Although mitochondrial alterations and moderate caspase-9 activation were observed, they did not result in effective apoptotic cell death. Instead, RA triggered a predominantly cytostatic response. Analysis of P53 and cyclin-dependent kinase inhibitor 1A (P21) revealed cell line-dependent changes in subcellular localization, with limited changes in overall protein levels. Notably, P21 redistribution patterns varied across models and were consistent with *CDKN1A* transcriptional induction. These findings indicate that RA primarily modulates cell cycle regulation through the P53–P21 axis and acts as a context-dependent regulator of proliferation rather than a cytotoxic agent.

Keywords: roburic acid, colorectal cancer, cytostatic activity, apoptosis, senescence

Introduction

Roburic acid (RA) is a plant-derived tetracyclic triterpenoid known to modulate signaling pathways associated with inflammation and cell fate regulation, including tumor necrosis factor- α (TNF- α)/nuclear factor kappa B (NF- κ B). Building on our previous investigations into the anticancer activity of RA, we have consistently observed a pronounced antiproliferative effect in colorectal cancer (CRC) cells, reflected by reduced DNA synthesis, impaired clonogenic potential, and decreased cell density, without a pronounced increase in cell death (Gielecińska et al., 2025). These findings indicate that RA exerts predominantly cytostatic rather than cytotoxic activity, associated with G₀/G₁ cell cycle arrest, downregulation of proliferation markers such as Ki-67, and reduced DNA replication capacity.

Importantly, these effects occur in the absence of oxidative stress and DNA damage, distinguishing RA from classical cytotoxic agents (Gielecińska et al., 2026).

Despite this clearly defined growth-inhibitory phenotype, the underlying cellular response to RA remains unresolved. Several observations from our previous analyses raised important questions regarding the potential involvement of cell death pathways. For instance, the consistent reduction in mitochondrial membrane potential pointed to early mitochondrial dysfunction, a hallmark often associated with the initiation of apoptosis. Furthermore, cell cycle analysis revealed the emergence of a sub-G₁ population under selected conditions, suggesting DNA fragmentation in a subset of cells. Annexin V-FITC assays, combined with the dual-staining method, indicated the presence of apoptotic features, predominantly at higher concentrations and prolonged exposure. Importantly, these approaches did not reveal a detectable increase in necrotic cell populations (Gielecińska et al., 2026).

Taken together, these findings indicate that apoptotic processes may be engaged, albeit in a limited or context-dependent manner. This prompted us to examine whether apoptosis constitutes a primary mechanism of RA action, or whether alternative cellular outcomes, such as other forms of regulated cell death (RCD) or cellular senescence, may account for the observed phenotype. Accordingly, in the present study, we aimed to directly address this question by defining the predominant cellular fate induced by RA in CRC.

Results

SA-β-Gal Staining

Since cellular senescence can represent an alternative response to anticancer treatments, we evaluated senescence after a 1-day treatment followed by 7 days of culture. Under these conditions, RA at concentrations corresponding to 0.5× IC₅₀ and IC₅₀ did not cause any changes in senescent cell fraction in DLD-1, HCT-116, or HT-29 (Figure 1). In contrast, doxorubicin (DOX; 100 nM), used as a positive control, induced senescence, as evidenced by a statistically significant increase ($p < 0.05$) in senescence-associated β-galactosidase (SA-β-Gal)-positive cells compared to the untreated controls. These changes were accompanied by morphological features characteristic of a senescent phenotype, including enlarged, flattened, and granular cells (Figure 2). Accordingly, under these conditions, RA does not induce senescence. Notably, its antiproliferative effect is transient, as cells resume proliferation following drug withdrawal, albeit at a modestly reduced rate.

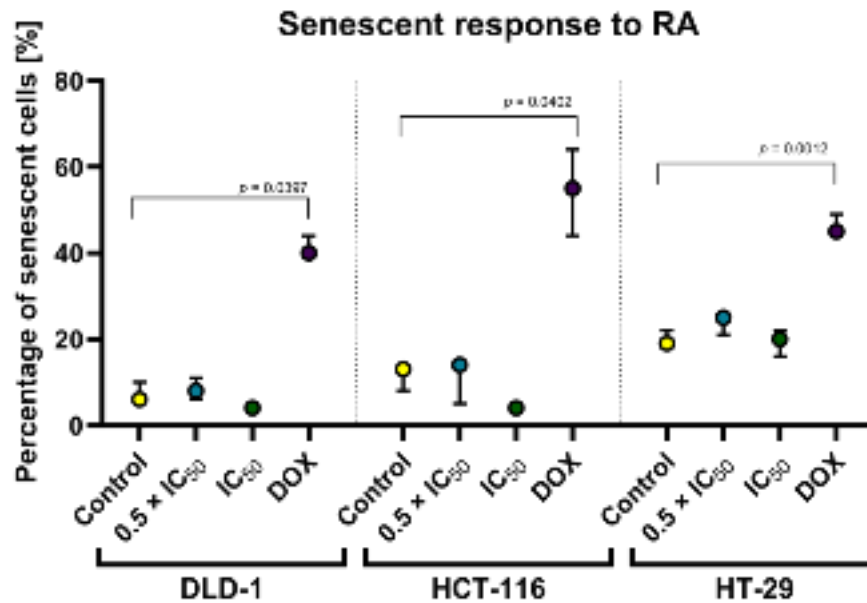


Figure 1. Senescence in DLD-1, HCT-116, and HT-29 colorectal cancer (CRC) cells was assessed by senescence-associated β -galactosidase (SA- β -Gal) staining after 1 day of exposure to roburic acid (RA) at concentrations corresponding to $0.5 \times \text{IC}_{50}$ and IC_{50} , or to doxorubicin (DOX; 100 nM), used as a positive control, followed by 7 days of culture. The data are presented as median with interquartile range. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn’s post hoc test; significant differences relative to untreated controls ($p < 0.05$), $N > 300$.

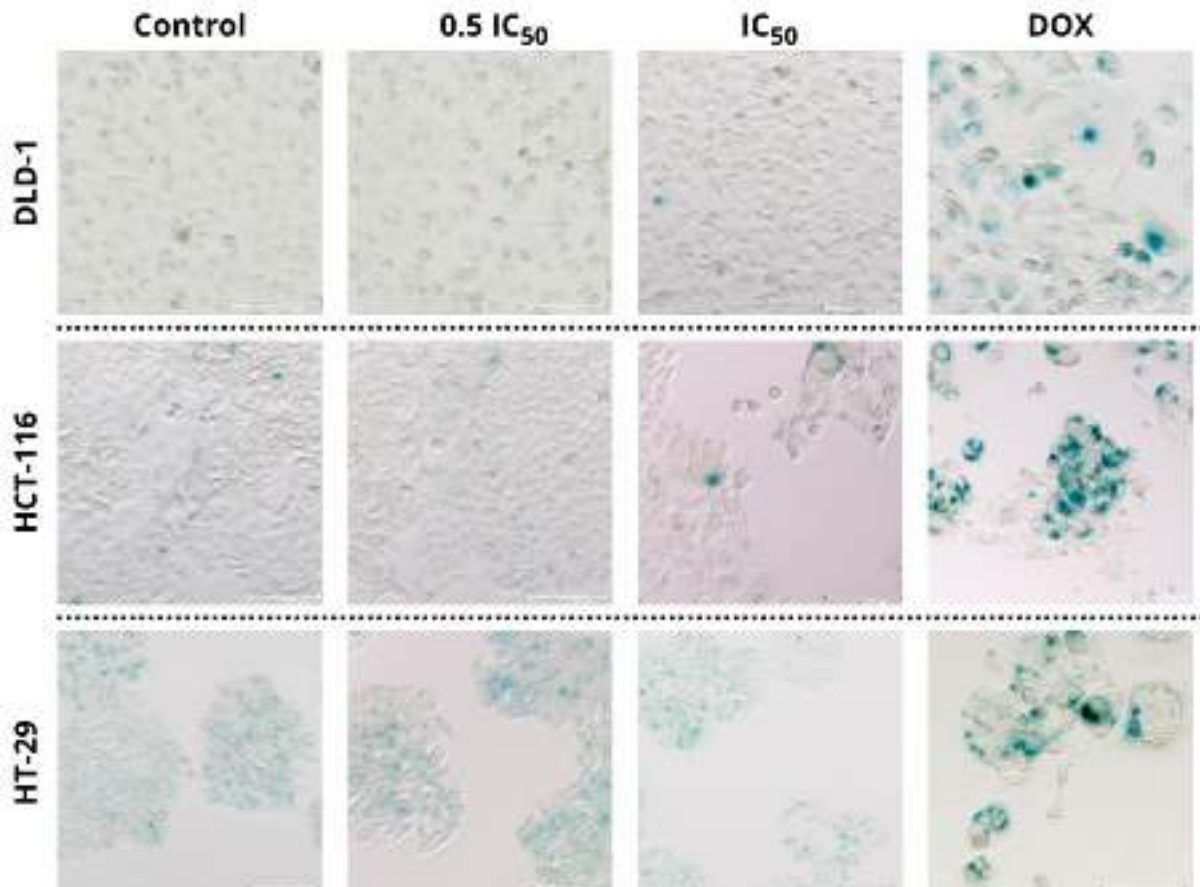


Figure 2. Representative images of senescence-associated β -galactosidase (SA- β -Gal) staining in DLD-1, HCT-116, and HT-29 colorectal cancer (CRC). Cells were untreated or treated with roburic acid (RA; $0.5 \times IC_{50}$ and IC_{50}) or doxorubicin (DOX; 100 nM) for 1 + 7 days. Magnification 20 $\times$.

Pharmacological inhibition of cell death pathways

To determine whether RA-induced growth inhibition involves the activation of specific cell death pathways, we performed co-treatment experiments using pharmacological inhibitors of apoptosis (Q-VD-OPh), necroptosis (necrostatin-1), and ferroptosis (ferrostatin-1). Experimental conditions were established based on previously determined IC_{50} values for RA (see Table 1 in the *Cell Treatment* section). As expected, treatment with RA at the IC_{50} resulted in approximately a 50% reduction in cellular metabolic activity compared with untreated control cells, consistent with previously established antiproliferative effects. However, co-treatment with any of the tested inhibitors did not lead to a significant increase ($p < 0.05$) in cellular metabolic activity compared to RA alone (Figure 3). Importantly, the inhibitors alone had no significant effect on cellular metabolic activity. The extended dataset, including conditions with inhibitors alone, is presented in Supplementary Figure S1.

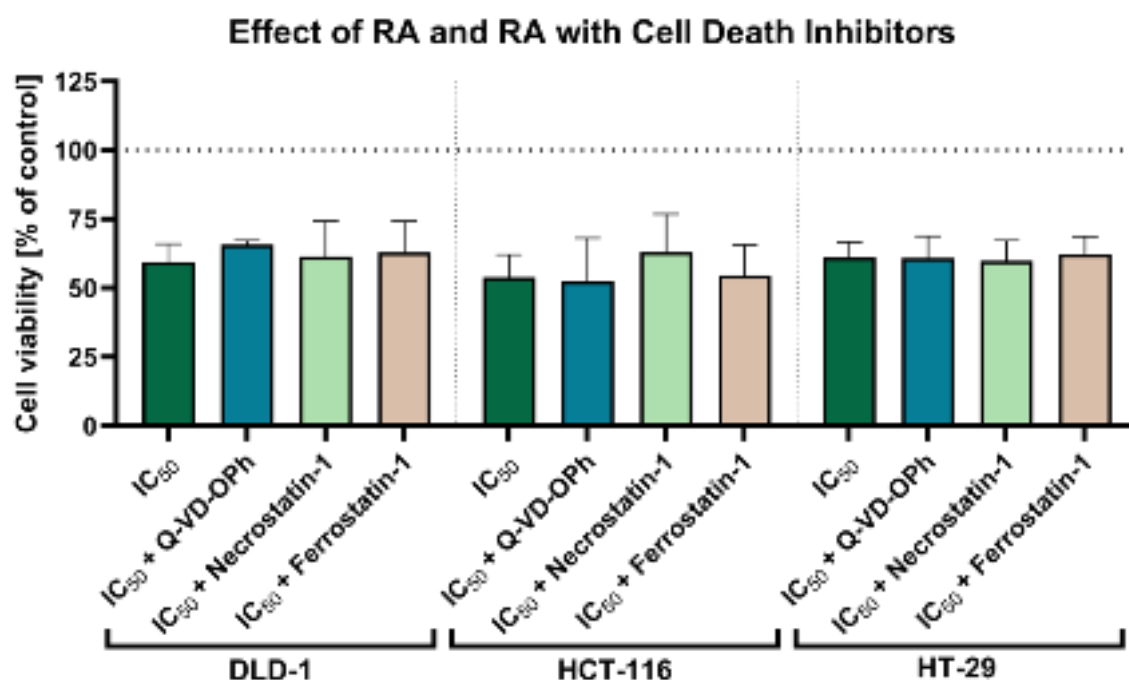


Figure 3. Effect of roburic acid (RA) and co-treatment with cell death inhibitors on cellular metabolic activity in colorectal cancer (CRC) cell lines. Cells (DLD-1, HCT-116, and HT-29) were treated with RA at IC_{50} alone or in combination with inhibitors of apoptosis (Q-VD-OPh), necroptosis (necrostatin-1), or ferroptosis (ferrostatin-1) for 24 hours. Cellular metabolic activity was assessed using an MTT assay and expressed as relative cell viability, normalized to untreated control (set as 100%). Data are presented as mean $\pm$ SD from three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Sidak's multiple comparisons test. Differences were considered statistically significant at $p < 0.05$.

In light of the previously observed reduction in mitochondrial membrane potential and the increased fraction of apoptotic cells under selected conditions, we next investigated caspase-9 activation in RA-treated CRC cells. The results were expressed as a luminescence ratio relative to untreated control

cells (set as 1.0). Treatment with RA at IC_{50} resulted in a significant increase ($p < 0.05$) in signal intensity in HCT-116 and HT-29 cells ($p = 0.026$ and $p = 0.005$, respectively) compared to untreated controls. In contrast, no significant changes were observed in DLD-1 cells at either tested concentration. DOX (0.2 μ M), used as a positive control, induced a more pronounced increase in luminescence across all cell lines, confirming the assay's reliability (Figure 4). Despite the observed increase in caspase-9 activity, the lack of a rescue effect upon pharmacological inhibition of cell death pathways suggests that caspase-dependent apoptosis does not represent the primary mechanism underlying the RA-induced growth inhibition.

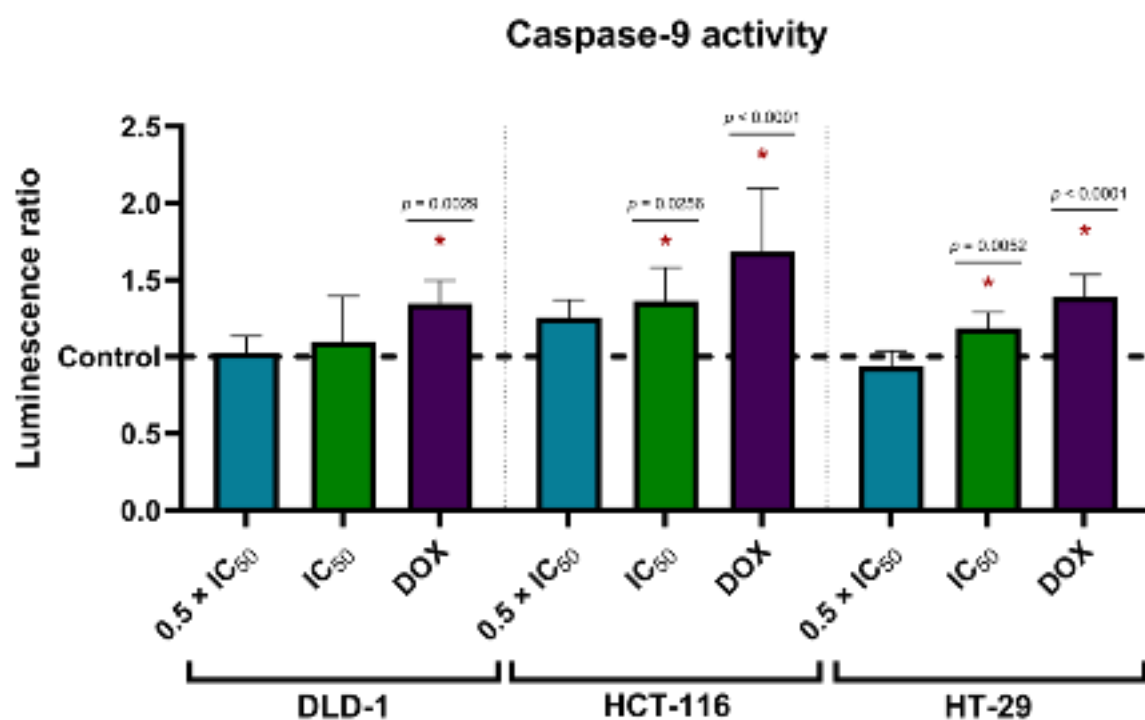


Figure 4. Effect of roburic acid (RA) on caspase-9 activation in colorectal cancer (CRC) cell lines. Cells (DLD-1, HCT-116, and HT-29) were treated with RA at $0.5 \times IC_{50}$ and IC_{50} concentrations for 24 hours. Doxorubicin (0.2 μ M) was used as a positive control. Caspase-9 activation was measured using a luminescence-based assay and expressed as a ratio relative to untreated control (set as 1.0). Data are presented as mean $\pm$ SD from two independent experiments. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons test; an asterisk indicates significant differences relative to untreated controls (*) ($p < 0.05$).

P21 and P53 Immunofluorescence Assay

To comprehensively evaluate RA-induced changes, both the subcellular distribution (nuclear-to-cytoplasmic ratio; N/C) and overall fluorescence intensity of P21 (Figure 5) and P53 (Figure 6) were assessed. Representative images illustrating these changes, along with alterations in cell morphology, are presented in Figures 7-9.

A consistent pattern of P21 redistribution in response to RA treatment was observed in both DLD-1 and HCT-116 cells. In these lines, exposure to RA at $0.5 \times IC_{50}$ led to a significant decrease ($p < 0.05$) in the P21 N/C ratio, reflecting a relative reduction in nuclear P21 accompanied by an increased contribution of the cytoplasmic signal. Treatment with DOX, used as a positive control, resulted in a

marked increase in the P21 N/C ratio, indicative of enhanced nuclear accumulation. In contrast to this shared response, HT-29 cells exhibited a distinct pattern. Here, RA treatment at both concentrations led to a significant increase in the P21 N/C ratio, consistent with an enrichment of P21 within the nuclear compartment. Notably, DOX produced the opposite effect, significantly decreasing the P21 N/C ratio and suggesting a shift toward cytoplasmic localization.

To determine whether these changes in subcellular distribution were associated with alterations in overall protein-associated fluorescence, total cellular P21 fluorescence intensity was analyzed. In DLD-1 cells, RA treatment at IC₅₀ resulted in a significant reduction ($p < 0.05$) in total P21 fluorescence intensity, whereas no changes were observed at the lower concentration. In HCT-116 cells, total P21 fluorescence intensity remained unchanged across all treatment conditions, despite the observed redistribution of the protein. A similar lack of effect of RA on total fluorescence was observed in HT-29 cells; however, in this case, DOX treatment led to a significant decrease in overall P21 fluorescence intensity.

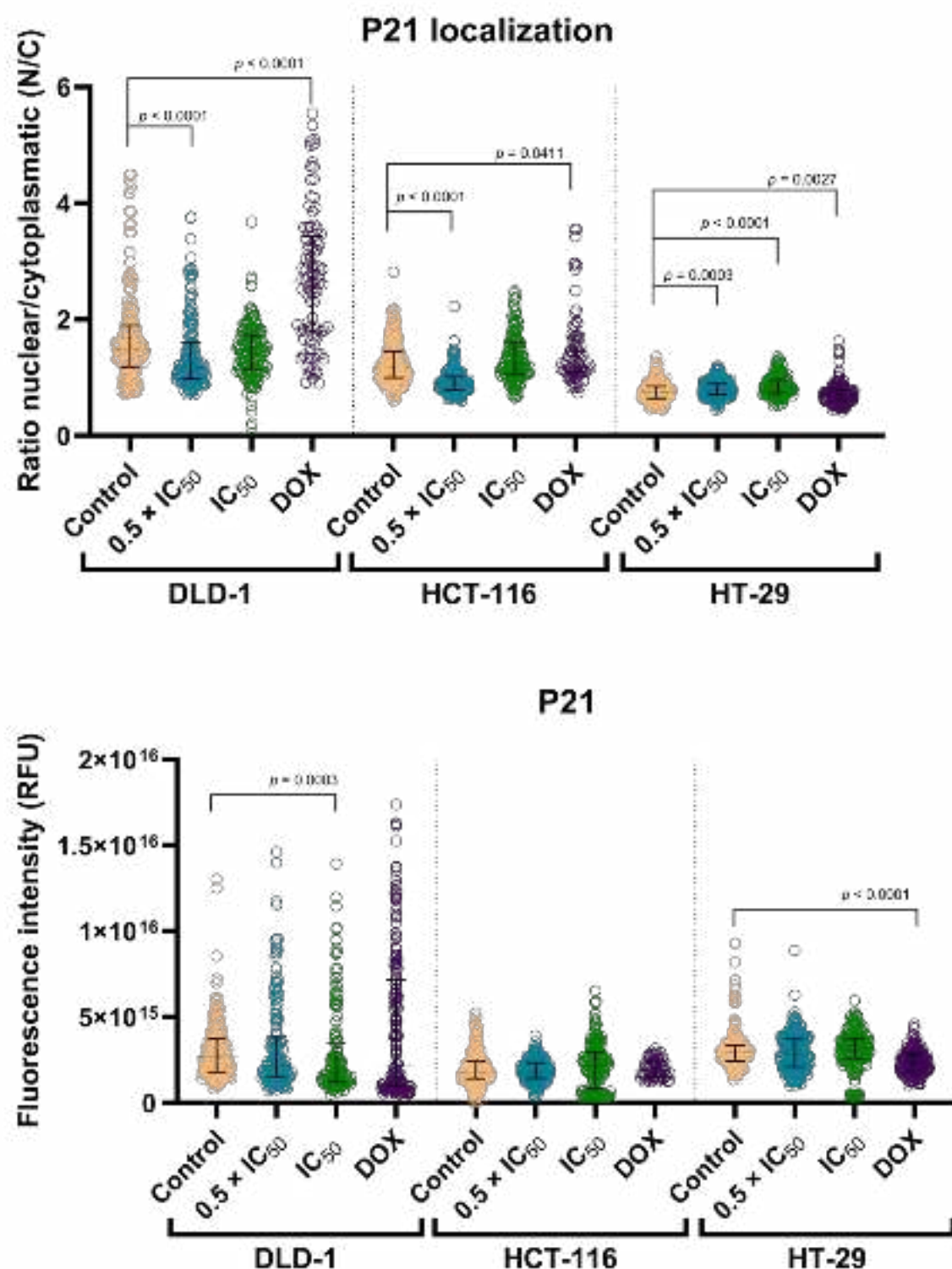


Figure 5. P21 subcellular localization and fluorescence intensity in colorectal cancer (CRC) cell lines following roburic acid (RA) and doxorubicin (DOX) treatment. Upper panel: nuclear-to-cytoplasmic (N/C) ratio of P21. Lower panel: total cellular P21 fluorescence intensity. Cells were treated with RA at $0.5 \times IC_{50}$ and IC_{50} concentration or DOX ($0.2 \mu M$). The plot represents individual data with median and interquartile range. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn’s post hoc test; significant differences relative to untreated controls ($p < 0.05$).

P53 subcellular distribution in response to RA treatment displayed a clear cell line-dependent pattern. In both DLD-1 and HCT-116 cells, treatment with DOX resulted in a significant increase ($p < 0.05$), consistent with enhanced nuclear accumulation. However, RA treatment led to a significant decrease in the N/C ratio at IC₅₀ in both cell lines, indicating reduced nuclear localization and a shift toward the cytoplasmic compartment. A distinct response was observed in HT-29 cells, where all treatments, both RA concentrations and DOX, resulted in a significant decrease compared to the untreated control, indicating reduced nuclear localization and a relative shift toward the cytoplasmic compartment. These differences are likely associated with *TP53* status, as HCT-116 cells harbor wild-type *TP53*, whereas DLD-1 and HT-29 cells carry mutant *TP53*.

To complement the analysis of subcellular distribution, total cellular P53 fluorescence intensity was evaluated. In DLD-1 cells, RA at IC₅₀ resulted in a significant reduction ($p < 0.05$) in total fluorescence intensity, whereas DOX treatment led to a significant increase of this parameter. In HCT-116 cells, a significant increase in fluorescence intensity was observed only following DOX treatment, while RA did not induce measurable changes. In contrast, in HT-29 cells, both RA concentrations increased total fluorescence intensity, whereas DOX treatment decreased it.

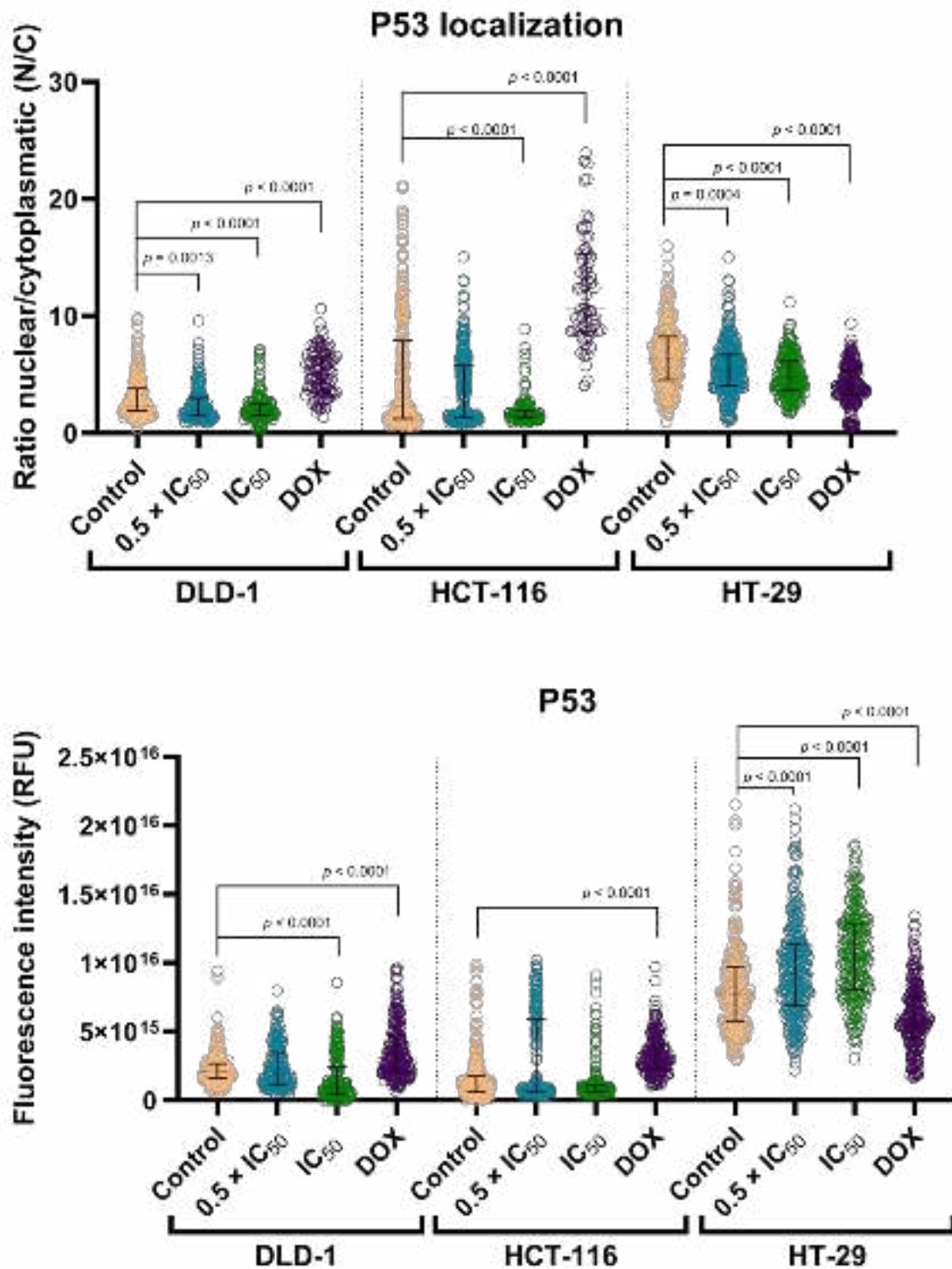


Figure 6. P53 subcellular localization and fluorescence intensity in colorectal cancer (CRC) cell lines following roburic acid (RA) and doxorubicin (DOX) treatment. Upper panel: nuclear-to-cytoplasmic (N/C) ratio of P53. Lower panel: total cellular P53 fluorescence intensity. Cells were treated with RA at $0.5 \times IC_{50}$ and IC_{50} concentration or DOX (0.2 μ M). The plot represents individual data with median and interquartile range. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn’s post hoc test; significant differences relative to untreated controls ($p < 0.05$).

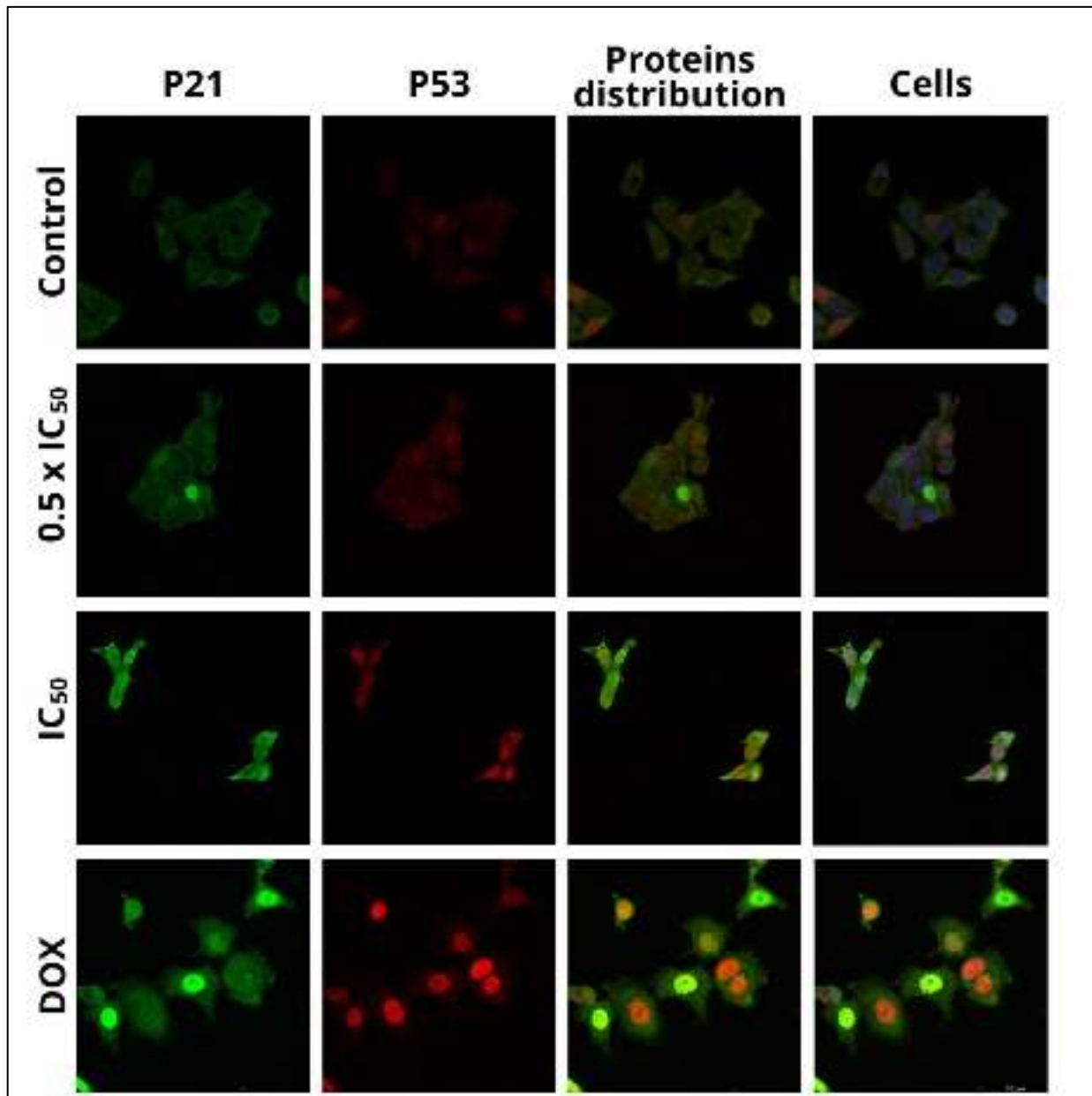


Figure 7. Representative immunofluorescence images showing the distribution and fluorescence intensity of P21 and P53 in DLD-1 cells following treatment with roburic acid (RA) at $0.5 \times \text{IC}_{50}$ and IC_{50} concentrations, as well as doxorubicin (DOX). Merged images illustrate protein colocalization, while the rightmost panels also include nuclear counterstaining with DAPI. Magnification 63 $\times$.

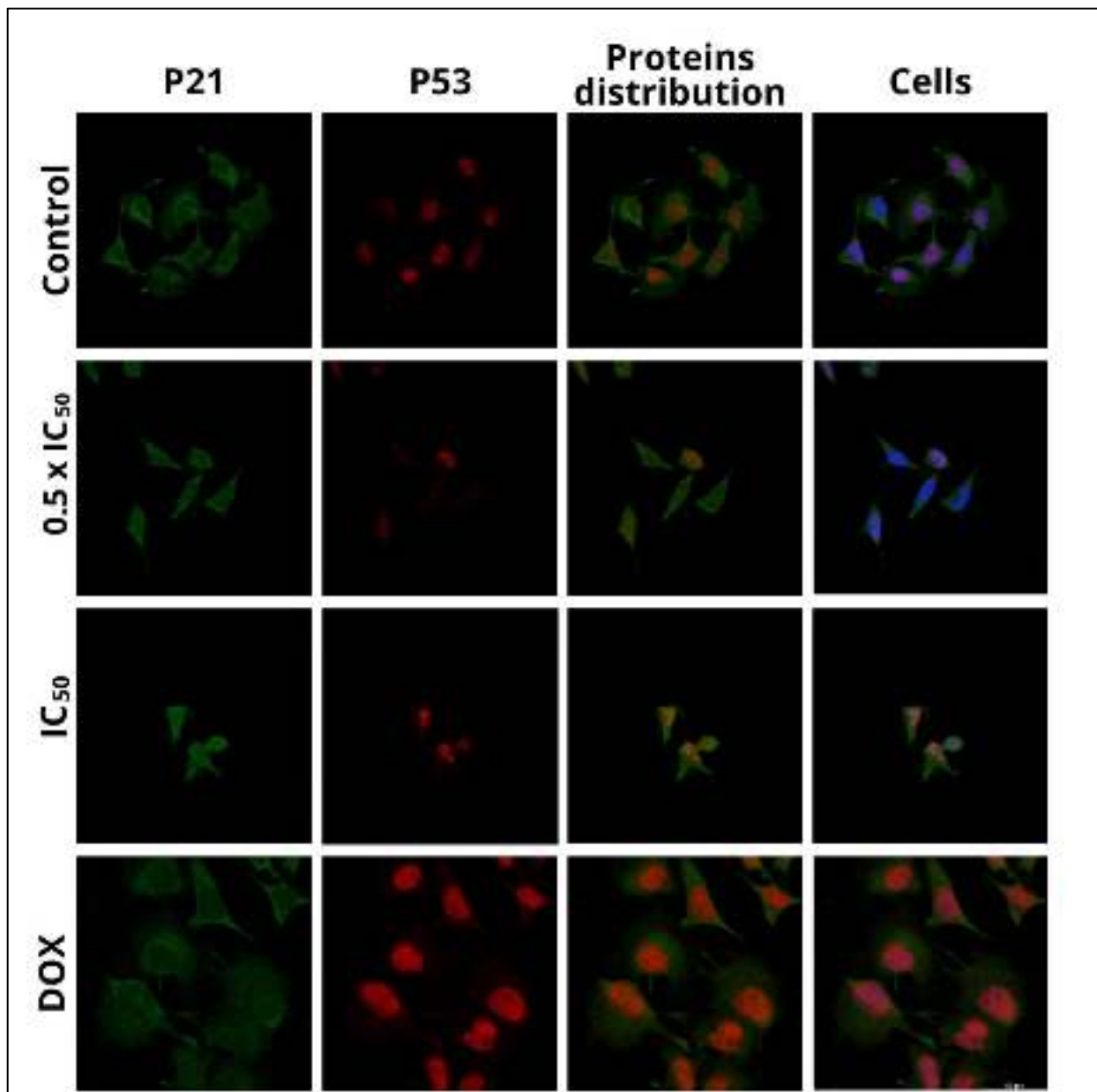


Figure 8. Representative immunofluorescence images showing the distribution and fluorescence intensity of P21 and P53 in HCT-116 cells following treatment with roburic acid (RA) at $0.5 \times \text{IC}_{50}$ and IC_{50} concentrations, as well as doxorubicin (DOX). Merged images illustrate protein colocalization, while the rightmost panels also include nuclear counterstaining with DAPI. Magnification 63 $\times$.

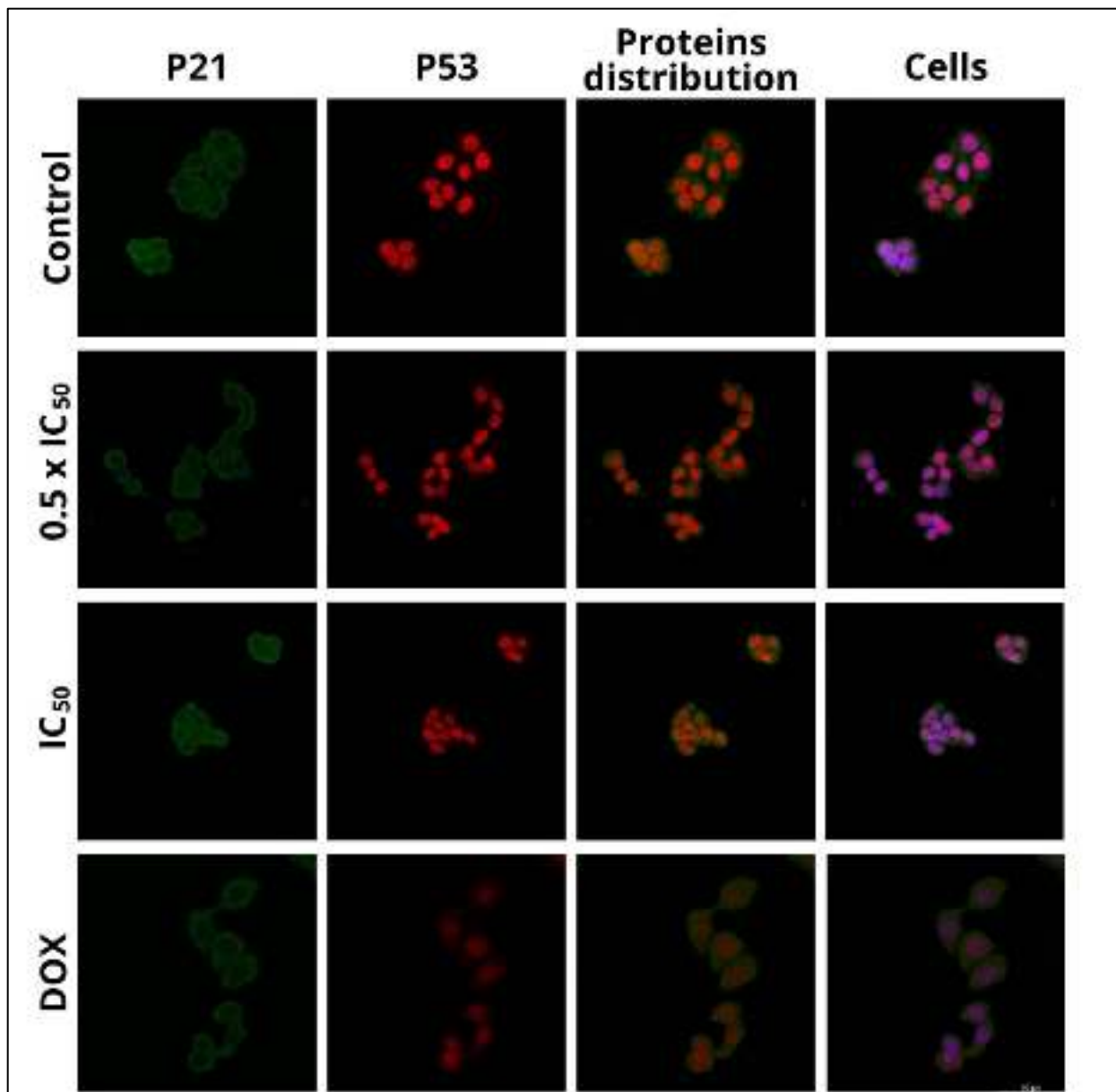


Figure 9. Representative immunofluorescence images showing the distribution and fluorescence intensity of P21 and P53 in HT-29 cells following treatment with roburic acid (RA) at $0.5 \times IC_{50}$ and IC_{50} concentrations, as well as doxorubicin (DOX). Merged images illustrate protein colocalization, while the rightmost panels also include nuclear counterstaining with DAPI. Magnification 63 $\times$.

Discussion

The present study builds upon our previous investigations into the biological activity of RA in CRC models, which were selected based on their high responsiveness to RA observed in earlier experiments. In our prior work, RA was shown to exert a pronounced antiproliferative effect characterized by reduced DNA synthesis, impaired clonogenic potential, and cell cycle arrest at the G_0/G_1 phase, accompanied by decreased expression of proliferation markers such as Ki-67, without evidence of genotoxicity or oxidative stress (Gielecińska et al., 2026, 2025). Several observations pointed toward the potential involvement of apoptotic processes. These included a reduction in mitochondrial membrane potential and the emergence of a sub- G_1 population of cells, as well as, most

importantly, a significant increase in the fraction of apoptotic cells detected under selected conditions using Annexin V-FITC binding and dual acridine orange/ethidium bromide staining, particularly at higher concentrations and prolonged exposures. In contrast, trypan blue exclusion assays did not reveal an increase in dead cells, indicating that RA did not induce necrosis or other forms of cell death associated with membrane integrity loss (Kroemer et al., 2009; Strober, 2015). Moreover, RA was reported to interfere with TNF- α signaling by preventing its interaction with the receptor, further indicating its ability to modulate pathways associated with cell fate regulation (Gielecińska et al., 2025; Xu et al., 2022).

In light of the observed inconsistencies in apoptosis-related responses, the present study was designed to determine whether RA induces cell death systematically and to resolve these ambiguities. To this end, pharmacological inhibitors targeting apoptosis, necroptosis, and ferroptosis were employed to assess the functional contribution of these pathways to the RA-induced phenotype. In parallel, caspase-9 activation, a key mediator of the mitochondrial apoptotic pathway, was evaluated to further investigate the potential involvement of intrinsic apoptosis and to determine whether it could explain the previously observed mitochondrial alterations. Considering that growth inhibition may also result from non-lethal cellular outcomes, cellular senescence was additionally examined. Finally, given the central role of P21 and P53 in regulating cell fate decisions, their expression and subcellular localization were analyzed to gain deeper insight into the molecular mechanisms underlying RA action.

We first examined the potential induction of cellular senescence to anticancer effects of RA. Cellular senescence is a state of irreversible cell cycle arrest triggered by various stressors, including DNA damage, oxidative stress, oncogene activation, and therapeutic interventions (Princilly et al., 2023; Subedi et al., 2025). Unlike apoptotic or necrotic cells, senescent cells remain metabolically active and preserve membrane integrity while permanently losing their proliferative capacity (Sikora et al., 2018, 2016). This phenotype is associated with characteristic morphological and biochemical features, such as enlarged and flattened morphology, increased cytoplasmic granularity, enhanced lysosomal activity, and expression of SA- β -gal, a widely used marker that enables the identification of senescent cells due to its elevated activity in these cells (Santos-Sousa et al., 2025; Sikora et al., 2018, 2016, 2014; Subedi et al., 2025). Importantly, therapy-induced senescence has been widely described for several classes of anticancer agents, including DOX, cisplatin, and etoposide (Lee et al., 2019; Milczarek, 2020; Mosieniak et al., 2015; Saleh et al., 2020; Subedi et al., 2025; Uruski et al., 2021; Wang et al., 2021). Despite the well-established ability of many anticancer agents to induce senescence, RA did not promote this phenotype under the tested conditions. No significant increase in the fraction of SA- β -gal-positive cells was observed in any of the examined CRC cell lines following RA treatment. In contrast doxorubicin, used in our experiment as a positive control, effectively induced senescence of CRC cells. This interpretation is further supported by the observation that RA-treated cells retain the capacity to resume proliferation following drug withdrawal, albeit at a slightly reduced rate, suggesting a reversible growth arrest rather than a permanent loss of proliferative capacity. Such behavior clearly distinguishes RA activity from classical senescence-inducing chemotherapeutics. It should be noted that long-term culture conditions required for senescence assessment may influence baseline levels of SA- β -gal-positive cells. Certain cell lines, such as HT-29, tend to grow in dense clusters and may reach high confluency over extended culture periods, which can promote senescence-like features independent of treatment. This may partially explain the relatively elevated baseline levels observed in control samples and highlights the importance of careful interpretation of senescence assays under prolonged culture conditions.

Having excluded cellular senescence, we next investigated whether RCD pathways contribute to the cellular response to RA. To this end, we examined the involvement of major forms of RCD, including apoptosis, necroptosis, and ferroptosis, using well-established pharmacological inhibitors. These

pathways represent three of the best-characterized RCD modalities in cancer biology, each defined by distinct molecular mechanisms and biochemical hallmarks (Galluzzi et al., 2016; Gielecińska et al., 2023; Kciuk et al., 2024; Kroemer et al., 2009). Notably, co-treatment with these inhibitors did not significantly restore cellular metabolic activity compared to RA treatment alone, indicating that the reduction observed at IC₅₀ concentration of the compound is unlikely to be primarily mediated by classical cell death pathways. This lack of protective effect was consistent across all tested inhibitors, suggesting that neither apoptosis, necroptosis, nor ferroptosis represents a dominant mechanism underlying RA-induced growth inhibition. In particular, the absence of a response to the pan-caspase inhibitor Q-VD-OPh argues against a major contribution of caspase-dependent apoptosis, while the lack of effect of necrostatin-1 and ferrostatin-1 excludes the involvement of necroptotic and ferroptotic pathways, respectively. In our earlier work, we observed an increase in Annexin V-positive cells under selected conditions, particularly at higher concentrations and prolonged exposure, while the overall apoptotic fraction remained limited (Gielecińska et al., 2026). These observations do not contradict our previous findings but rather refine their interpretation, indicating that apoptotic responses to RA are highly dependent on experimental context. Apoptotic features following RA treatment have also been reported by other groups. Xu et al. described pronounced apoptosis at relatively low RA concentrations (Xu et al., 2022). However, as we discussed previously, their experiments were conducted under serum-deprived conditions, which are known to sensitize cells to stress and lower the threshold for apoptotic activation (Gielecińska et al., 2026). Under such conditions, cancer cells are more prone to undergo apoptosis even in response to stimuli that would otherwise elicit non-lethal outcomes in standard culture environments. Beyond serum availability, differences in baseline cellular stress, metabolic state, and cell line intrinsic properties may further influence responsiveness to RA. These factors can shift the balance between adaptive and death-inducing pathways, thereby modulating the extent of apoptotic signaling. Accordingly, rather than reflecting a fundamental mechanistic discrepancy, these differences are more likely attributable to context-dependent variability in cellular responses, highlighting that RA-induced apoptosis may occur only when cellular stress exceeds a critical threshold.

Given the observed reduction in mitochondrial membrane potential and the presence of apoptotic features under selected conditions, we next focused on caspase-9 activation as a key initiator of the intrinsic apoptotic pathway. This approach was guided by the central role of mitochondrial dysfunction in intrinsic apoptosis, where mitochondrial outer membrane permeabilization leads to cytochrome c release and subsequent caspase-9 activation (Gielecińska et al., 2023). Consistent with this rationale, RA treatment at IC₅₀ resulted in a moderate but significant increase in caspase-9 activity in HCT-116 and HT-29 cells, but not in DLD-1 cells. However, the magnitude of this activation was markedly lower than that induced by DOX, suggesting that although mitochondrial apoptotic signaling is engaged, it does not lead to robust apoptotic execution under these conditions. Caspase-8 activation was not specifically assessed, as our data did not indicate a prominent contribution to the extrinsic apoptotic pathway. Activation of this pathway is typically mediated by death receptors, including tumor necrosis factor receptor 1 (TNFR1), and depends on extracellular signaling cues. Notably, RA has been reported to modulate TNF α -TNFR1 signaling, which may influence the activation threshold of receptor-mediated apoptosis (Gielecińska et al., 2025; Xu et al., 2022). While this axis was not directly investigated in the present study, such modulation could potentially limit engagement of the extrinsic pathway. Importantly, despite the observed increase in caspase-9 activity, our data did not indicate a substantial induction of apoptosis under the tested conditions. It raises the question of how caspase activation should be interpreted in this context. One possible explanation is that the detected increase reflects a partial or transient activation of the intrinsic apoptotic machinery that does not reach the threshold required for full apoptotic execution. Alternatively, caspase-9 activation may occur in a non-lethal context, as caspases have been implicated in processes beyond cell death, including cellular remodeling and stress responses (Hollville and Deshmukh, 2018; Shalini et al., 2015). It is also conceivable that the observed signal arises from a subpopulation of cells

undergoing apoptosis, which remains insufficient to affect overall population-level readouts significantly. Taken together, these considerations suggest that while RA can engage components of the intrinsic apoptotic machinery, caspase-9 activation alone does not necessarily translate into effective apoptotic cell death in this model.

To further elucidate the mechanisms underlying RA's cytostatic effects, we analyzed the expression and subcellular localization of P53 and P21, two key regulators of cell cycle arrest and stress responses. The tumor suppressor P53 plays a central role in coordinating cellular responses to stress, including DNA damage and oncogenic signaling, by regulating transcription of target genes involved in cell cycle arrest, apoptosis, and senescence. Among its downstream effectors, P21 (cyclin-dependent kinase inhibitor 1A; *CDKN1A*) is a well-established mediator of cell cycle inhibition, primarily by blocking cyclin-dependent kinases and enforcing G₁/S arrest (Huang et al., 2026; Kciuk et al., 2022; Zheng et al., 2025). In our previous study, we examined changes in the expression of genes encoding these proteins (Gielecińska et al., 2026). Here, we extended this analysis by focusing directly on protein-associated fluorescence and subcellular distribution, allowing us to assess not only their abundance but also their localization, which critically determines their functional output. While nuclear localization is generally associated with transcriptional activity and cell cycle arrest, cytoplasmic localization, particularly in the case of P21, has been linked to non-canonical functions, including modulation of apoptosis and cell survival (Huang et al., 2014; Karimian et al., 2016). Therefore, in addition to total protein-associated fluorescence, we quantified the N/C ratio to capture potential shifts in protein distribution that may reflect functional changes. Importantly, HCT-116 cells, which harbor wild-type tumor antigen P53 (*TP53*), served as a functional reference model for intact P53 signaling, allowing us to interpret RA-induced effects in the context of a competent P53 pathway. In contrast, DLD-1 and HT-29 cells carry mutant *TP53*, providing a comparative framework to assess how P53 status influences the cellular response to RA.

The interpretation of these results proved particularly challenging, reflecting the complex, cell-line-dependent alterations in both protein localization and overall fluorescence intensity. Notably, changes in subcellular distribution were more consistent than variations in total fluorescence intensity, suggesting that RA primarily affects protein relocalization rather than expression levels. For P21, RA treatment induced a redistribution toward the cytoplasmic compartment in DLD-1 and HCT-116 cells, whereas in HT-29 cells, the opposite pattern was observed, with increased nuclear accumulation. This divergence indicates that the functional outcome of P21 regulation by RA is strongly context dependent. Importantly, cytoplasmic localization of P21 has been associated with anti-apoptotic functions and may contribute to cell survival despite growth inhibition, whereas nuclear P21 is typically linked to cell cycle arrest (Asada et al., 1999; Karimian et al., 2016; Qiu et al., 2011). In the case of P53, RA-induced effects were also cell line-dependent and appeared to correlate with *TP53* status. In HCT-116 cells, which harbor wild-type *TP53*, RA treatment at IC₅₀ resulted in a significant decrease in nuclear localization, indicating shift toward the cytoplasmic compartment. A similar pattern was observed in DLD-1 cells, which carry mutant *TP53*. In HT-29 cells, all treatments led to a consistent reduction in nuclear localization, suggesting impaired transcriptional activity. These findings are consistent with the notion that mutant P53 proteins often exhibit altered subcellular dynamics and may fail to properly regulate downstream targets (Wang et al., 2022; Xu et al., 2008).

Based on our previous transcriptomic data, it is evident that RA consistently induces *CDKN1A* expression across all tested cell lines, with distinct temporal dynamics depending on *TP53* status. In HCT-116 cells, *CDKN1A* upregulation was rapid and sustained, whereas in DLD-1 and HT-29 cells, induction became more evident following prolonged exposure. This observation supports the notion that P21 regulation can occur through both *TP53*-dependent and -independent mechanisms, allowing effective checkpoint control even in cells with compromised P53 functions. However, the lack of a direct correlation between *CDKN1A* transcription and P21 protein localization observed here

highlights the importance of post-transcriptional and post-translational regulation. In particular, the cytoplasmic redistribution of P21 in DLD-1 and HCT-116 cells suggests that increased transcription does not necessarily translate into enhanced nuclear activity but may instead reflect altered functional compartmentalization of the protein. In HCT-116 cells, which retain functional *TP53*, RA treatment was associated with sustained *CDKN1A* induction despite a reduction in nuclear P53 localization. This suggests the activation of the P53–P21 axis may occur without pronounced nuclear accumulation of P53, potentially reflecting transient or non-canonical regulation of this pathway. Notably, similar responses have been reported in this model, in which activation of the P53–P21 pathway preferentially leads to cell cycle arrest rather than apoptosis, particularly under moderate cellular stress (Mosieniak et al., 2015). This is consistent with our observations, in which RA-induced signaling engages components of the stress response machinery without triggering robust cell death. Taken together, these findings indicate that RA modulates the P53–P21 axis at multiple regulatory levels, including transcription, protein localization, and functional activity. Rather than inducing a uniform response, RA appears to reprogram cellular behavior in a context-dependent manner, favoring cytostatic outcomes over apoptotic cell death (Fig. 10).

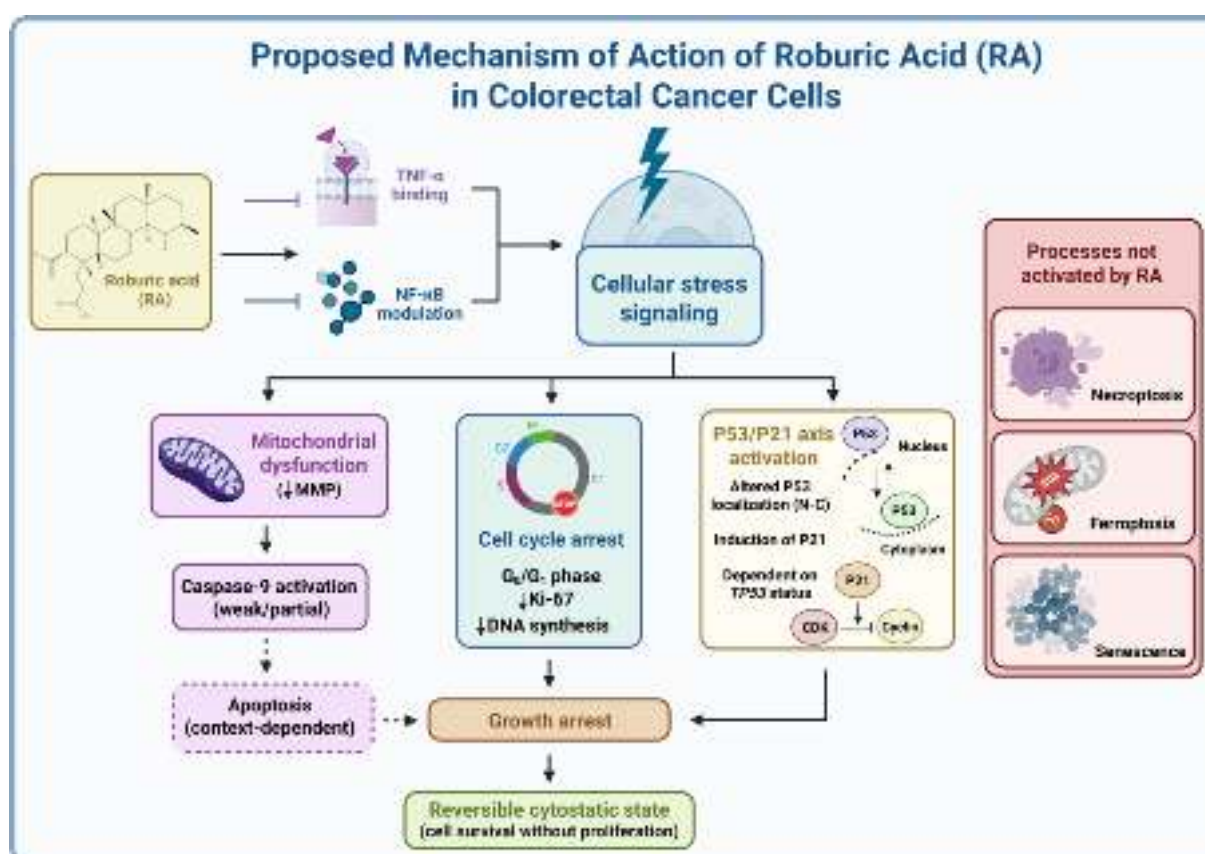


Figure 10. Proposed mechanism of action of roburic acid (RA) in colorectal cancer cells. RA induces a cytostatic response through modulation of cellular stress signaling, potentially involving interaction with tumor necrosis factor- α (TNF- α) and regulation of nuclear factor kappa B (NF- κ B)-associated pathways. These upstream effects lead to cell cycle arrest, mitochondrial dysfunction, and alterations in P53/P21 signaling. Although partial activation of caspase-9 and apoptotic features may occur, apoptosis appears to be limited and context-dependent. Notably, necroptosis, ferroptosis, and senescence are not significantly involved. Overall, RA promotes a reversible cytostatic state rather than irreversible cell death.

Materials and methods

Chemicals

Cell culture reagents: Fetal bovine serum (FBS), phosphate-buffered saline (PBS), and RPMI-1640 culture medium were obtained from Biowest (CytoGen, Zgierz, Poland). Stabilized penicillin-streptomycin solution and trypsin-ethylenediamine tetraacetic acid (EDTA) were purchased from Merck/Sigma Aldrich Chemical Co. (Burlington, MA, USA). Tissue culture plates (12-, 24-, and 96-well, including clear-bottom plates) were obtained from Nest Biotechnology (Wuxi, China), while white 96-well plates for luminescence assays were purchased from Greiner Bio-One (Kremsmünster, Austria). Roburic acid (RA), doxorubicin (DOX), and the inhibitors Q-VD-OPh (pan-caspase inhibitor), necrostatin-1, and ferrostatin-1 were obtained from MedChemExpress (Monmouth Junction, NJ, USA). For senescence assays, 16% formaldehyde was purchased from Thermo Fisher Scientific (Waltham, MA, USA), while 8% glutaraldehyde was obtained from Polysciences Inc. (Warrington, PA, USA). Sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate dodecahydrate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$), and potassium ferrocyanide ($\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$) were purchased from Warchem Sp. z o.o. (Zakręt, Poland), whereas potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$) was obtained from Sigma Aldrich Chemical Co. Sodium chloride and magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) were supplied by Chempur (Piekary Śląskie, Poland), and X-gal was purchased from Invitrogen (by Thermo Fisher Scientific, Waltham, USA). For immunofluorescence assays, Triton X-100, Tween 20, and bovine serum albumin (BSA) were obtained from Merck/Sigma-Aldrich (Burlington, MA, USA). Fluoromount-G™ Mounting Medium with DAPI was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Cell Culture

Human CRC cell lines DLD-1 (adenocarcinoma; ATCC® CCL-221™), HCT-116 (adenocarcinoma; ATCC® CCL-247™), and HT-29 (carcinoma; ATCC® HTB-38™) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin–streptomycin. Cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 . The medium was refreshed every 24–48 hours, and cells were passaged at approximately 80–90% confluence using 0.25% trypsin-EDTA. Mycoplasma contamination was routinely assessed monthly using the MycoBlue™ Mycoplasma Detector kit (Vazyme Biotech, Nanjing, China).

Cell Treatment

The concentrations of RA used in this study were determined based on previously reported cytotoxic and antiproliferative effects assessed by MTT, crystal violet, and neutral red assays. The working concentrations were established using consensus IC_{50} values obtained from these methods. Depending on the experimental setup, cells were exposed to RA at $0.5 \times \text{IC}_{50}$ or IC_{50} . Detailed concentrations applied in each assay are provided in Table 1.

Table 1. Concentrations of roburic acid (RA) used in the individual experimental assays.

Concentration [μM]	Cell line		
	DLD-1	HCT-116	HT-29
$0.5 \times \text{IC}_{50}$	8.12	10.17	11.2
IC_{50}	16.23	20.33	22.39
$2 \times \text{IC}_{50}$	32.47	40.66	44.78

SA-β-Gal Staining

Cellular senescence was assessed using SA-β-Gal staining according to the protocols described by Mosieniak et al. and Dimri et al., with minor modifications (Dimri et al., 1995; Mosieniak et al., 2015). Briefly, DLD-1, HCT-116, and HT-29 cells were seeded at a density of 18,000 cells/well in 24-well plates and allowed to attach overnight. The following day, cells were treated with RA at concentrations corresponding to $0.5 \times IC_{50}$ and IC_{50} , or with DOX (100 nM) as a positive control, for 24 hours. After treatment, the medium was removed, cells were washed with PBS, and cultured in fresh drug-free medium for an additional 7 days. At the end of the culture period, cells were washed with PBS and fixed in 2% formaldehyde/0.2% glutaraldehyde for 3 min at room temperature. After washing, cells were incubated with SA-β-Gal staining solution (pH 6.0) containing 1 mg/mL X-gal, 40 mM citric acid/sodium phosphate buffer, 150 mM NaCl, 2 mM $MgCl_2$, 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide. Staining was performed at 37°C in a CO₂-free environment for 12–16 hours. Senescent cells were identified by the presence of blue-green staining in the cytoplasm. At least 300 cells per condition were counted in randomly selected fields, and the percentage of SA-β-Gal-positive cells was calculated. Images were acquired using a Leica MICA imaging system equipped with 20 × 0.5 Plan Fluotar objective (Leica Microsystems, Wetzlar, Germany). Cell analysis was performed using Leica LAS X Office version 1.4.9 (Leica Microsystems, Wetzlar, Germany).

Pharmacological inhibition of cell death pathways

To evaluate the potential involvement of distinct cell death pathways in the cellular response to RA, co-treatment experiments were performed using the inhibitors of apoptosis, necroptosis, or ferroptosis. DLD-1, HCT-116, and HT-29 cells were seeded in 96-well plates at a density of 10,000 cells/well and allowed to adhere for 24 hours. Cells were subsequently treated with RA at the previously determined IC_{50} concentration, either alone or in combination with the pan-caspase inhibitor Q-VD-OPh (10 μM), necrostatin-1 (30 μM), or ferrostatin-1 (0.5 μM). Control groups included untreated cells and cells treated with each inhibitor alone. The selected concentrations were based on commonly reported literature ranges for effective inhibition of the respective cell death pathways (Ito et al., 2017; Tariq et al., 2025; Vechalapu et al., 2024). Following 24 hours of incubation, cellular metabolic activity was assessed using the MTT assay as described in our earlier study (Gielecińska et al., 2025). Absorbance was measured at 570 nm using a microplate reader (Power Wave XS, BioTek Instruments, Winooski, VT, USA). The results were expressed as a percentage relative to the untreated control (set as 100%).

Caspase-9 activity assay

Caspase-9 activation was assessed using the Caspase-Glo® 9 Assay System (Promega; Lot No. 0005909111) according to the manufacturer's instructions. DLD-1, HCT-116, and HT-29 cells were seeded in white opaque 96-well plates at a density of 10,000 cells/well in 100 μL of complete medium and allowed to adhere for 24 hours. Cells were then treated with RA at $0.5 \times IC_{50}$ and IC_{50} concentrations. DOX (0.2 μM) was used as a positive control. After 24 hours of treatment, an equal volume of Caspase-Glo® 9 reagent (1:1, v/v) was added to each well, and the plates were incubated at room temperature in the dark for 30–60 min. Luminescence was measured using a SpectraMax® i3x Multi-Mode Detection Platform (Molecular Devices, San Jose, CA, USA). To account for differences in baseline luminescence between cell lines, results were normalized to untreated controls and expressed as a relative luminescence ratio (the control set at 1.0). Data are presented as mean ± SD from two independent experiments.

P21 and P53 Immunofluorescence Assay

P21 and P53 immunofluorescence staining was performed according to our previously described protocol with minor modifications (Gielecińska et al., 2026). Briefly, cells were seeded onto glass coverslips placed in 12-well plates at a density of 30,000 cells/well in 1 mL of complete medium and allowed to adhere for 24 hours. Cells were then treated with RA at $0.5 \times IC_{50}$ and IC_{50} concentrations, or with DOX (0.2 μM) as a positive control, for 24 hours. Following treatment, cells were washed with PBS and fixed in 70% ethanol at $-20^{\circ}C$. After washing, cells were permeabilized with 0.5% Triton X-100 in PBS and blocked in PBS containing 3–5% BSA and 0.1% Tween 20. Samples were incubated for 1 hour with a rabbit anti-P21 polyclonal antibody (Proteintech, cat. no. 10355-1-AP, 1:400 dilution) and a mouse anti-P53 monoclonal antibody (Proteintech, cat. no. 60283-2-Ig, 1:800 dilution) diluted in PBS supplemented with 1% bovine serum albumin and 0.5% Tween-20. This was followed by a 1-hour incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Thermo Fisher, A-11008; 1:500 dilution) and CoraLite594-conjugated goat anti-mouse IgG secondary antibody (Proteintech, cat. no. SA00013-3, 1:500 dilution). Coverslips were mounted using Fluoromount-G medium containing 4,6-diamidino-2-phenylindole (DAPI; 1 $\mu g/mL$) for nuclear counterstaining. Fluorescence images were acquired using a Leica TCS SP8 confocal laser scanning microscope, equipped with a $63 \times /1.40$ HC PL APO CS2 objective (Leica Microsystems, Wetzlar, Germany). Cell analysis was performed using Leica LAS X Office. For each experimental condition, at least 200 cells were analyzed. Quantitative analysis was performed using Leica imaging software by measuring the region of interest (ROI) area and fluorescence intensity (intensity sum) in nuclear and cytoplasmic compartments. Nuclear regions were defined based on DAPI staining. The N/C ratio was calculated as the mean fluorescence intensity in the nucleus divided by the mean fluorescence intensity in the cytoplasm, where the cytoplasmic signal was obtained by subtracting the nuclear signal from the total cellular signal. In addition, total cellular fluorescence intensity was quantified for each cell by measuring the fluorescence signal within the entire cell area, allowing assessment of overall protein-associated fluorescence independently of subcellular localization. Data are presented as median values and expressed relative to untreated controls.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA) and Statistica version 13.3 (TIBCO Software Inc., Palo Alto, CA, USA). Data distribution normality was evaluated using the Shapiro–Wilk test. Data obtained from the SA- β -Gal staining and P21 and P53 immunofluorescence assays are presented as medians with interquartile ranges, whereas results from the pharmacological inhibition of cell death pathways and the caspase-9 activity assay are expressed as means $\pm$ standard deviation (SD). For comparisons involving more than two groups, one-way analysis of variance (ANOVA) followed by Dunnett's or Sidak's multiple comparisons test was applied. When data did not follow a normal distribution, the Kruskal–Wallis test with Dunn's post hoc test was used. A p -value of less than 0.05 was considered statistically significant.

Conclusions

In conclusion, the present study demonstrates that RA exerts a pronounced antiproliferative effect in CRC cells, which is not primarily mediated by classical cell death pathways or by the induction of cellular senescence. Instead, RA induces a predominantly cytostatic response characterized by reversible cell cycle arrest and modulation of key regulatory proteins. Although elements of apoptotic

signaling, including mitochondrial dysfunction and caspase-9 activation, were observed, these responses remained limited and did not translate into effective apoptotic cell death. Mechanistically, RA was found to modulate the P53–P21 axis at multiple levels, including transcription, protein localization, and functional activity. Importantly, alterations in subcellular distribution, particularly the cytoplasmic relocation of P21, suggest that RA influences not only protein abundance but also their functional compartmentalization. The observed effects were strongly dependent on *TP53* status, highlighting the importance of cellular context in determining treatment response. Overall, our findings indicate that RA primarily acts as a regulator of proliferative capacity, promoting adaptive cellular responses that limit cell cycle progression without inducing widespread cell death.

Supplementary Materials

The supporting information can be downloaded at.....

Author Contributions

Conceptualization, A.G., S.W., B.M., M.K., A.W., and R.K.; collected literature, A.G.; wrote the manuscript, A.G. and S.W.; supervised project and proofread the paper, A.G., S.W., B.M., M.K., A.W., and R.K. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

The data presented in this study are available in the main text of this article, the Supplementary Materials, or by request from the corresponding author.

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Conflicts of Interest

The authors declare no conflicts of interest.

Abbreviations

BSA	bovine serum albumin
CDKN1A	cyclin-dependent kinase inhibitor 1A
CRC	colorectal cancer
DAPI	4,6-diamidino-2-phenylindole
DOX	doxorubicin
EDTA	ethylenediamine tetraacetic acid
FBS	fetal bovine serum
N/C	nuclear-to-cytoplasmic ratio

NF-κB	nuclear factor kappa B
PBS	phosphate-buffered saline
RA	roburic acid
RCD	regulated cell death
ROI	region of interest
SA-β-Gal	senescence-associated β-galactosidase
TNF-α	tumor necrosis factor-alpha
TNFR1	tumor necrosis factor receptor 1
TP53	tumor antigen P53
TRIS	tris(hydroxymethyl)aminomethane

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Supplementary Information

Revisiting Apoptosis: What Lies Behind the Antiproliferative Activity of Roburic Acid in Colorectal Cancer Cells?

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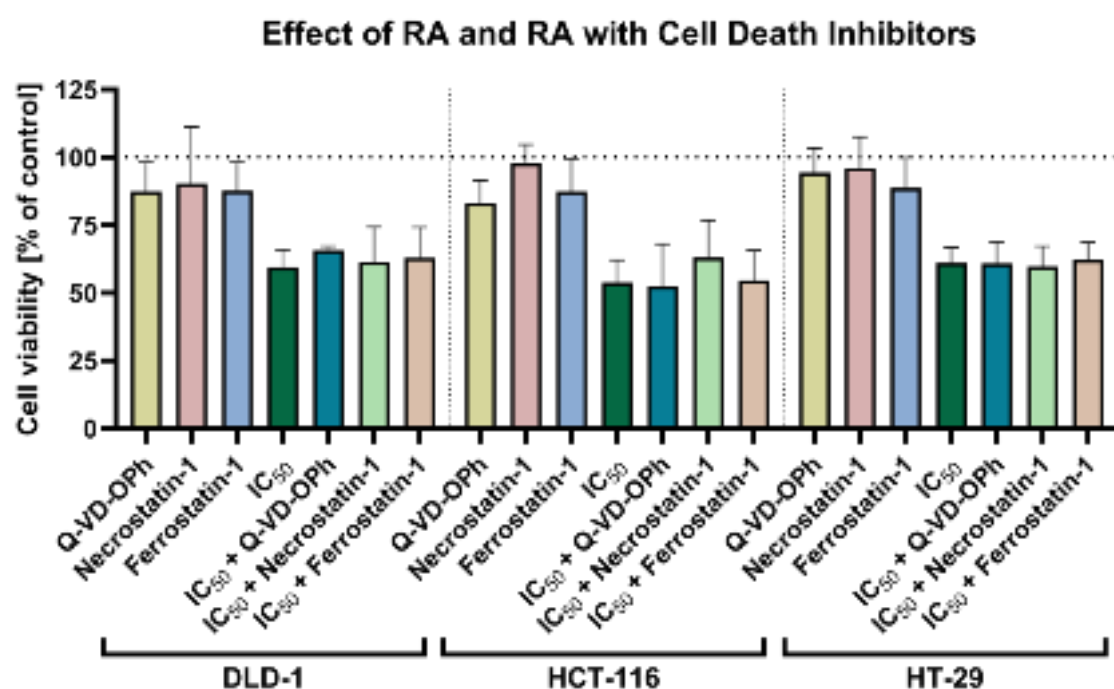
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Supplementary Figure S1. Extended analysis of the effects of RA and cell death inhibitors on cellular metabolic activity. Cells (DLD-1, HCT116, and HT-29) were treated with RA at IC₅₀ alone or in combination with inhibitors of apoptosis (Q-VD-OPh), necroptosis (necrostatin-1), or ferroptosis (ferrostatin-1), as well as with inhibitors alone, for 24 hours. Cellular metabolic activity was assessed using an MTT assay and expressed as relative cell viability, normalized to untreated control (set as 100%). Data are presented as mean ± SD from three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Sidak's multiple comparisons test.