lek. Piotr Strus

Pochodne podofilotoksyny i benzotiazolu jako leki przeciwnowotworowe – optymalizacja struktury i badanie mechanizmu działania

Rozprawa na stopień doktora nauk medycznych i nauk o zdrowiu w dyscyplinie nauki medyczne

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1. **Strus P**, Lisiecki K, Czarnocki Z, Młynarczuk-Biały I, Biały Ł. Novel podophyllotoxin derivatives as Anticancer Agents: Design, Synthesis, and Biological Screening. W: Advances in Biomedical Research - selected topics. Wydawnictwo Naukowe TYGIEL sp. z o. o. ISBN: 978-83-65932-55-6. 2018:48-61

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2. **Strus P**, Borensztejn K, Szczepankiewicz AA, Lisiecki K, Czarnocki Z, Nieznanska H, Wojcik C, Bialy LP, Mlynarczuk-Bialy I. Novel podophyllotoxin and benzothiazole derivative induces transitional morphological and functional changes in HaCaT cells. Toxicol In Vitro. 2021 Jun;73:105144. doi: 10.1016/j.tiv.2021.105144. Epub 2021 Mar 13. PMID: 33722735.

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   IF: 3,685

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3. **Strus P**, Sadowski K, Kostro J, Szczepankiewicz AA, Nieznańska H, Niedzielska M, Zlobin A, Nawar Ra'idah P, Molęda Z, Szawkało J, Czarnocki Z, Wójcik C, Szeleszczuk Ł, Młynarczuk-Biały I. Cellular Distribution and Ultrastructural Changes in HaCaT Cells, Induced by Podophyllotoxin and Its Novel Fluorescent Derivative, Supported by the Molecular Docking Studies. Int J Mol Sci. 2024 May 29;25(11):5948. doi: 10.3390/ijms25115948. PMID: 38892135; PMCID: PMC11172492.

   Rodzaj publikacji: praca oryginalna

   IF: 4,900

   MNiSW: 140

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4. **Strus P,** Sadowski K, Ploch W, Jazdzewska A, Oknianska P, Raniszewska O, Mlynarczuk-Bialy I. The Effects of Podophyllotoxin Derivatives on Noncancerous Diseases: A Systematic Review. Int J Mol Sci. 2025 Jan 23;26(3):958. doi: 10.3390/ijms26030958. PMID: 39940726; PMCID: PMC11816842.

   Rodzaj publikacji: praca poglądowa

   IF: 4,900

   MNiSW: 140

   Liczba cytowań: 0

	Suma IF	**Suma MNiSW**	**Liczba cytowań**
Publikacje wchodzące w skład doktoratu	13, 485	380	16
Wszystkie publikacje doktoranta	16,875	520	130

Spis treści

Wykaz stosowanych skrótów

PPT – Podofilotoksyna

HaCaT – nienowotworogenna, unieśmiertelniona linia komórkowa keratynocytów człowieka

HeLa – linia ludzkich komórek raka szyjki macicy

MDA-MB-231 – linia ludzkich komórek raka piersi (Human breast cancer cell line)

MCF-7 – linia ludzkich komórek raka piersi (Human breast cancer cell line)

PC-3 – linia ludzkich komórek raka prostaty (Human prostate cancer cell line)

DU-145 – linia ludzkich komórek raka prostaty (Human prostate cancer cell line)

CFPAC-1 – linia ludzkich komórek raka trzustki (Human pancreatic cancer cell line)

NIH-3T3 – linia mysich fibroblastów (Mouse embryonic fibroblast cell line)

KL3 – 3-(4-((3,4,5-trimetoksyfenylo)amino)benzotiazol-2-ylo)-4-(3,4,5-trimetoksyfenylo) furan-2(5H)-on

IC50 – stężenie, przy którym wzrost komórek jest hamowany o 50% w stosunku do kontroli (Half maximal inhibitory concentration)

MTT – test MTT (3-(4,5-dimetylo-2-tiazolilo)-2,5-difenylo-tetrazoliowy bromek) - metoda oceny żywotności komórek

DNA – kwas deoksyrybonukleinowy

RNA – kwas rybonukleinowy

ATP – adenozynotrójfosforan

EDTA – kwas etylenodiaminotetraoctowy

DMSO – dimetylosulfotlenek

PBS – fosforanowy roztwór soli

SDS – siarczan dodecylu sodu

HCl – kwas solny

NaOH – wodorotlenek sodu

FBS – płodowa surowica bydlęca

DMEM – pożywka do komórek Dulbecco's Modified Eagle Medium

RPMI 1640 – pożywka do komórek RPMI 1640

Annexin V – aneksyna V

PI – jodek propidyny

FITC – izotiocyjanian fluoresceiny

PE – fikoerytryna

APC – allofikocyjanina

WB – Western blotting

RT-qPCR – reakcja łańcuchowa polimerazy z odwrotną transkrypcją w czasie rzeczywistym

SEM – skaningowa mikroskopia elektronowa

TEM – transmisyjna mikroskopia elektronowa

Streszczenie w języku polskim

W niniejszej pracy doktorskiej przedstawiono cykl czterech publikacji dotyczących projektowania, syntezy i badań aktywności przeciwnowotworowej nowych pochodnych podofilotoksyny i benzotiazolu z analizą mechanizmu działania zarówno macierzystej substancji, jak i zsyntetyzowanych przez nasz zespół nowych pochodnych.

Podofilotoksyna (PPT) jest związkiem pochodzenia roślinnego o silnych właściwościach przeciwnowotworowych, jednak jej zastosowanie kliniczne jest ograniczone ze względu na wysoką toksyczność. Już od połowy zeszłego wieku PPT była eksperymentalnie używana do leczenia nowotworów, między innymi nosa, gardła i krtani związanych z HPV. Mimo obiecującej skuteczności, ze względu na dużą toksyczność, w wielu przypadkach nie wydano rekomendacji. Obecnie jest stosowana wyłącznie miejscowo w leczeniu kłykcin kończystych odbytu i narządów płciowych.

PPT służy jednak jako rusztowanie dla rozwoju mniej toksycznych i bardziej skutecznych substancji do ogólnoustrojowej terapii przeciwnowotworowej. Przykłady obejmują leki przeciwnowotworowe, takie jak etopozyd i tenipozyd, które są pochodnymi PPT i weszły do codziennego użytku w onkologii.

Celem badań było uzyskanie nowych pochodnych o lepszym profilu terapeutycznym, a zatem wyższej skuteczności przeciwnowotworowej i niższej toksyczności w kierunku zdrowych komórek, a także poznanie mechanizmu działania nowych leków oraz porównanie efektów ich działania do PPT w modelu komórkowym.

W ramach pracy zsyntetyzowano serię nowych związków KL1, KL2 i KL3, które następnie przebadano *in vitro* na różnych liniach komórkowych, w tym: w liniach komórek nienowotworowych HaCaT, NIH 3T3 oraz w liniach komórek nowotworowych: HeLa, MDA-MB-231, MCF-7, PC-3, DU-145, CFPAC-1. Najbardziej obiecującą pochodną jest KL3, która jest połączeniem PPT i benzotiazolu. W naszych badaniach związek KL3 wykazał działanie przeciwnowotworowe równe lub bardziej efektywne niż PPT i był mniej toksyczny dla komórek nienowotworowych niż macierzysta PPT. Indeks selektywności dla większości badanych linii komórkowych wykazał, że KL3 jest bardziej selektywny niż PPT. Przeprowadzono eksperymentalną analizę mechanizmu działania KL3 oraz PPT, z uwzględnieniem badania cyklu komórkowego, indukcji procesu apoptozy oraz analizy ultrastruktury komórek w transmisyjnej mikroskopii elektronowej.

Wyniki zostały opublikowane w trzech artykułach oryginalnych. Po analizie dostępnej bibliografii na temat zastosowań pochodnych PPT w chorobach innych niż nowotworowe, doktorant podjął się przygotowania przeglądu systematycznego w celu uzupełnienia luki w wiedzy na ten temat. W tym celu, wykorzystując wiedzę pozyskaną w szkole doktorskiej, utworzony został przegląd systematyczny w 2025 roku.

Abstract

„New derivatives of podophyllotoxin and benzothiazole as anticancer agents - structure optimization and mechanism of action study."

This dissertation presents a series of four publications on the design, synthesis and study of the anticancer activity of new podophyllotoxin and benzothiazole derivatives with an analysis of the mechanism of action of both the parent substance and the new derivatives synthesized by our team.

Podophyllotoxin (PPT) is a plant-derived compound with potent anticancer properties, but its clinical use is limited due to its high toxicity. Since the middle of the last century, PPT has been used experimentally to treat HPV-related cancers of the nose, throat and larynx, among others. Despite its promising efficacy, due to its high toxicity, it has not been recommended in many cases. Currently, it is only used topically to treat anal and genital condylomas.

However, PPT serves as a scaffold for the development of less toxic and more effective substances for systemic anticancer therapy. Examples include anticancer drugs such as etoposide and teniposide, which are derivatives of PPT and have entered everyday use in oncology.

The aim of the study was to obtain new derivatives with a better therapeutic profile and therefore higher anti-cancer efficacy and lower toxicity toward healthy cells, as well as to learn more about the mechanism of action of the new drugs and compare their effects to PPT in a cellular model.

In this work, a series of new compounds KL1, KL2, and KL3 were synthesized and tested *in vitro* on various cell lines, including: non-cancer cell lines HaCaT, NIH 3T3, and cancer cell lines: HeLa, MDA-MB-231, MCF-7, PC-3, DU-145, CFPAC-1. The most promising derivative is KL3, which is a combination of PPT and benzothiazole. In our study, compound KL3 showed antitumor activity equal to or more effective than PPT and was less toxic to non-cancer cells than the parent PPT. The selectivity index for most of the cell lines tested showed that KL3 was more selective than PPT. An experimental analysis of the mechanism of action of KL3 and PPT was carried out, including cell cycle assay, induction of apoptosis process and analysis of cell ultrastructure by transmission electron microscopy.

The results were published in three original articles. After analyzing the available bibliography on the applications of PPT derivatives in non-cancerous diseases, the doctoral student undertook the preparation of a systematic review to fill the gap in knowledge on this subject. To this end, and using the knowledge gained in doctoral school, a systematic review was created in 2025.

1. WSTĘP

1.1. Podofilotoksyna i zarys historyczny

Choroby nowotworowe są, poza chorobami układu krążenia, drugą przyczyną skracającą oczekiwany czas życia pacjentów, szczególnie młodszych [1, 2]. We współczesnej onkologii opracowano wiele opcji terapii przeciwnowotworowej i udostępniono je komercyjnie. W wielu przypadkach pacjent nowotworowy wchodzi w wieloletnią remisję. Wciąż istnieją jednak przypadki, które źle odpowiadają na rutynowe opcje terapeutyczne, albo z powodu poważnych działań niepożądanych stosowanych terapii, albo z powodu progresji i niewrażliwości komórek nowotworowych [3-6]. Takie przypadki stanowią wyzwanie współczesnej onkologii, którego rozwiązań poszukuje się najpierw w badaniach podstawowych, w tym w modelu komórkowym i przedklinicznym. Nieustanny wysiłek współczesnych badań nad terapiami przeciwnowotworowymi jest napędzany dążeniem do osiągnięcia triady rozwoju leków: wysokiej skuteczności, przystępnej ceny i bezpieczeństwa [7-10]. Droga do idealnego leku przeciwnowotworowego jest jednak wypełniona wyzwaniami, gdyż komórki nowotworowe wciąż się zmieniają. Jedną z cząsteczek, która służy za szkielet do syntez chemicznych, jest PPT [11].

PPT to naturalnie występująca substancja. Jest lignanem typu aryltetraliny występującym w etanolowym ekstrakcie z kłącza *Podophyllum peltatum*, wieloletniej rośliny zielnej. Ten gatunek rośliny, określany również jako mandragora amerykańska lub mayapple i znany w Polsce jako „Stopkowiec tarczowaty", jest jednym z dwóch głównych źródeł PPT. Drugim powszechnie zidentyfikowanym źródłem jest *Sinopodophyllum hexandrum* (Royle), znany również jako *Podophyllum hexandrum* lub „Stopkowiec himalajski" w Polsce. Oba te gatunki należą do rodziny Berberidaceae, która szczyci się szerokim zasięgiem geograficznym w Ameryce Północnej i Azji [12-14].

Historyczna literatura naukowa obfituje w badania wyjaśniające potencjalne zastosowania PPT, ze wskazaniami obejmującymi różnorodne schorzenia, w tym dnę moczanową, gruźlicę, rzeżączkę, kiłę, zaburzenia miesiączkowania, puchlinę, kaszel, łuszczycę, brodawki weneryczne, a nawet nowotwory. Od lat pięćdziesiątych XX wieku była nawet stosowana jako potencjalny lek w chorobach gardła, krtani i nosa w laryngologii. Jednak kliniczna użyteczność PPT jest znacznie utrudniona przez jej nieodłączną toksyczność. W konsekwencji wiele z jej potencjalnych wskazań terapeutycznych musiało zostać usuniętych z oficjalnych wytycznych medycznych [11, 15-24].

Obecnie jedynym zatwierdzonym wskazaniem terapeutycznym dla PPT są kłykciny kończyste, charakteryzujące się brodawkami anogenitalnymi wywołanymi przez wirusa brodawczaka ludzkiego. Podawanie PPT jest ściśle miejscowe, a pacjentom odradza się stosowanie produktu na dużych powierzchniach ciała ze względu na ryzyko ogólnoustrojowych reakcji toksycznych [25-27].

Działania niepożądane związane ze stosowaniem PPT są liczne i mogą być poważne. Ponad 10% pacjentów zgłasza objawy bólu, rumienia i owrzodzenia powierzchni w ciągu dwóch do trzech dni od rozpoczęcia leczenia. Przypadkowe podanie doustne lub ogólnoustrojowe może wywołać poważne ogólne objawy zatrucia, obejmujące nudności, wymioty, biegunkę, tachykardię, niedociśnienie i przyspieszoną czynność oddechową, potencjalnie kończącą się niewydolnością oddechową. Zgłaszano również zaburzenia neurologiczne, takie jak zawroty głowy, demencja, śpiączka i neuropatia obwodowa, co dodatkowo podkreśla potrzebę ostrożnego podawania i monitorowania tego silnego leku przeciwnowotworowego [13, 14, 28, 29].

Ponieważ synteza PPT de novo jest trudna i kosztowna, jej szkielet jest podstawą do syntezy licznych aktywnych potencjalnych leków [13, 14, 17, 30]. Jedną z obiecujących strategii opracowaną w pracowni profesora Zbigniewa Czarnockiego z Uniwersytetu Warszawskiego, z którą współpracujemy, jest łączenie PPT z innymi farmakoforami, takimi jak benzotiazol [31]. Benzotiazol jest heterocyklicznym związkiem o szerokim spektrum aktywności biologicznej, w tym przeciwnowotworowej. Pochodne benzotiazolu wykazują zdolność do hamowania wzrostu komórek nowotworowych poprzez różne mechanizmy, takie jak indukcja apoptozy, hamowanie proliferacji oraz wpływ na szlaki sygnałowe związane z rozwojem nowotworów [32-34]. Połączenie PPT z benzotiazolem może prowadzić do synergistycznego działania przeciwnowotworowego [31, 35-37].

1.2. Założenia i cel pracy

Głównym celem naukowym naszego projektu jest opracowanie i przetestowanie nowych pochodnych PPT, które mają być bardziej skuteczne i mniej toksyczne dla ludzkich komórek, niż macierzysta PPT. W związku z tym pytania badawcze obejmują:

Pytanie 1. Czy badane nowe pochodne PPT (oznaczone jako KL1, KL2, KL3) są mniej toksyczne dla normalnych komórek i bardziej skuteczne przeciwko komórkom nowotworowym niż macierzysta PPT?

Hipoteza: KL1, KL2 i KL3 są mniej toksyczne dla normalnych komórek i bardziej skuteczne przeciwko komórkom nowotworowym, niż macierzysta PPT.

Pytanie 2. Czy badane substancje mają ukierunkowane mechanizmy działania?

Hipoteza: KL1, KL2 i KL3 wpływają na dystrybucję faz cyklu komórkowego, zmieniają proteostazę, wpływają na organella komórkowe, indukują apoptozę.

1.3. Znaczenie projektu

Ze względu na toksyczność, a tym samym ograniczenia dla PPT – potrzebne są nowe alternatywy terapeutyczne, które byłyby skuteczne jako leki przeciwnowotworowe i nie byłyby toksyczne dla normalnych komórek.

Niedogodności związane z toksycznością prowadzą do zmniejszenia przestrzegania zaleceń przez pacjentów podczas terapii [38]. Dlatego istnieje potrzeba syntezy nowego leku o co najmniej tak wysokiej skuteczności w terapii, jak macierzysta PPT i znacznie lepszym profilu bezpieczeństwa. Spełnienie tych potrzeb jest głównym celem. Planowane jest uzyskanie nowego leku przeciwnowotworowego, który będzie bardziej specyficzny dla komórek nowotworowych i mniej toksyczny dla ludzkich komórek nienowotworowych, niż macierzysta PPT.

Drugim, równie ważnym, jak główny, celem jest sprawienie, aby synteza naszego leku była przyjazna dla środowiska, szybka i tania (tak zwana „zielona synteza"). Dlatego od 5 lat współpracujemy z profesorem Zbigniewem Czarnockim z Uniwersytetu Warszawskiego. Uzyskano proces syntezy oparty na fotocyklizacji i przyłączeniu benzotiazolu do węgla C3 pierścienia C rusztowania PPT [39]. Synteza ta jest przyjazna dla środowiska ze względu na brak toksycznych produktów ubocznych produkcji, które musiałyby być utylizowane oraz brak konieczności stosowania katalizatorów z metali ciężkich, a tym samym brak ryzyka zanieczyszczenia otrzymanych związków metalami ciężkimi (których zawartość w leku musi być na bardzo niskim poziomie). Synteza jest również efektywna i tania dzięki zastosowaniu tanich i łatwo dostępnych substratów oraz metodologii warunków przepływu w procesie fotocyklicznym, które pozwalają na otrzymanie wielu pochodnych o różnie zdobionych pierścieniach. Dzięki temu możliwe jest wpływanie na parametry takie jak: rozpuszczalność, biodostępność, stabilność, z zapewnieniem możliwości prowadzenia syntezy na dużą skalę. Podsumowując, uzyskano innowacyjny proces syntezy, który pozwala na produkcję dużych ilości nowych leków w czystej postaci bez kosztownych metod i toksycznych produktów ubocznych.

Inspiracją do syntezy i badania pochodnych PPT był fakt, że kilka pochodnych PPT jest powszechnie stosowanych w medycynie, takich jak etopozyd, etopofos (fosforan etopozydu) i tenipozyd, które są obecnie stosowane klinicznie na całym świecie [40, 41].

Chociaż PPT jest inhibitorem polimeryzacji tubuliny, etopozyd i tenipozyd działają jako inhibitory topoizomerazy II DNA, co pokazuje, że różne pochodne mogą mieć różne mechanizmy działania [40, 41]. To uzasadnia badania nad mechanizmem działania nowych leków.

1.4. Połączenie serii publikacji

W niniejszej rozprawie doktorskiej przedstawiono cykl 4 publikacji w jednej linii tematycznej, w których autorzy zajmowali się projektowaniem, syntezą i badaniem aktywności przeciwnowotworowej nowych pochodnych PPT z benzotiazolem. Pierwsze wyniki zostały opublikowane w 2018 roku oraz pogłębione i uzupełnione w kolejnych dwóch publikacjach w 2021, oraz 2024 roku [31, 36, 37]. Ukazała się też praca poglądowa na temat działań pochodnych PPT innych niż leczenie przeciwnowotworowe, gdyż takiego podsumowania w literaturze nie było. W tym celu utworzony został oryginalny przegląd systematyczny w 2025 roku [35].

W badaniach *in vitro* wykorzystano różne linie komórkowe, w tym komórki HaCaT, HeLa, MDA-MB-231, MCF-7, PC-3, DU-145, CFPAC-1 oraz NIH-3T3, wywodzące się odpowiednio z ludzkich keratynocytów, raków sutka, raków prostaty, raka trzustki oraz z mysich fibroblastów. Wykazano, że niektóre z otrzymanych pochodnych, w tym związek KL3, wykazują aktywność przeciwnowotworową, często porównywalną lub wyższą od aktywności PPT. Ponadto, badania mechanizmu działania wykazały, że związki te wpływają na różne szlaki molekularne zaangażowane w rozwój nowotworów, takie jak szlaki związane z apoptozą, autofagocytozą, stresem siateczki śródplazmatycznej, proliferacją komórek oraz cyklem komórkowym.

Sumaryczne wyniki (szczegółowo opisane w dalszej części rozprawy) z 3 prac oryginalnych i jednego przeglądu systematycznego pozwoliły na otrzymanie szerokiej wiedzy na temat nowych pochodnych PPT.

Wyniki poszczególnych etapów badań nad pochodnymi PPT prezentowane były również na licznych konferencjach naukowych ogólnopolskich i międzynarodowych. Część wystąpień zdobyła nagrody za najlepsze wystąpienia na konferencji. Najważniejszą z nich była nagroda podczas Międzynarodowej konferencji lekarskiej Rhinoforum 2023 (1. miejsce). Osiągnięcia

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2. Kopie opublikowanych prac

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2.1. Novel podophyllotoxin derivatives as Anticancer Agents: Design, Synthesis, and Biological Screening

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Introduction

The monograph you hold is the result of the meetings and hard work of young scientists from various universities and institutes in Poland. These meetings have been held in Warsaw from 9 years under the auspices of the Department of Histology and Embryology of the Medical University of Warsaw and for the last two years also the Warsaw Branch of the Polish Society for Histochemistry and Cytochemistry.

In the monograph we present the most important topics in biomedical research from 2017. All presented works have passed the peer-review process positively. These works come from various fields of biomedicine from medical biology throughout biophysics to clinical sciences.

Wishing you enjoyable and productive reading

Editors,

Łukasz Biały

Izabela Młynarczuk-Biały

Novel podophyllotoxin derivatives as Anticancer Agents: Design, Synthesis, and Biological Screening

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Abstract

The systemic treatment of advanced cancer, where local surgery is ineffective, still offers numerous difficulties due to therapy resistance, side effects and relapse. Thus, novel therapeutics are ongoing. The scaffolds for novel drugs often derive from naturally occurring substances. One of such example is podophyllotoxin – a plant derived substance. Podophyllotoxin itself is indicated in local anticancer treatment and due to its' substantial toxicity is not recommend for systemic therapy. However, its analogues like etoposide is an element of systemic anticancer therapy administered in many malignancies. Limiting for etoposide is bone marrow depression and secondary leukemia induced in susceptible individuals. Thus, less toxic and more effective substances are needed for cancer therapy. Due to complexity of podophyllotoxin molecule it's scaffold is nowadays intensively studied as a source for novel therapeutic substances. Within this project our team aimed to modify the molecule of podophyllotoxin to obtain novel derivatives that were screened for anticancer potential. Furthermore, one of new compound was conjugate of podophyllotoxin and benzothiazole. Benzothiazole is widely used in research because of antitumor, antibacterial, anticonvulsant, anti-inflammatory and other activities of its derivatives. The obtained derivates turned out to be less toxic for normal fibroblasts in comparison to parental podophyllotoxin.

Keywords: podophyllotoxin, benzothiazole, cancer, cell viability,

Introduction

A number of studies and many clinical examples demonstrate that the treatment of cancer often encounters numerous difficulties. The lack of therapy response, relapse, or even toxic side effects are a few of such examples (Miller, Siegel et al. 2016; Siegel, Miller et al. 2016). Moreover, in developed countries, the prevalence of cancer shows increasing tendency and application of cancer medicals makes its treatment one of the most costly (Miller, Siegel et al. 2016; Siegel, Miller et al. 2016). In advanced stages of malignant disease the existing treatment remains often ineffective or relapse is diagnosed (Miller, Siegel et al. 2016; Siegel, Miller et al. 2016). Hence, to overcome these difficulties new drugs that are effective, specific, less toxic and relatively cheap are ongoing.

Thus, the ideal anti-cancer drug – would be one that combined two features: nontoxic for normal cells and effectiveness for tumor cells (Gewirtz, Holt et al. 2007; Mattson, Calabrese 2009). It also should be cost-effective and have uncomplicated application.

Over last years, natural products continued to play a highly significant role in the drug discovery and development process. Thus in the area of anticancer drugs, according to published data including 25-year period of drug development, 47% of

all approved antitumor drugs worldwide were either natural products or directly derived therefrom (Newman, Cragg 2007).

Our team was inspired by the ideas above. Therefore, we designed and synthesized derivatives of podophyllotoxin (PTOX) which is the anti-cancer, plant-derived drug (Ardalani, Avan et al. 2017; Zhang, Rakesh et al. 2018). PTOX belongs to the class of aryltetralinlactone cyclolignans and it is purer and more stable form of podophyllin (Gordaliza, Garcia et al. 2004; Saraiva, Vega et al. 2007; Silva, Coimbra et al. 2007; Silva, Martins et al. 2009; Srivastava, Negi et al. 2005; Yousefzadi, Sharifi, Behmanesh et al. 2010; Yousefzadi, Sharifi, Chashmi et al. 2010).

The crude *Podophyllum peltatum* plant extract is named podophyllin. However, podophyllin as a mixture of different active substances contains little active compound and numerous harmful ingredients of high toxicity, thus does not comply with the WHO guidelines for plant derived treatments and is not recommended for clinical treatment protocols (von Krogh, Longstaff 2001).

The purified form of podophyllin-derived active compound is named podophyllotoxin. This polycyclic compound was first isolated in 1880 from the *Podophyllum peltatum* species. However, there are few other species, such as *Berberidaceae*, *Apocynaceae*, *Polygalaeae*, *Apiaceae*, *Linaceae* and other, which contain PTOX and analogs. PTOX contains five rings, which are methylenedioxy, two tetrahydronaphthalene, lactone and aryl rings (Scheme 1). PTOX is common and the most effective cure for anogenital warts; especially for condyloma acuminatum caused by human papilloma virus (HPV) (Gilson, Ross et al. 2009; Keri, Patil et al. 2015; Seth 2015).

The exact mechanism of antineoplastic action of PTOX is unknown. However, it has been shown that PTOX due to binding tubulin, a subunit of microtubules, prevents the polymerization of tubulin into microtubules. Affinity and site of binding is similar to colchicine, well-known plant-derived (*Colchicum autumnale*) drug with similar mechanism (Spasevska, Ayoub et al. 2017), although PTOX binds more rapidly and reversibly whereas colchicine bind irreversibly. Thereby, this mechanism could include the cell cycle arrest at mitosis and impede the formation of the mitotic-spindle (Ardalani, Avan et al. 2017). Interestingly, PTOX competitively inhibits the binding of colchicine (Lu, Chen et al. 2012). As mentioned above, PTOX alone is used in a local treatment of anogenital warts (Lewis, Goldmeier 1995; Longstaff, von Krogh 2001). Clinical results with systemic application of PTOX were disappointing due to severe gastrointestinal side effects (Imbert 1998), therefore PTOX won't be approved for systemic treatment. However, continuous efforts concerning the synthesis of PTOX analogues led to the discovery of new anticancer drugs. For example its derivative, etoposide is currently used in the clinic for the treatment of a variety of malignancies including lung and testicular cancers, glioblastoma multiforme, lymphoma and nonlymphocytic leukemia (Bohlin, Rosen 1996). Teniposide another PTOX derivate, is applied for the treatment of childhood acute lymphocytic leukemia (Bohlin, Rosen 1996). In contrast to the parent podophyllotoxin, which binds to tubulin and inhibits microtubule assembly, etoposide has a distinct mechanism of action (Kumar, Kumar et al. 2011). In fact, molecules such as etoposide, amsacrine, and mitoxantrone are topoisomerase II inhibitors that induce cell death by enhancing topoisomerase II-mediated DNA cleavage through stabilization of the transient DNA/topoisomerase II cleavage complex (Kumar, Kumar et al. 2011; Wang 1996).

Like numerous anticancer drugs, also etoposide is not free of toxic side effects. Bone marrow depression is a serious, dose-limiting side effect diagnosed in patients receiving etoposide (Leroy, Kajava et al. 2001). The use of effective doses of etoposide is also associated with an increased risk of secondary acute myelogenous leukemia. For this reason, there exist an urgent need for development of more potent analogues of podophyllotoxin characterized by less toxic side effects.

Within this study we aimed to modify the molecule of phodophyllotoxin by binding it with functional groups to obtain novel compounds with potential for systemic application.

Therefore, three new cyclolignans were synthesized using photocyclization or acid-catalyzed cyclization strategy (Lisiecki, Krawczyk et al. 2017; Lisiecki, Krawczyk et al. 2016). One of the new compounds, named KL3, is a conjugate of two molecules with anti-tumor activity, podophyllotoxin and benzothiazole. The analogues of benzothiazole and its derivates are widely used in pharmaceutical research, because of their biological and pharmacological properties. Benzothiazole is a class of heterocyclic compounds having 2 hetero atoms: sulphur and nitrogen (Seth 2015). Benzothiazole is a privileged bicyclic ring system with multiple applications. In the 1950s, 2-aminobenzothiazoles were intensively studied as central muscle relaxants. Several years later riluzole was found to interfere with glutamate neurotransmission (6-trifluoromethoxy-2-benzothiazolamine, PK-26124, RP-25279, Rilutek). After that benzothiazole derivatives have been also studied as anticancer agents (Youssef, Noaman 2007). The potential of benzothiazole and related compounds was examined in breast tumors, regardless of estrogen receptor status, and against ovarian, renal, lung, and colon cancer cells (Kashiyama, Hutchinson et al. 1999).

Derivatives of benzothiazole serve as scaffolds for experimental drug design without technical difficulties (Ahmed, Yellamelli Valli Venkata et al. 2012; Ali, Siddiqui 2013; Bhuva, Kini 2010; Bolelli, Musdal et al. 2017; Henriksen, Hauser et al. 2007; Kok, Gambari et al. 2008; Liu, Lin et al. 2008). This versatile and unique compounds have received remarkable attention because of antitumor activity against breast (both estrogen receptor-positive and estrogen receptor-negative cell lines), ovarian, colon lung and renal cell lines and their interesting pharmacological activities, including anticonvulsant, analgesic, anti-tumor, antibacterial, anti-microbial, skeletal muscle relaxant and other activities such as: antidiabetic, anti-inflammatory, anticonvulsant, antiviral, antioxidant, antitubercular, antimalarial, antiasthmatic, antihelmintic, photosensitizing and diuretic (Amnerkar, Bhusari 2010; Bondock, Fadaly et al. 2009, 2010; Bradshaw, Wrigley et al. 1998; Caleta, Grdisa et al. 2004; Charris, Monasterios et al. 2002; Duggan, Lewis et al. 2009; Gurupadayya, Gopal et al. 2008; Hutchinson, Jennings et al. 2002; Kashiyama, Hutchinson et al. 1999; Kumbhare, Kosurkar et al. 2012; Sharma, Sinhmar et al. 2013; Shi, Bradshaw et al. 1996; Yoshida, Hayakawa et al. 2005).

In 2016 we have developed methodology for the stereoselective synthesis of cyclolignans related to podophyllotoxin (Lisiecki, Krawczyk et al. 2016). It involves the use of L-prolinol as a chiral auxiliary and continuous flow irradiation of a chiral atropoisomeric 1,2-bisbenzylidenesuccinate amide ester. Based on this discovery, a formal synthesis of (-)-podophyllotoxin and total synthesis of (+)-epigalcatin were

completed (Lisiecki, Czarnocki 2018; Lisiecki, Krawczyk et al. 2017; Lisiecki, Krawczyk et al. 2016).

New compounds should be soluble and stable in water solution. They need to have acceptable bioavailability, easily pass through cancer cells membrane and they should provide the evidence of high selectivity against cancer cells. An unsophisticated and cheap way to fast screening of new compounds is their incubation with selected cell lines in a cell culture. We can observe the effects of novel compounds on proliferation of cells, cell morphology and identify signs for degeneration using microscopic imaging as well as we can study cytotoxic/cytostatic effects within viability assays (Hejchman, Taciak et al. 2015; Mlynarczuk-Bialy, Doeppner et al. 2014; Mlynarczuk-Bialy, Roeckmann et al. 2006).

An assessment of cell survival is essential to test if compounds have cytotoxic/cytostatic effects. Cytotoxic effect results from direct harmful effect of examined compound on cells and cytostatic effect is a consequence of decreased cell proliferation that secondly may also lead to cell death. One of the best established viability assays is crystal violet decolorization assay (CVDA). In this method staining of attached, living cells with crystal violet dye, which binds to proteins and DNA, allow to detect the amount of remaining alive cells. Dead cells lose their adherence and by washing with PBS are eliminated from the population of cells, reducing the amount of bound crystal violet in a culture in comparison to the control, vehicle-treated group. This is a quick and if validated, adequate screening method that can be used for the estimation of cytostatic/cytotoxic effects after treatment with potential anticancer compounds (Hejchman, Taciak et al. 2015; Mlynarczuk-Bialy, Doeppner et al. 2014; Mlynarczuk-Bialy, Roeckmann et al. 2006). Therefore, to estimate the number of living cells we used CVDA.

To improve our results and determine cytopathic effects of novel compounds we also used light microscope. Due to specification of our Juli-Stage (NanoEnTek) microscope we were able to verify proliferation and amount of cells and also take photographs of cells incubated with new compounds.

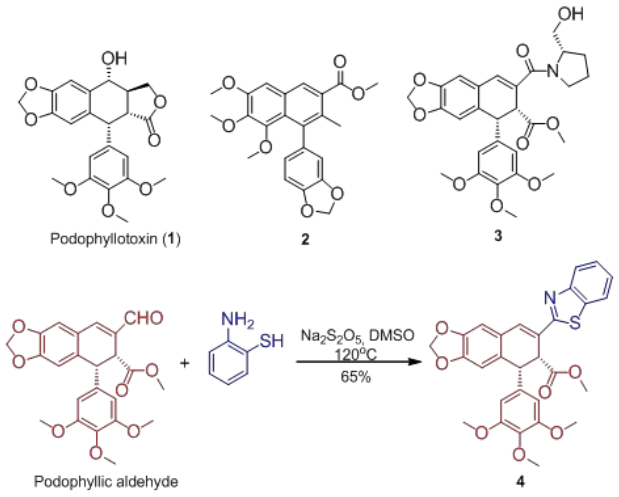
Results and discussion

Design of PTOX derivates

As shown in the available literature, cyclolignans are compounds with remarkable potential. However, most of derivatives are obtained by modifying natural compound (mainly podophyllotoxin). This does not allow for accessing other stereoisomers because some stereochemical features in the main carbon skeleton of podophyllotoxin cannot be changed during synthesis. What is more, the structural complexity of podophyllotoxin, derived from the presence of four stereogenic carbons in ring C has limited most of the structural activity relationship obtained by derivatization of the parent natural product rather than by the total synthesis. These features make it necessary to search for derivatives of PTOX with simplified structures, which can be obtained *via* short synthetic pathway from simple starting materials. Such are needed, since the therapeutic potential of PTOX and its derivatives is often limited by problems of drug resistance (Gupta 1983), hydrophobicity and low selectivity (Gordaliza, Garcia et al. 2004). Although many methods of stereoselective synthesis of cyclolignans are known, their main drawback is the lack of generality and often

high cost of synthesis. To meet this, we developed new methodology (Yu, Che et al. 2017) based on the use of cheap and readily available starting materials such as simple aromatic aldehydes, succinic acid ester, and L-prolinol as a chiral auxiliary. The key step of the synthesis is photocyclization. It has been shown that this process occurs at much higher yield when carried out under flow conditions. This approach has an additional advantage, it allows for unlimited scale of synthesis, which is an important issue in the multi-step syntheses. In addition, avoiding the use of sophisticated organocatalysts or catalysts containing heavy metals, will be extremely important because of the elimination of the possibility of contamination with toxic metals. Our method is therefore extremely advantageous in the context of the synthesis of compounds with potential pharmaceutical application.

In this study, we investigated anticancer properties of three new derivatives of PTOX - **2**, **3**, **4**. Compound **2** is a derivative of 1-arylnaphthalene which poses anti-cancer activity (Hui, Zhao et al. 2011). Derivative **3** contains in its structure L-prolinol moiety which is a H-bond donor and its conformationally restricted main chain may improve receptor binding. Compound **4** is a conjugate of podophyllotoxin (red) and benzothiazole moiety (blue). In recent years, benzothiazoles have been recognized as promising pharmacophores with diverse biological properties including anti-cancer activity (Sharma, Sinhmar et al. 2013). All of those compounds are in good accordance with Lipinski's rule of five (Lipinski, Lombardo et al. 2001) – less than 5 hydrogen bond donors, less than 10 hydrogen bond acceptors and molecular mass less than 500 daltons or not much above. Compounds **2** and **3** were obtained by us previously (Lisiecki, Krawczyk et al. 2017; Lisiecki, Krawczyk et al. 2016) and compound **4** was synthetized from podophyllic aldehyde (total synthesis of this compound was already reported by us (Lisiecki, Krawczyk et al. 2016)) and 2-aminothiophenol (Scheme 1). The synthesis of presented compounds is highly efficient and starts from cheap and readily available substrates.



Scheme 1. Chemical structures of podophyllotoxin (**PTOX**), compounds 2 -**KL1** and 3 -**KL2** (top) and synthesis of compound 4 -**KL3** (bottom).

Cytotoxicity assay

We performed crystal violet viability assay to examine the sensitivity of tumor and non-tumor cells towards novel derivates in comparison to the parental one PTOX.

Compound KL1 did not induce any significant cytotoxic/cytostatic effect against HeLa, MDA-MB, DU145 and CFPAC. Also, in comparison to PTOX – a model toxic drug for NIH-3T3 cells, the novel KL1 compound was 100 times less toxic. Similar results were obtained for KL2 compound. The values of IC50 for this substance on all but one cell line (MCF7) was good above 140 μM and ranged from 140 μM to 490 μM (Table 1). In addition, in PC3 cell line the IC50 for KL1 was 48 μM. Also, non-tumor NIH-3T3 cells turned out to be 90 times less susceptible to the action to KL2 in comparison to the parental PTOX.

These results showed that compounds KL1 and KL2 by chemical modifications of parental PTOX exhibited lower cytotoxicity than PTOX itself. Moreover, both compounds turned out to be highly effective in induction of cytotoxic/cytostatic effects on MCF7 cells and in comparison to non tumor mouse fibroblasts they were tumor cell specific with selectivity index (SI) of 7.9 and 4.5 for KL1 and KL2, respectively (Table 2).

Compound KL3 turned out to be most active in induction of cytotoxic/cytostatic effects from all of three novel compounds. The IC50 values for tumor cells were form 0.5 μM to 10 μM. Only MCF7 breast cancer cells remained relatively resistant to KL3 with IC50 of 116 μM.

Such a good anti tumor profile of KL3 was accompanied with less toxicity towards NIH-3T3 cells in comparison to the parental PTOX.

As suspected – combination of two anti tumor agents within one molecule (KL3) improved the antitumor activity, in comparison to KL1 and KL2, and what is also very desiderated this drug was more toxic for most cancer cell lines than to no-tumor fibroblasts.

Table 1 Compilation of IC50 value of novel compounds and of the parental POTX estimated in 7 cell lines. IC50 – Concentration of compound corresponding to 50% growth inhibition after 48-hour incubation.

	Inhibitory concentration (IC 50) [μM]						
Compound	**Immortalized non tumorogenic cells**	**Tumor derived cells**					
	Fibroblast	**Cervix cancer**	**Breast cancer**		**Prostate cancer**		**Pancreas cancer**
	NIH-3T3	HeLa	MDA-MB	MCF7	PC3	DU145	CFPAC
KL1	55	165	300	7	48	425	370
KL2	45	140	490	10	144	290	400
KL3	1	0.5	0.7	116	0.7	6.8	10
PTOX	0.5	0.5	125	123	10	1	100

The standard error bars did not extend 10% and for more transparency are not included in the table. Results are from 3 independent experiments.

The selectivity index is helpful for comparison between different tumor cell lines. However, when IC50 values are high, the comparison between substances does not matter. Obtained substances KL1 and KL2 are less toxic. Thus, we have obtained leading structures of lower cytotoxicity than parental PTOX. In turn, KL3 as compound represented by covalently bound of two anti tumor substances turned out to be the most effective with the highest anti tumor activity from all studied substances. The IC50 value for KL3 was below 1 μM for three cancer cell lines.

Table 2 Tumor cell selectivity

	Selectivity Index (SI)					
Compound	**Tumor derived cells**					
	Cervix cancer	**Breast cancer**		**Prostate cancer**		**Pancreas cancer**
	HeLa	MDA-MB	MCF7	PC3	DU145	CFPAC
KL1	0.3	0.2	7.9	1.1	0.1	0.1
KL2	0.3	0.1	4.5	0.3	0.2	0.1
KL3	2.0	1.4	0.009	1.4	0.1	0.1
PTOX	1.0	0.004	0.004	0.1	0.5	0.005

Microscopy analysis

A series of images was recorded and representative regions of particular groups is shown at Fig 1. Microscopic experiments were performed on NIH-3T3 cells conducted from disaggregated BALB/c mouse embryos. They are extremely sensitive to contact inhibition and are highly susceptible to transformation by SV40 VIRUS and murine sarcoma virus (Labruère, Gautier et al. 2010).

In comparison to the control group, demonstrating spindle-shaped morphology of NIH-3T3 cells that are elongated with long processes and share 95% of confluence, both treated groups vary in cell count and shape from the control group. Cells treated with 10 μM or 100 μM PTOX demonstrate 60 and 50% of confluence, respectively. In comparison to the controls, their processes are shortened and thickened, the perinuclear region is enlarged, the total surface occupied by a single cell is enlarged. We can observe also signs of cell degeneration like small rounded cells and pyknotic cells that's number increases by increasing concentration of PTOX.

Compound KL3 induced shortness of NIH-3T3 processes similarly to parental PTOX, also the perinuclear region was enlarged. Moreover, this enlargement was more pronounced than in PTX treated group. The ratio of degenerating cells was also higher than in PTX treated cells. We observed numerous pyknotic cells in KL3 100

μM treated group and some amount of big rounded cells – suggesting mitotic catastrophe as a reason of such perturbations (Figure 1).

Even if such initial compounds might have diminished cytotoxic potencies compared with the parent PTOX, the ease of preparation of carefully designed libraries of analogues would lead to more informative studies and expeditious structure optimization. In this regard we have obtained less toxic scaffolds represented by KL1 and KL2 and a highly effective KL3 that is twice less toxic for NIH-3T3 cells in comparison to PTOX and turned out to be selective for tumor cells. To improve the molecules, further modifications should be designed and performed combined with studies on detailed mechanisms of action of these compounds.

Materials and methods

Synthesis

Methyl 4-(benzo[d][1,3]dioxol-5-yl)-5,6,7-trimethoxy-3-methyl-2-naphthoate (2) was prepared in accordance with the procedure previously described.3

(5R,6R)-Methyl 7-((S)-2-(hydroxymethyl)pyrrolidine-1-carbonyl)-5-(3,4,5-trimethoxy-phenyl)-5,6-dihydronaphtho[2,3-d][1,3]dioxole-6-carboxylate (3) was prepared in accordance with the procedure previously described.4

(5R,6R)-Methyl 7-(benzo[d]thiazol-2-yl)-5-(3,4,5-trimethoxyphenyl)-5,6-dihydronaphtho [2,3-d][1,3]dioxole-6-carboxylate (4). A mixture of podophyllic aldehyde (prepared in accordance with the procedure previously described4) (64 mg, 0.15 mmol, 1 equiv.), 2-aminothiophenol (19.7 mg, 0.16 mmol, 1.05 equiv.) and Na2S2O5 (30 mg, 0.16 mmol, 1.05 equiv.) in 1 mL of DMSO was heated at 120˚C for 2 h. The reaction mixture was allowed to cool to room temperature, excess water was added, and yellow solid precipitate was collected by filtration. The precipitate was washed with water, dried and purified on a silica gel column, using ethyl acetate in n-hexane (gradient, from 0% to 10%) as an eluent. A yellow solid was obtained (52 mg, 0.098 mmol, 65%). M.p. 165–166°C. Rf (50% AcOEt/n-hexane) 0.54. [α]D25 = 281.0 (c 0.1, CHCl3). 1H NMR (CDCl3, 300 MHz): δ 7.95 (dd, J1 = 8.1 Hz, J2 = 0.6 Hz, 1H), 7.84 (dd, J1 = 7.8 Hz, J2 = 0.6 Hz, 1H), 7.43 (m, 2H), 7.35 (dt, J = 7.8, 1.2 Hz, 1H), 6.86 (s, 1H), 6.63 (s, 1H), 6.56 (s, 2H), 5.98 (d, J = 1.5 Hz, 1H), 5.96 (d, J = 1.5 Hz, 1H), 4.61 (d, J = 7.5 Hz, 1H), 4.44 (d, J = 7.8 Hz, 1H), 3.89 (s, 3H), 3.84 (s, 6H), 3.43 (s, 3H). 13C NMR (CDCl3, 75 MHz): δ 171.7, 167.1, 153.8, 153.3, 148.6, 146.5, 137.4, 134.8, 134.4, 133.4, 131.7, 128.5, 127.0, 126.2, 125.3, 123.1, 121.4, 108.8, 108.8, 106.6, 101.4, 77.4, 77.0, 76.6, 60.9, 56.1, 51.7, 49.1, 48.5. HRMS (ESI) m/z: calcd for C29H25NO7SH [M+H]+, 532.1424; found, 532.1440.

Reagents

PROX (SIGMA) and Novel compounds were dissolved in DMSO (SIGMA) at 100 mM and kept in the fridge until examination.

Cell Culture

Mouse NIH-3T3 fibroblasts (ATCC, USA) were cultured in Dulbeco (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated FCS, penicillin (100 U/ml), streptomycin 100 µg/ml.

For analysis of antitumor activity of examined compounds, following cell lines were used: MDA-MB-431, HeLa, PC3 and DU145. All of cell lines was obtained from ATCC. All cells were cultured in media recommended by suppliers. Then supplemented with standard antibiotics and 10% FCS all from Sigma-Aldrich. Cells were kept in 25 cm2 tissue flasks (Greiner, Berlin, Germany) and passaged every 2-3 days.

Cell Proliferation Assay

The cytotoxic/cytostatic effects of novel compounds on culture cells were examined in vitro using the crystal violet assay, as previously described (Mlynarczuk-Bialy et al., 2006). Briefly, cells (5 x 103 cells/well) were seeded in 96-well microtiter plates (BD, Biosciences, San Jose, California, USA) and incubated with serial dilutions of examined compounds. Inhibitors were added in quadruplicate to a final volume of 200 µL. Appropriate volumes of culture medium, supplemented with DMSO (<0.1%) were added as controls. After an incubation period of 24, 48 or 72 hours, cells were washed once with PBS, fixed with 70% ethanol for 30 min and finally stained with 0,1% crystal violet in PBS for 30 min and washed carefully with water to remove unbound dye. The remaining dye was eluted by 1% SDS in water and determined at 550 nM. Cytostatic/cytotoxic effect was expressed as relative viability of treated cells (% of control cells incubated with medium only) and was calculated as follows: relative viability = (Ae-Ab) x 100/(Ac-Ab), where Ab is background absorbance, Ae is experimental absorbance and Ac is the absorbance of untreated controls.

Light microscopy

In order to assess the influence of novel derivates on morphology of examined cells, after 48 h of incubation period, cells form cell culture were directly imaged by a phase contrast microscope (Juli, NanoEnTek) at magnification of 100x.

In comparison to the controls (A,B) we observe dose-dependent cytopathic effects including shortness of cytoplasmic processes and thickness of perinuclear region. In the higher drug concentration, we observe also cell rounding and detachment from the bottom. In comparison to PTOX (C,D) the effects induced by KL3 (E,F) are similar but more pronounced (advanced).

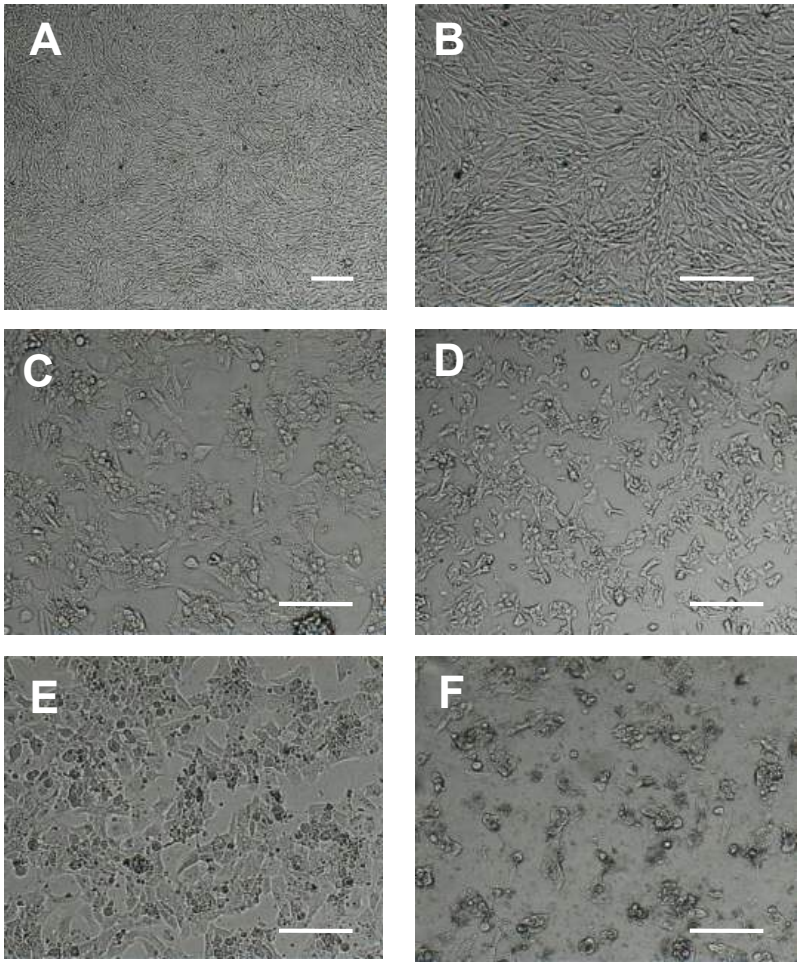


Figure 1: Photomicrographs of NIH-3T3 cells:
Untreated cells (A, B smaller and bigger magnification, respectively), C,D: cells treated with PTOX 10 μM or 100 μM – respectively, or E,F groups treated with KL3 10 μM or 100 μM – respectively. Scale bars 50 μM

Summary and conclusion

We aimed to create novel compounds of anti-cancer drug – PTOX. We planned and designed the synthesis of more effective as well as less toxic compounds. Furthermore, we were also focused on economic aspects and would like to reduce costs of synthesis. Due to these assumptions we planned to use cheap and easily available substrates. Moreover, in the process of synthesizing we don't need to use heavy metal catalysts, and thus there is no risk of contamination of compounds

obtained with heavy metals (whose content in the drug must be at a very low level). The next advantage form the used methodology, thanks to the use of flow conditions in the photocyclic process, it was easy to conduct synthesis on a large scale. This methodology also allowed obtaining numerous derivatives with differently decorated rings, thanks to which it was possible to influence parameters such as: solubility, bioavailability, stability. What is interesting, when it comes to the KL3 compound itself, it was the first synthesis of the PTOX and benzothiazole congeners with the cis configuration at the C1 and C2 carbon atoms.

For in vitro tests of new substances, we applied low-cost and well-standardized viability assays in combination with bio-imaging of the tested cells. We showed that the applied methods are able to verify in an unambiguous manner whether the examined chemical structures exhibit biological effects.

Result of cytotoxicity assay and microscopic analysis proved, novel compounds are less toxic and more effective in comparison to parental PTOX. That is a promising result because the toxicity of PTOX excludes its systemic application. Anti cancer KL3 compound with better effectiveness and lover toxicity can be useful in more applications as the initial substances.

In summary, we obtained KL3 derivative, that is less toxic than the parental one, in an innovative synthesis process that allows to synthesize large quantities of the product in its pure form - this work is therefore a comprehensive description of the compound in cell culture-based model. Its future testing in animal models can verify pre-clinical potential of KL3 compound.

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2.2. Novel podophyllotoxin and benzothiazole derivative induces transitional morphological and functional changes in HaCaT cells.

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Novel podophyllotoxin and benzothiazole derivative induces transitional morphological and functional changes in HaCaT cells

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ABSTRACT

Podophyllotoxin (PPT) is an antimitotic drug used topically in the treatment of anogenital warts. Due to its toxicity it cannot be administered systemically as an anticancer agent. However, modified PPT derivatives such as etoposide and teniposide are used clinically as systemic agents. Thus, we invented novel PPT derivative KL3 that was synthesized by photocyclization. Earlier we have shown that KL3 has an anticancer effect in various cell lines. Here we compared the toxicity of KL3 vs PPT on non-cancerous normal human keratinocytes (HaCaT) and peripheral blood mononuclear cells (PBMC) showing that KL3 is less toxic than PPT to non-cancerous cells. At concentrations that neither induced cell death, nor affected cell cycle, KL3 in HaCaT cells evoked transient ultrastructural features of ER stress, swelling of mitochondria and elongation of cytoplasmic processes. Those changes partially reversed with prolonged incubation while features of autophagy were induced. PPT in equivalent concentrations induced HaCaT cell death by cell cycle arrest, intrinsic apoptosis and finally disintegration of cell membranes followed by secondary necrosis. In conclusion, we show that the KL3 derivative of PPT in contrast to PPT allows repair of normal keratinocytes and triggers mechanisms that restore non-tumor cell homeostasis

1. Introduction

Molecules of natural origin and their derivatives serve as scaffolds for the invention and synthesis of novel drugs. Podophyllotoxin (PPT) is the main constituent of roots and rhizomes of the plants from the genus *Podophyllum* belonging to *Berberidaceae* family (Lewis and Goldmeier, 1995; Longstaff and von Krogh, 2001; von Krogh and Longstaff, 2001).

The exact mechanism of PPT action in human cells is not fully known. PPT stabilizes microtubules and stops replication of cellular DNA (Ardalani et al., 2017; Gupta, 1983) (Screpanti et al., 2010; Wolff et al., 1991), thus is considered an antitumor drug. PPT is further involved in the enzymatic activation of Cytochrome P450—2C19 and P450-3A4 important in NADPH-dependent electron transport pathway in mitochondria (Preissner et al., 2010).

Due to its high toxicity and adverse effects, PPT is not used systemically, only for the topical treatment of anogenital warts (Gilson et al., 2009; Komericki et al., 2011; Lacey et al., 2003) as a 0.5% solution or gel, which is used for up to 16 weeks three times a week and washed 6–10 h after the application to avoid adverse effects. (Council, Europe, o, 2008). Local adverse effects include erosion and scarring of treated skin. Systemic adverse effects of PPT include: nausea, vomiting, diarrhea, abdominal pain, thrombocytopenia, leukopenia, abnormal liver tests, sensory ataxia, altered consciousness (Chang et al., 1992; Kao et al., 1992). A fatal case was described after topical application of 90 ml of a 17.5% podophyllin solution to the vulva (Council, Europe, o, 2008). When taken orally, a 350 mg dose of PPT may already be lethal (Council,

Abbreviations: NaCac, sodium cacodylate; PPT, podophyllotoxin.

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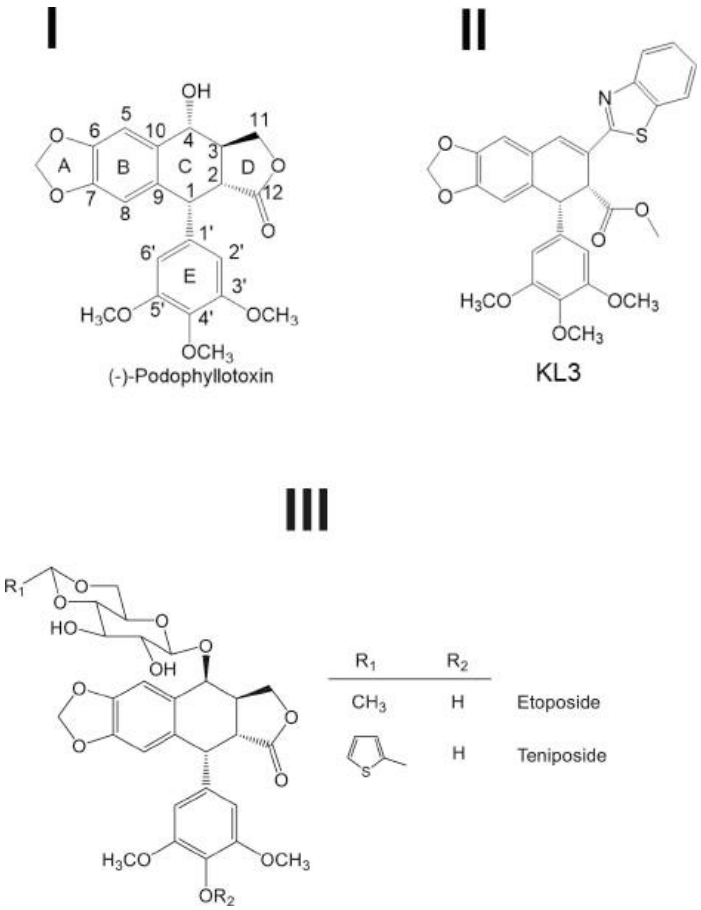

Fig. 1. The diagram shows the structures of PPT (I) and its derivatives KL3 (II), etoposide and teniposide (III). In the diagram (I) letters indicate rings and Arabic numbers indicate particular carbon atoms of the leading structure that is the same for I, II and III. The KL3 derivative has benzothiazole moiety attached to the carbon atom C3 of ring C. This modification resulted in opening of ring D. Etoposide and teniposide have a glucose group at the carbon atom C4 of ring C and side groups at positions R1 and R2 are indicated at III. There is a certain analogy in the structure of KL3 and etoposide and teniposide. However both molecules are differential arranged by different spatial localization of sugar residue of etoposide and benzothiazole moiety of KL3.

Europe, o, 2008). Fatal outcomes are generally due to CNS effects leading to coma, respiratory depression and cardiovascular crisis. Several cases of poisoning have been reported after ingestion of products containing PPT. The lowest published lethal dose in women is 6 mg/kg. LD 50 for PPT given orally in mouse is estimated to be 68 mg/kg (Council, Europe, o, 2008).

After topical application on skin in the treatment of anogenital warts PPT targets mainly keratinocytes (Henseleit et al., 1996), therefore keratinocytes derived non-cancerous HaCaT cell line are commonly used in research on various topically used drugs including PPT (Wei et al., 2019)' (Bohr et al., 1986) (Zhang et al., 2017). There are no reports showing ultrastructural changes in PPT treated HaCaT cells and very few reports describing PPT effects on other cell types.

PPT derived antitumor agents are etoposide and teniposide (Fig. 1) (Ardalani et al., 2017; Kumar et al., 2011; Liu et al., 2015; Zhang et al., 2018). Both of them inhibit DNA-topoisomerase II and are used for the treatment of germ-cell malignancies, small-cell lung cancer, non-Hodgkin's lymphoma, leukaemia, Kaposi's sarcoma, neuroblastoma, and soft tissue sarcoma.

Comparing the structure of PPT with both derivatives it is evident that attachment of additional moiety to the highly toxic molecule of PPT results in creation of effective antitumor drugs with lower toxicity to non-cancerous tissues.

In our study we aimed to examine safety profile of novel PPT derivative (KL3) in non-cancerous keratinocytes in comparison to PPT. KL3 is synthesized by attachment of benzothiazole to C3 carbon of C Ring of PPT scaffold as described elsewhere (Lisiecki and Czarnocki, 2018; Lisiecki et al., 2017; Lisiecki et al., 2016). Previously, we have shown that KL3 induced cell death in various cancer cells (originating from pancreas, cervix, prostate) (Strus et al., 2018).

In the previous work, we compared the effects of PPT and KL3 on viability, cell cycle phases distribution, apoptosis/necrosis induction, and mitochondrial status by measuring intracellular ATP levels in HaCaT cells. In particular, we analyzed for the first time the ultrastructure and morphometric parameters of selected organelles (like mitochondria, RER, podipodia) following treatment with KL3 versus the parental PPT. Moreover, we tested viability of human peripheral blood mononuclear cells (PBMC) treated with both compounds as an in vitro system to predict in vivo toxicity.

KL3 turned out to be less toxic than PPT to HaCaT cells and PBMC and therefore, we believe that it is a good candidate for further assessment of its safety and utility as potential antitumor drug.

2. Materials and methods

2.1. Chemicals

The compound KL3, chemical name: (5*R*,6*R*)-Methyl 7-(benzo[*d*] thiazol-2-yl)-5-(3,4,5-trimethoxyphenyl)-5,6-dihydronaphtho [2,3-d] [1,3]dioxole-6-carboxylate (KL3) was prepared in accordance with the procedure previously described (Lisiecki and Czarnocki, 2018; Lisiecki et al., 2017; Lisiecki et al., 2016; Strus et al., 2018). Briefly, a mixture of podophilic aldehyde, 2-aminothiophenol and $Na_2S_2O_5$ in DMSO was heated at 120 °C for 2 h. The mixture was cooled to ambient temperature and yellow precipitate was collected by filtration. The product was dried and purified on a silica gel column, using ethyl acetate in n-hexane as an eluent. PPT was purchased from Sigma-Aldrich. Both substances were dissolved in DMSO at 100 mM stock solution and kept at −20 °C until use.

2.2. Cell culture

HaCaT cells (ATCC), not exceeding 5 passages, were cultured in DMEM (Gibco, CO, UK) containing stable glutamine and supplemented with 10% FBS and a standard antibiotic antimycotic solution (Gibco). Cells were grown in a humidified atmosphere at 37 °C and passaged every three days with standard Trypsin-EDTA solution (Gibco). Cells were tested for mycoplasma contamination by staining with HOECHST (Sigma-Aldrich) and followed by confocal microscopy analysis by means of Leica-SP4 (Leica, Germany) microscope.

PBMC were isolated as described elsewhere (Mlynarczuk et al., 2004) and treated with tested compounds for 24- or 48 h, followed by viability assay with PrestoBlue (Thermo Fisher Scientific. Waltham, MA) according to manufacturer's instructions.

2.3. Viability assays

To determine the cytotoxic/cytostatic effects of compounds involved in this study, we performed crystal violet assay as described in (Mlynarczuk-Bialy et al., 2006). The amount of the crystal violet dye absorbed at the adherent cell surface is directly proportional to the number of living cells. For this assay, cells were seeded in a 96 well plates (Nest Biotechnology, Wuxi, China) at the density of 10^4 cells/well, treated in quadruplicates with increasing concentrations of PPT or KL3 for 24 h or 48 h. After the incubation period, cells were washed with PBS followed by staining with 0.05% crystal violet water solution (ChemPur, Piekary Slaskie, Poland). The excess of crystal violet was washed out with distilled water. The stained cells were solubilized in 1% SDS water solution. The absorbance of particular probe was read in Fluostar plate reader at 550 nM (BMG Labtech, Ortenberg, Germany). Cell viability was expressed as a percentage of that of the control (vehicle treated) cells. All results in the study were based on at least three experiments.

The experimental compounds were dissolved in DMSO that could affect cell viability. Therefore, a preliminary experiment was performed in order to investigate the effects of DMSO. Increasing concentrations of DMSO, ranging from 0.1% to 5%, for 48 h of cell culture were tested. The maximum concentration of DMSO which did not decrease cell viability, measured by crystal violet assay, was found to be 0.5%. In our experiments maximal DMSO concentration as a solvent was 0.1%. This was applied as a control for cell-based assays.

2.4. Colony growth assays

The sensitivity of HaCaT cells to prolonged treatment with KL3 or PPT was assessed with colony growth assay as described elsewhere (Mlynarczuk-Bialy et al., 2006). Cells were seeded at 20 cells per well in a 48 well plate (Nest). After 10 days of incubation, colonies were counted after staining with 0.5% crystal violet in water (Sigma-Aldrich Saint Louis MO USA). Treatment was done in quadruplicate at each concentration. The control group is treated with culture medium containing compounds' solvent solution and is assigned as 100% of relative cells viability.

2.5. ATP synthesis

In order to quantify ATP within metabolically active cells, the luminescent CellTiter-Glo Assay G7570 (Promega Corporation Madison, WI) was done according to the manufacturer' instructions. Briefly, cells were treated with KL3 or PPT for 24 or 48 h (1–100 μM) in 200 μl of medium at 96-well white plates (PerkinElmer).

2.6. Caspase 9 activity

For measurement of active caspase 9, that is activated by cytochrome *c* within intrinsic apoptosis pathway, the Caspase-Glo 9 assay (Promega Corporation Madison, WI) was performed according to manufacturer's instructions. Vehicle treated cells served as negative controls and HaCaT cells treated with UV for 15 min 24 h before the assay served as positive controls.

2.7. Kinetic determination of apoptotic and/or necrotic cells

Live cell kinetic apoptosis and necrosis assays were performed in order to estimate the dynamics and quantity of cell death induced by both substances. For this purpose, RealTime-Glo Apoptosis and necrosis assay was performed according to manufacturer's instructions. As positive apoptosis control, cells were treated with UV for 15 min, as positive necrosis control, cells were treated with cell lysis agent (Promega).

2.8. Annexin V apoptosis and necrosis assay

The extent of apoptosis/necrosis was measured using the Real Time Apoptosis/necrosis assay (Promega) according to the manufacturer's instructions. Briefly, HaCaT cells were plated at a seeding density of 3×10^4 cells/100 μl in 96 well plates and treated with tested drugs (at 0, 1, 10 and 100 μM; KL3 or PPT, respectively). After 24 h or 48 h of incubation, test reagent was added to the plate. For internal validation of the assay as positive control, cells were treated with cell lysis agent (Promega) or with UV (apoptosis induction).

2.9. Transmission electron microscopy

HaCaT cells were plated on tissue culture dishes, treated with KL3 or PPT for 24 h or 48 h, then washed in PBS, trypsinized, and harvested. The cell pellet was fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate (NaCac) buffer (pH 7.4), postfixed in 2% osmium tetroxide in NaCac, stained en bloc with 2% uranyl acetate, dehydrated with a graded ethanol series, and embedded in epon resin (all from Sigma Aldrich). Ultrathin sections were cut with a diamond knife on a Leica EM UC6 ultramicrotome (Leica Microsystems, Bannockburn, IL), collected on copper grids, and stained with uranyl acetate and lead citrate. Cells were observed in a JEM 1400 transmission electron microscope (JEOL Co., Japan 2008) at 80 kV and imaged with 11 Megapixel TEM Morada G2 (EMSIS GmbH, Germany) camera.

2.10. Morphometric analysis of TEM images

Images of cells recorded by electron microscopy were evaluated by two independent researchers. By comparing the scale bar with the length of a given structure, its median longitudinal and transverse dimensions and electron density were calculated. For rough endoplasmic reticulum, the distance between its membranes in visible cross sections was calculated. The average of at least 20 calculations and standard deviation was calculated. For autophagy the surface area of the region

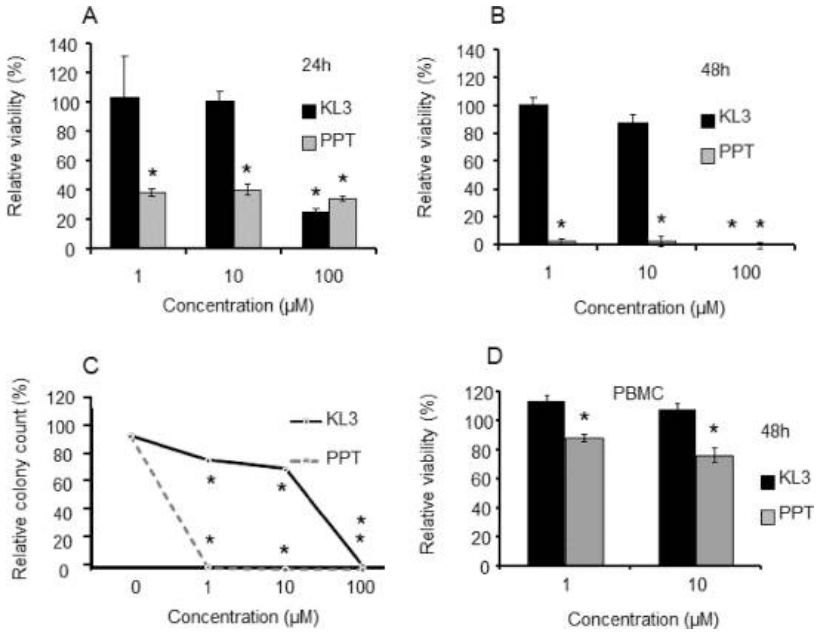

Fig. 2. Viability of HaCaT cells after incubation with KL3 or PPT at 24 h (A) or 48 h (B), (C) Colony formation assay of HaCaT cells after incubation with KL3 (solid line) or PPT (dotted line). (D) Viability of PBMC (Peripheral Blood Mononuclear Cells) treated for 48 h with KL3 or PPT. Stars: $p < 0.05$ at Student's *t*-test.

with visible autophagic vesicles was measured. Usually phagocytic vesicles formed a cluster in the perinuclear region. If more than 1 cluster was visible, the area of all clusters was calculated and added.

2.11. Statistics

The Student's *t*-test was used in the statistical significance calculations. A p value <0.05 was considered statistically significant.

3. Results

3.1. KL3 is less toxic than PPT to HaCaT cells and to PBMC

PPT and KL3 both induce time dependent cytotoxic/cytostatic effects in HaCaT cells (Fig. 2). After 24 h incubation, KL3 at 1 μM and 10 μM had a negligible effect on HaCaT cell viability, while at the highest concentration of 100 μM, it induced a 60% reduction in viability leaving 40% of live cells relative to controls (Fig. 2A-black columns). PPT at 24 h reduces HaCaT viability to about 40% in all concentrations (1 μM- 39.6%, 10 μM – 41.3%,100 μM – 36%). (Fig. 2A–grey columns).

After 48 h KL3 is toxic to HaCaT only in the highest concentration 100 μM by reducing cell viability to 1% (Fig. 2B black columns), but at concentrations of 1 μM and 10 μM has no significant effect on cell viability (90% and 80% respectively). In contrast, at 48 h PPT turned out to be highly toxic to HaCaT cells reducing the cell amount to about 1% in all concentrations (1 μM- 4.5%, 10 μM- 5%, 100 μM- 0.5%, respectively) (Fig. 2B grey columns). Therefore in apoptosis/necrosis experiments data from HaCaT groups treated with KL3 100 μM and PPT 1–100 μM for 48 h and 100 μM for 24 h were not shown. Treatment of PBMC with KL3 at 1 and 10 μM for 48 h does not affect its viability while slight but statistically significant reduction of PBMC's viability was noted after treatment with PTX 1 μM and 10 μm, 88% and 75% of control viability, respectively (Fig. 2D).

3.2. KL3 but not PPT allow formation of colonies of HaCaT cells

To determine the potential of HaCaT keratinocytes to grow when treated with KL3 in comparison to PPT, single seeded cells were incubated for 10 days with given substances at the concentrations selected in the viability assay and were subjected to colony formation assay.

As displayed in Fig. 2c (solid line) KL3 inhibits colony formation slightly at 1 μM and 10 μM (80%, 70%, respectively) in comparison to the vehicle controls, while at 100 μM it completely abolishes colony formation by HaCaT cells. In contrast, PPT completely inhibited colony growth in all tested concentrations (1–100 μM), (Fig. 2c – dotted line).

3.3. At low concentration KL3 does not affect ATP generation

To evaluate the effect of KL3 and PPT on mitochondrial ATP generation, ATP level was measured after 24 h and 48 h of incubation. As shown in Fig. 3A, 1 μM KL3 slightly but do not significantly affects cellular ATP level (95%-24 h, 90%-48 h), while higher KL3 concentrations strongly diminish ATP level (10 μM 20% and 15% for 24 h and 48 h, respectively). PPT (Fig. 3B) lowers ATP level in all tested concentrations at 24 h (1 μM 68% 10 μM 70%, respectively).

3.4. KL3 at low concentration does not induce either apoptosis or necrosis

For evaluation of apoptosis and necrosis, we performed an assay based on phosphatidylserine externalization and loss of membranes integrity. As shown in Fig. 3. 1 μM KL3 does not induce either apoptosis or necrosis after 24 h and 48 h. In contrast, 1 μM PPT induces both apoptotic phosphatidylserine externalization and necrotic loss of membrane integrity. Similar, both KL3 and PPT at 10 μM lead to apoptotic

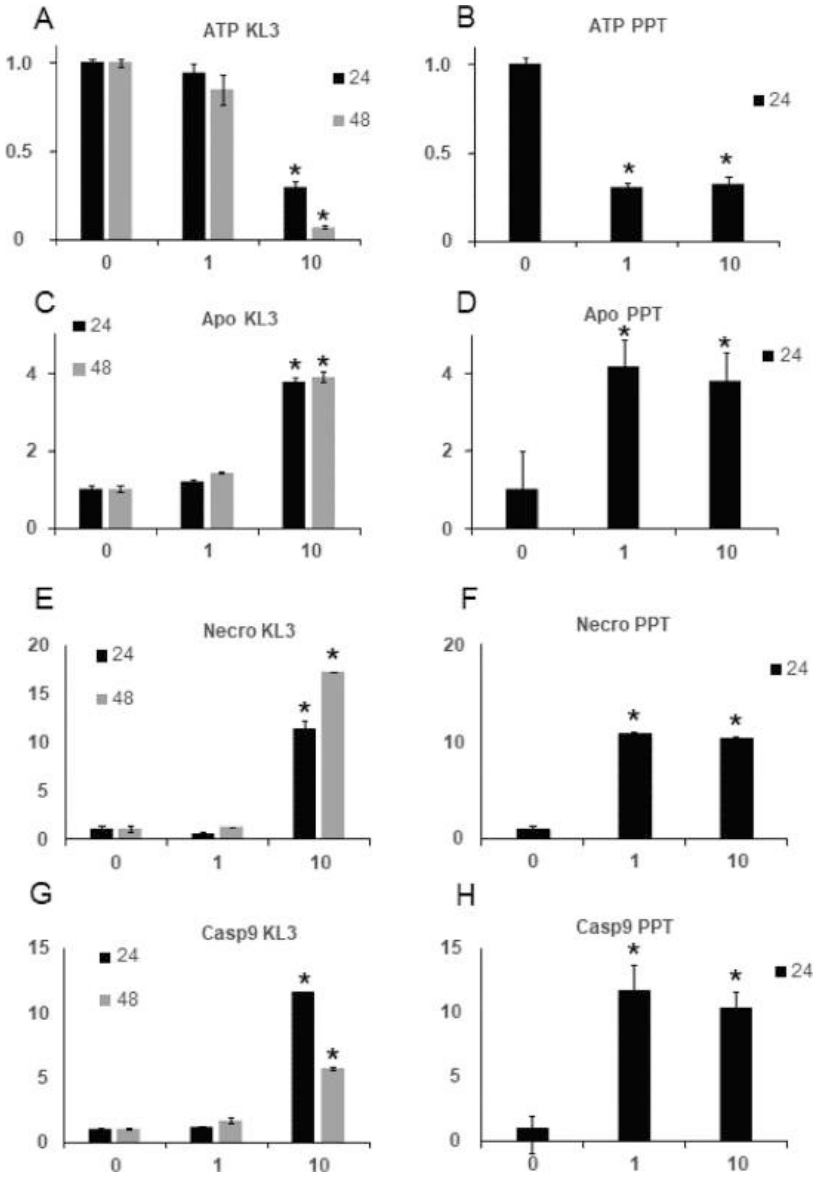

Fig. 3. Biochemical analysis of ATP production (A, B), Phosphatidylserine externalization (C, D), Necrosis induction (E, F) caspase 9 activation (G, H) in HaCaT cells after incubation with KL3 (left panel), or PPT (right panel). x-axis: luminescence in RLU*10^4 (A-D and G-H) or fluorescence in RFU*10^4, y-axis: concentration (μM). Boxes: incubation time 24 h (black) and 48 h (grey). Stars: $p < 0.05$ at Student's *t*-test. Abbreviations: Apo: apoptosis, Necro: necrosis, Casp9 – caspase 9.

phosphatidylserine externalization accompanied by increase in markers of necrosis.

3.5. Both KL3 and PPT activates the intrinsic apoptotic Caspase 9

Activity of caspase 9 was measured by luminescent assay. While 1 μM KL3 does not activate caspase 9 (Fig. 3B), in higher KL3 concentrations (10 μM) as well as all PPT concentrations (1- and 10 μM) caspase 9 gets activated. In KL3 10 μM, caspase 9 activity is higher at 24 h than after 48 h of incubation. However, the level of caspase 9 is still significantly elevated in comparison to the controls.

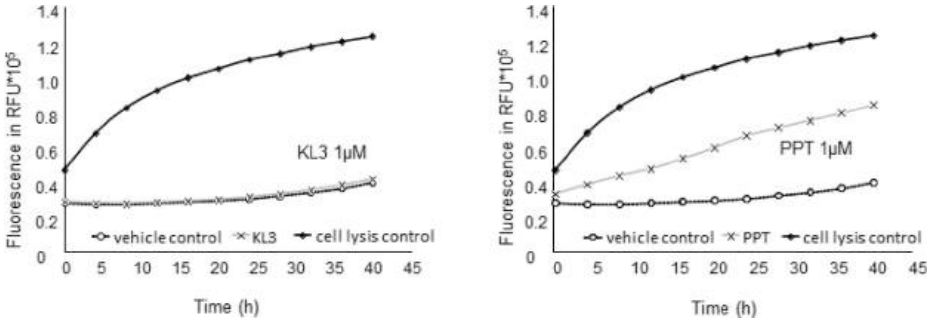

Fig. 4. Kinetic analysis of necrosis in KL3 or PPT treated HaCaT keratinocytes. Black line – cell lysis controls, dotted line: vehicle treated cells, grey line: cells treated with KL3 or PPT both at 1 μM (left and right panel, respectively).

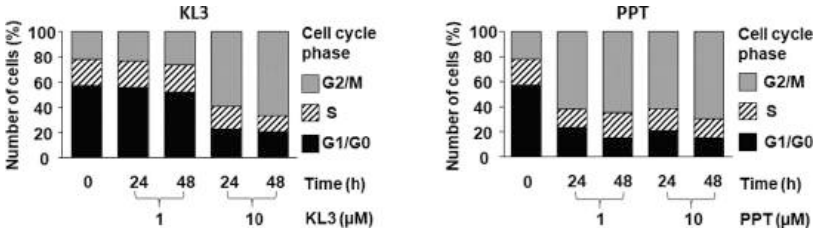

Fig. 5. Flow cytometric analysis of cell cycle in HaCaT cells after incubation with KL3 or PPT, respectively.

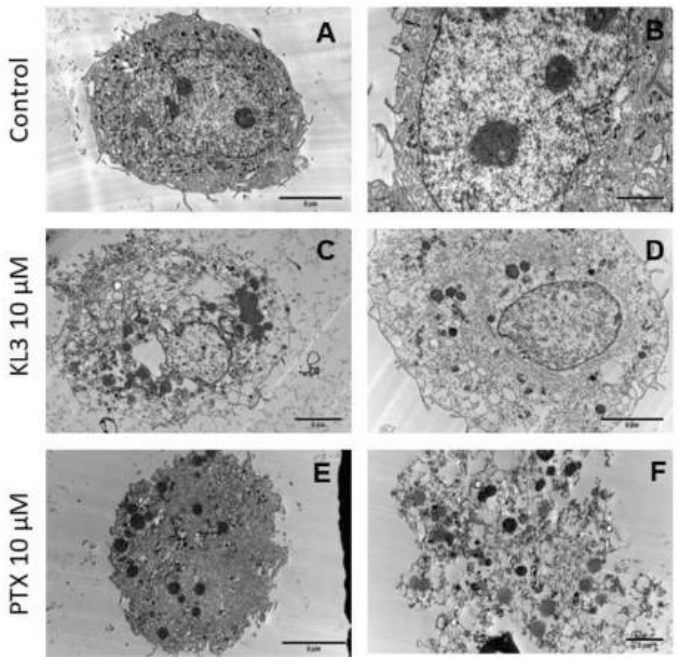

Fig. 6. The effects of indicated substances on the ultrastructure of HaCaT cells. A-B – control cells – cell nuclei as well as the cytoplasmic region demonstrate normal interphase HaCaT cell morphology. C—D KL3 at 10 μM – Reduced electron density of the cytoplasm, advanced ER distension, vacuolization of the cytoplasm – Non ER associated rich in protein vacuoles are observed. The cell nucleus has a reduced electron density and a diffuse not visible nucleolus. Cell membranes are preserved. *E*-F PTX at 10 μM – In cells, advanced degeneration - loss of cell membrane continuity (E), accumulation of electronically dense vacuoles (black vesicles at E and F). Scale bar: left panel 5 μm (A,C,E,), right panel 2 μm (B,D,F).

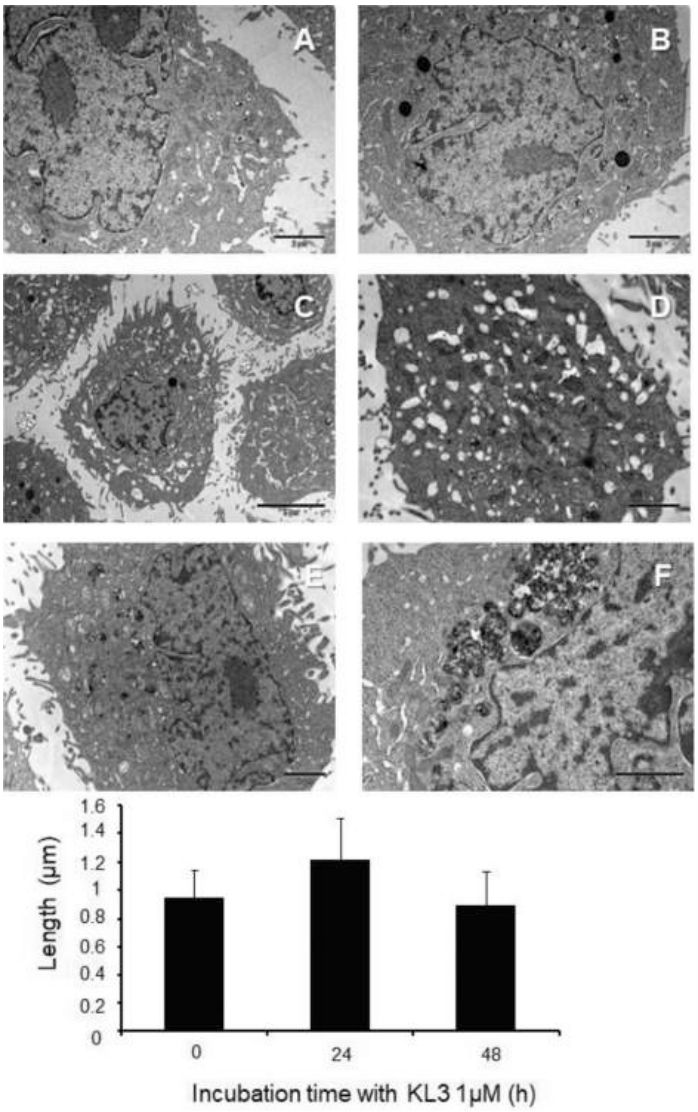

Fig. 7. Upper panel: Ultrastructure of HaCaT control cells treated with vehicle (A-B) or treated with KL3 at 1 µM for 24 h (C—D) or KL3 at 1 µM for 48 h (E-F). After 24 h distension of RER is visible, mitochondria become wider and longer. After 48 h of treatment with KL3 autophagy is visible (F). Scale bar: 5 µm A, C, E, 2 µm B, D, F.
Lower panel: Diagram displaying the length of cytoplasmic processes in control or treated cells.

3.6. KL3 does not induce direct necrosis in HaCaT cells

For evaluation of kinetic effects of examined substances, we performed real time dynamic analysis of fluorescent signal induced by disruption of cell membranes (Fig. 4). There is an increase in necrosis in cell lysis controls indicated by the black lines, while the dotted lines represent negative vehicle treated controls. In comparison with KL3, PPT at 1 µM induced necrosis (Fig. 4 left panel), while KL3 did not induce necrosis at 1 µM (Fig. 4 right panel).

3.7. Both KL3 and PPT induce cell cycle block at G2/M

To analyze distribution of cell cycle phases in HaCaT cells treated with KL3 vs PPT we performed flow cytometric analysis (Fig. 5). KL3 at 1 µM did not affect the distribution of cell cycle phases, while at 10 µM KL3 induced G2/M cell cycle arrest (58% for 24 h and 64% for 48 h). In

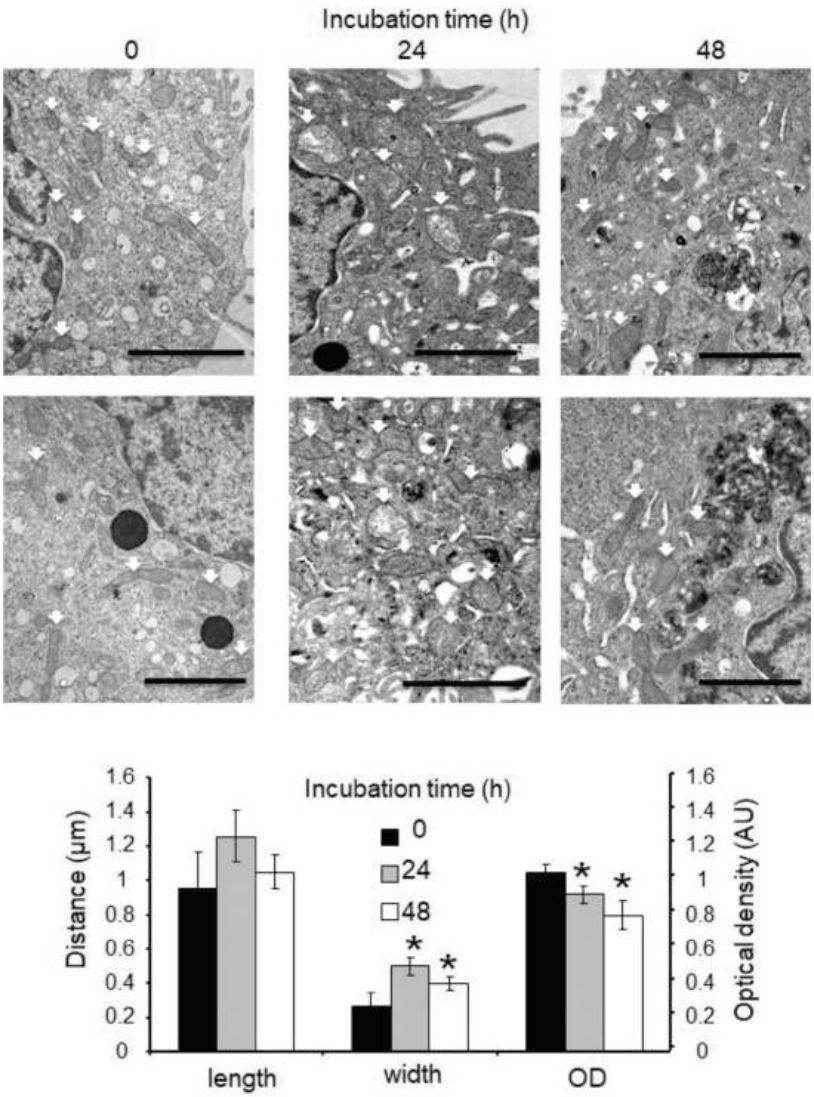

Fig. 8. Mitochondria in HaCaT cells Upper panel: Representative cytoplasmic regions displaying mitochondria in every group. Arrows indicate mitochondria. Scale bars: 2 μm.
Lower panel: Calculated length, width and optical density of mitochondria in control cells as well as in cells treated with KL3 at 1 μM for 24 or 48 h. Stars $p < 0.05$ at Student's *t*-test.

contrast, PPT evokes G2/M cell cycle arrest in both concentrations (1 μM-62%; 10 μM- 64% for 24 h, and 1 μM-63%; 10 μM- 67% for 48 h).

3.8. Ultrastructural analysis of HaCaT cells

To assess the effect of KL3 versus PPT on the ultrastructure of human keratinocytes following treatment with the same doses of KL3, we have performed ultrastructural analysis using transmission electron microscopy. In comparison to control groups we observed dose-dependent changes in cellular morphology (Figs. 6, 7). The changes were more advanced in cells treated with PPT, demonstrating disintegration of cell membrane that was first apparent at 1 μM and widespread at 10 μM. In contrast, KL3 at 1 μM did not induce such changes in cell membrane. Therefore, we have chosen KL3 at 1 μM concentration for advanced morphological studies including morphometric analysis of selected organelles.

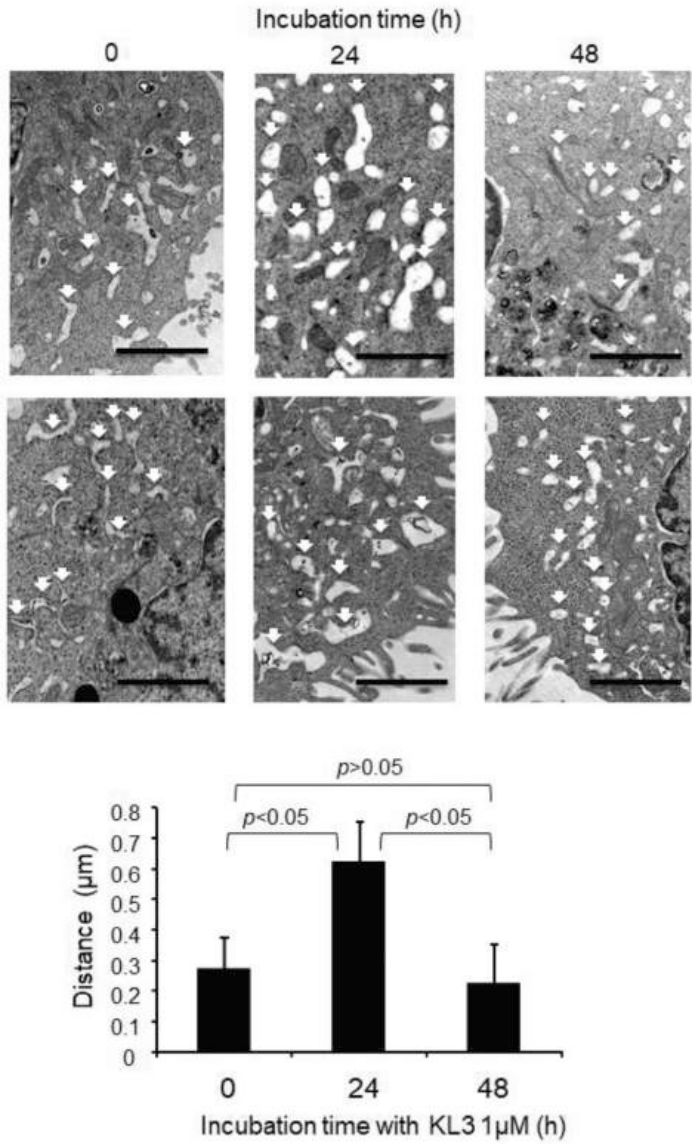

Fig. 9. Endoplasmic reticulum (ER) in HaCaT cells. Upper panel: ER (arrows) in representative cytoplasmic regions in control or treated cells (with KL3 at 1 µM at 24 or 48 h). Scale bars 2 µm. Lower panel: Diagram with calculated distance between the rough endoplasmic reticulum membranes of cisterns. There is a transient ER dilation after 24 h of incubation. This increase was significant. After 48 h of incubation, the distance between cisterns decreases and is comparable to the control group.

KL at the lowest concentration tested of 1 µM did not significantly inhibit growth of HaCaT cells as demonstrated in the viability (Fig. 2 A, B) and colony formation tests (Fig. 2 C). However, in comparison to control cells, after incubation with 1 µM KL3, changes in the ultrastructure of cytoplasmic organelles like cellular processes, mitochondria, ER and autophagic vesicles were observed (Fig. 6-8).

Initially, after 24 h of incubation, KL3 appears to induce an elongation of the cytoplasmic processes of HaCaT cells. However, after extended 48 h incubation this process seems reversed and those processes are shortened again (Fig. 7).

In the control cells, the mitochondria have visible combs and a defined width, length and electron density. In comparison, 24 h incubation of HaCaT cells with 1 µM KL3 disintegration of mitochondrial combs is observed with widening and elongation of mitochondria and decreased electron density. Nevertheless, KL3 does not appear to disrupt the integrity of the mitochondrial membranes, which remain continuous and well delimited from the cytoplasm (Fig. 8).

24 h incubation with 1 µM KL3 causes RER distension, which is a

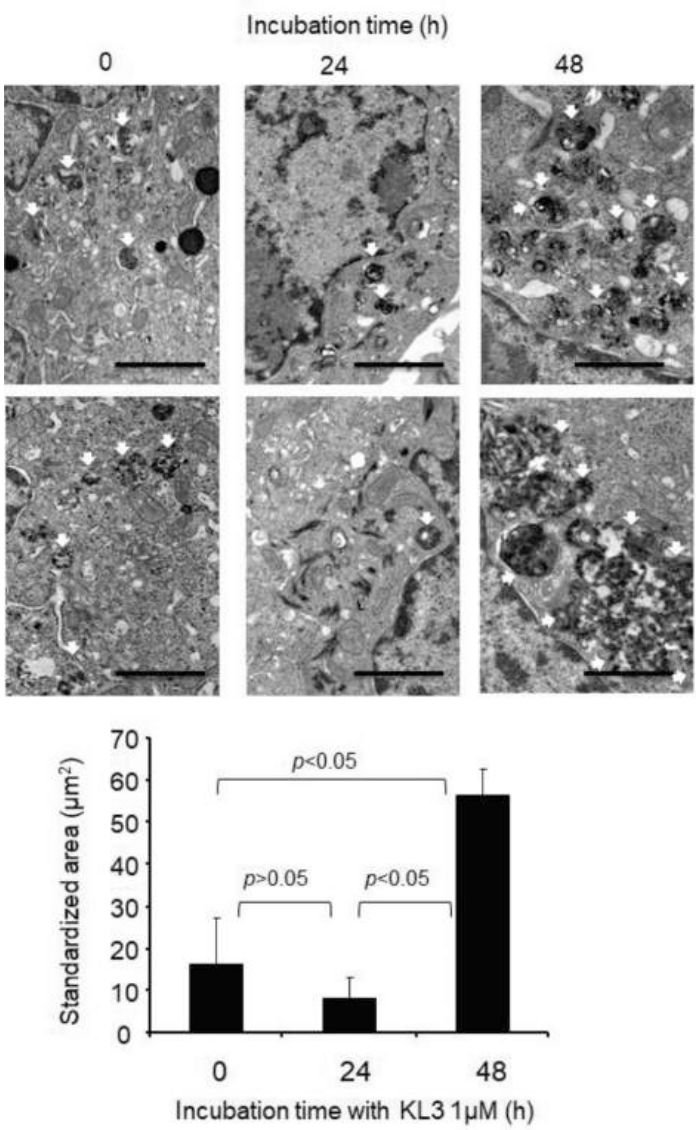

Fig. 10. Autophagy in HaCaT cells. Upper panel: Representative cytoplasmic regions display subnuclear region in control or treated cells (with KL3 at 1 μM at 24 or 48 h). Autophagic structures are marked with arrows. Scalebar: 2 μm. Lower panel: Diagram with calculated area of autophagy structures. There is an insignificant reduction in autophagy at 24 h of treatment with KL3 at 1 μM and a significant increase of autophagy after 48 h of treatment.

known manifestation of ER stress. However, this process seem reversed after 48 h, with ER having similar appearence to the controls (Fig. 9). At the same time the double-membrane encapsulated autophagic vesicles containing differentiated protein-lipid material turn out to be visible (Fig. 7). A decrease in the calculated surface of autophagosomes in the cytoplasm is observed at 24 h of incubation with 1 μM KL3. In contrast, extended 48 h incubation with1 μM KL3 causes a significant increase in the surface of the cytoplasm occupied by autophagosomes (Fig. 7 lower panel). (See Fig. 10.)

Incubation with a1μM KL3 for either 24 h or 48 h does not appear to induce any changes in the ultrastructure of cell nucleus, such as e.g. chromatin condensation, pyknosis or cell nucleus fragmentation (Fig. 7).

In summary, 24 h treatment with 1 μM KL3 induced transient ER stress, mitochondrial swelling as well as elongation of cytoplasmic

processes. The cells appear to adapt to extended exposure to KL3, and after 48 h most of those changes appear to be reversed with simultaneous induction of autophagy (Fig. 10).

4. Discussion

Due to significant toxicity of systematically administered PPT, various derivatives of this compound have been obtained in order to have decreased toxicity to non-cancerous cells and enhanced bioavailability (Zhao et al., 2019).

In this context we studied the toxicity of a novel benzothiazole containing PPT derivative referred as KL3, using the model of human keratinocytes (HaCaT) in order to assess its toxicity to noncancerous tissues. We have shown elsewhere that KL3 exerts toxic and antiproliferative effects in many cancer cell lines (Strus et al., 2018).

Here we show that in contrast to PPT low dose of KL3 (1 μM) is only slightly toxic to noncancerous epithelial HaCaT cells. This is significant, since the 1 μM concentration is higher than IC50 of KL3 for HeLa (0.5 μM), MDA-MB (0.7 μM), PC3 (0.7 μM) cancer cells (Strus et al., 2018). Therefore, in a concentration that is lethal to several cancer cell lines, KL3 did not affect significantly noncancerous HaCaT cells. Also the BPMC were not affected by KL3 at 48 h, while PPT reduced relative viability of PBMC – mainly by direct cytotoxic effect, since isolated PBMC do not proliferate in the cell culture.

KL3 appears to induce transient ER stress, mitochondrial swelling as well as elongation of cytoplasmic processes in HaCaT cells after the initial 24 h of incubation. However, in sharp contrast to the toxic PPT effects, those changes do not appear to lead to cell death and are reversed after 48 h with simultaneous induction of autophagy. This is consistent with the pro-survival role of autophagy, especially following proteotoxic stress, such as the one caused by proteasome inhibitors, also known to induce ER stress. However, in contrast to the described effects of proteasome inhibition, we have not observed the formation of aggresomes in treated cells (Wójcik et al., 1996a; Wójcik et al., 1996b).

At higher KL3 concentrations (10 μM and 100 μM) induced cell death (apoptosis and necrosis) similarly to its parent compound PPT. Activation of caspase 9 in cells treated with PPT as well as higher KL3 concentrations demonstrates that both substances induce apoptosis via the intrinsic pathway, likely downstream from ER stress induced unfolded protein response. Maximal caspase activation was observed at 24 h, that is characteristic for initiator caspases.

Similar to PPT, higher KL3 concentrations induced G2/M cell cycle arrest. Such arrest is characteristic of compounds affecting the microtubular transport mechanism, it can be predicted that novel KL3 acts in this way. Intriguingly, the same type of arrest is caused by proteasome inhibition, which also causes ER stress and increase in autophagy (Wójcik et al., 1996a; Wójcik et al., 1996b). Interestingly, proteasome inhibitors demonstrate significant synergy with topoisomerase inhibitors in terms of enhanced anti-tumor activity (Lee et al., 2016).

No previous studies were done comparing the ultrastructural effects of KL3 vs PPT on HaCaT cells. We have found only one publication reporting that PPT induces acantholysis and cytolytic changes in skin model that were described as prominent and mimicking the effect of cantharidin (Hermanns-Le et al., 1998). Cantharidin is a reference substance that induces the formation of skin blisters for toxicological studies (Hermanns-Le et al., 1998; Medina et al., 2003; Scheinfeld and Lehman, 2006; Yokel et al., 1987).

Another study was performed with a different PPT derivative using the HaCaT model, demonstrating that similarly to the KL3 compound, new derivatives were effective against T24 cells with decreased activity against HaCaT keratinocytes (Wei et al., 2019).

In our previous publications we have described the synthesis of KL3. It is worth to mention that KL3 represent a novel fusion molecule containing modification of PPT and benzothiazole. Benzothiazole was shown to have some antitumor activity on its own (Mistry et al., 2015; Reddy et al., 2019; Uremis et al., 2017). The innovatory method of synthesis of KL3 allows for unlimited scale of synthesis, with elimination of toxic substituents of donors resulting in safe, productive and clean synthesis (Lisiecki and Czarnocki, 2018; Lisiecki et al., 2017; Lisiecki et al., 2016).

Our method is therefore extremely advantageous in the context of potent big scale synthesis for potential pharmaceutical application.

5. Conclusions

An ideal anticancer drug should kill cancer cells and leave normal cells intact. Here we show that non-cancerous cells such as HaCaT cells respond with metabolic stress to the action of the new substance KL3, which is manifested by mitochondrial edema and endoplasmic reticulum stress. However, the cell's regulatory mechanisms allow recovery of vital functions since the changes were transient with tendency to normalize after prolonged incubation time.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Disclosures

C.W. is an employee of Amgen. The work presented in this manuscript was an independent project initiated prior to his employment and is not scientifically reviewed by Amgen.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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2.3. Cellular Distribution and Ultrastructural Changes in HaCaT Cells, Induced by Podophyllotoxin and Its Novel Fluorescent Derivative, Supported by the Molecular Docking Studies.

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Cellular Distribution and Ultrastructural Changes in HaCaT Cells, Induced by Podophyllotoxin and Its Novel Fluorescent Derivative, Supported by the Molecular Docking Studies

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Abstract: Podophyllotoxin (PPT) is an active pharmaceutical ingredient (API) with established antitumor potential. However, due to its systemic toxicity, its use is restricted to topical treatment of anogenital warts. Less toxic PPT derivatives (e.g., etoposide and teniposide) are used intravenously as anticancer agents. PPT has been exploited as a scaffold of new potential therapeutic agents; however, fewer studies have been conducted on the parent molecule than on its derivatives. We have undertaken a study of ultrastructural changes induced by PPT on HaCaT keratinocytes. We have also tracked the intracellular localization of PPT using its fluorescent derivative (PPT-FL). Moreover, we performed molecular docking of both PPT and PPT-FL to compare their affinity to various binding sites of tubulin. Using the Presto blue viability assay, we established working concentrations of PPT in HaCaT cells. Subsequently, we have used selected concentrations to determine PPT effects at the ultrastructural level. Dynamics of PPT distribution by confocal microscopy was performed using PPT-FL. Molecular docking calculations were conducted using Glide. PPT induces a time-dependent cytotoxic effect on HaCaT cells. Within 24 h, we observed the elongation of cytoplasmic processes, formation of cytoplasmic vacuoles, progressive ER stress, and shortening of the mitochondrial long axis. After 48 h, we noticed disintegration of the cell membrane, progressive vacuolization, apoptotic/necrotic vesicles, and a change in the cell nucleus's appearance. PPT-FL was detected within HaCaT cells after ~10 min of incubation and remained within cells in the following measurements. Molecular docking confirmed the formation of a stable complex between tubulin and both PPT and PPT-FL. However, it was formed at different binding sites. PPT is highly toxic to normal human keratinocytes, even at low concentrations. It promptly enters the cells, probably via endocytosis. At lower concentrations, PPT causes disruptions in both ER and mitochondria, while at higher concentrations, it leads to massive vacuolization with subsequent cell death. The novel derivative of PPT, PPT-FL, forms a stable complex with tubulin, and therefore, it is a useful tracker of intracellular PPT binding and trafficking.

Keywords: podophyllotoxin; HaCaT ultrastructure; podophyllotoxin with attached fluorescein; podophyllotoxin docking to tubulin; human keratinocytes; cell death

1. Introduction

Podophyllotoxin (PPT—(−)-podophyllotoxin) is an active pharmaceutical ingredient (API) that is clinically used as one of the few topical therapies to treat anogenital warts. Most cases of anogenital warts are caused by Human Papillomavirus (HPV) types 6 or 11 [1,2].

PPT was first isolated from *Podophyllum* spp. (family *Berberidaceae*). It is obtained mainly from the roots and the rhizomes of *Podophyllum peltatum* (American mayapple) and *Sinopodophyllum hexandrum* Royle (Barberry family) [3,4].

PPT has antimitotic activity and inhibits the polymerization of tubulin [5,6]. The exact mechanism underlying the antitumor effects of PPT remains unclear. Nevertheless, research indicates that PPT binds tubulin subunits, thus hindering its polymerization and formation of microtubules. PPT has a similar affinity and binding site to colchicine although PPT binds m—ore rapidly and reversibly than the irreversible binding of the latter [7]. PPT can also act as a competitive inhibitor of colchicine binding [8,9]. PPT binding leads to a G2/M arrest of the cell cycle by influencing the formation of the mitotic spindle [10]. Without the ability to divide, cells are arrested in the cell cycle and eventually die [11,12]. Due to its high toxicity, PPT can only be used topically—as either a 0.5% solution or a 0.15% cream. Even with topical use, the treatment schedule requires interruptions due to local and systemic side effects [2,13,14]. Local side effects include tenderness, erythema, erosion, and scarring of the skin in the place of application [2,15]. Less common are systemic side effects such as nausea, vomiting, diarrhea, abdominal pain, thrombocytopenia, leukopenia, abnormal liver function tests, sensory ataxia, and altered mental state [15–17].

PPT is frequently used as a scaffold for the synthesis of new derivatives that are created in the search of safer drugs with enhanced activity and less toxicity [10,18,19].

PPT is formed by five rings with four chiral centers, a trans-lactone, and an aryl tetrahydrogenated naphthalene backbone (Figure 1). Due to challenging de novo-synthesis of PPT, modifications of naturally derived PPT are a common route to produce PPT analogs. In this process, the PPT structure is modified by adding functional groups or conjugation with another molecule. In this way, new PPT derivatives are synthesized [20–23]. The anticancer PPT derivatives used in the clinic include etoposide and teniposide [24,25]. They form part of protocols for the treatment of several forms of solid tumors, leukemias, and lymphomas. In contrast to the PPT mechanism of action, they inhibit DNA-topoisomerase II and thus stop the cell cycle during mainly S and G2 phases [3,26]. Other PPT derivatives also have immunosuppressive, antioxidant, antiviral, hypolipemic, and anti-inflammatory effects, thus showing great potential for possible future therapeutic use beyond oncology [27]. Many other PPT scaffold-based agents are being investigated (e.g., etopophos, etoposide phosphate, GL331, NK-611, TOP53, and NPF). They all have demonstrated possible anticancer activity [3,28]. In particular, our research group studied KL3—another PPT derivative that was found to be less toxic for non-cancerous cells and more specific to cancer cells than the parent PPT compound [15].

Since PPT is widely used in search of new active derivatives, we believe that a better understanding of its effects at the ultrastructural level is needed; this may give us new perspectives and ideas for pharmaceutical research related to aryltetralinlactone cyclolignans.

In this manuscript we analyzed the ultrastructural changes induced by PPT in HaCaT cells, and examined the cellular distribution of fluorescein-labeled PPT. We have then used molecular docking to compare the affinity of both PPT and PPT-FL towards tubulin.

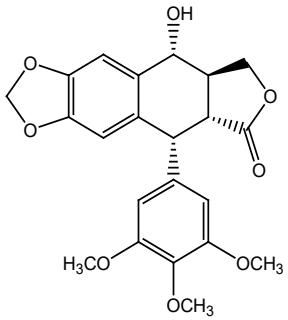


Figure 1. The structural formula of (−)-podophyllotoxin. Podophyllotoxin is an aryltetralin lignan lactone derived from *Podophyllum* spp. It consists of five rings with four chiral centers, one trans-lactone, and one aryl tetrahydronaphthalene skeleton with multiple modification sites.

2. Results

2.1. Properties of PPT-FL

According to the methodology described in Material and Methods, a new fluorescent derivative of PPT was synthesized (PPT-FL). It is a stable compound, well soluble in DMSO, and easily resolubilized in cell culture media. Next, we checked and compared the molecular docking of both PPT and PPT-FL to confirm a similar mechanism of action. Finally, both substances were tested in HaCaT keratinocytes in order to visualize their impact on cell viability and cellular distribution. PPT-FL at a concentration of 100 μM (1 μL/mL) gave bright intra-cellular fluorescence with a speckled pattern that was stable for 24 h (see results of cell-based assays).

2.2. Molecular Docking and MM/GBSA Calculations

As stated in the Materials and Methods Section 4, a careful review of the previously deposited crystal structures of tubulin bound to various ligands allowed us to choose 4 structures representing 4 different binding sites of this protein. The 1SA1 site presented the structure of the complex between PPT and tubulin; therefore, we have first docked the studied ligand to 1SA1 to check the accuracy of the molecular docking in predicting the structure of the complex and the conformation of PPT at the active site (Figure 2). This approach is known as re-docking.

The accuracy of the molecular docking in predicting the geometry of the PPT at the active site of tubulin is evidenced by the alignment of theoretically predicted and experimental structures. The rings of the PPT molecule overlap almost completely. Minor differences are seen between the angles formed by the methoxy groups.

We have also performed the molecular docking and MM/GBSA using PPT-FL instead of the parent PPT molecule (Table 1). Despite using grids of a size sufficient enough to fully accommodate PPT-FL, this ligand did not fit into the active sites of 1SA1, 4O2B, and 3HKD, which was mostly caused by steric hindrance. This suggests that PPT-FL most likely binds at another active site, which is different from the site of PPT binding. Indeed, PPT-FL formed a complex with tubulin binding at the "epothilone binding site", presented in 1TVK. This complex is stabilized by three hydrogen bonds formed between the PPT-FL and tubulin, involving residues THR274, ARG282, and GLY360.

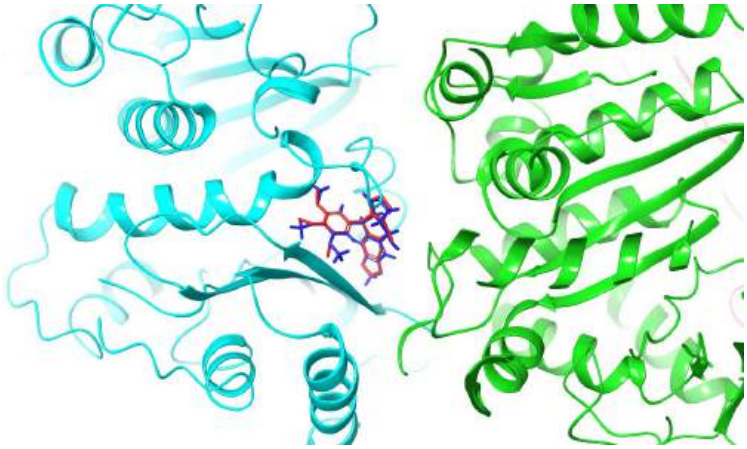


Figure 2. Comparison of the conformation and location of the PPT in the active site of tubulin (1SA1) from the crystal structure (red) and molecular docking studies (blue).

Table 1. Molecular docking and MM/GBSA calculations result for the complexes formed between tubulin and PPT/PPT-FL.

Tubulin Structure Refcode	Ligand	Glide GScore [kcal/mol]	MM/GBSA ΔG Binding [kcal/mol]	Residues Forming Hydrogen Bonds
1SA1	PPT	−8.11491	−61.9107	THR179
	PPT-FL	*	*	*
1TVK	PPT	−5.13944	−47.1387	ASN101, CYS241
	PPT-FL	−3.85146	−51.9734	THR274, ARG282, GLY360
4O2B	PPT	−7.59811	−15.5007	THR274
	PPT-FL	*	*	*
3HKD	PPT	*	*	*
	PPT-FL	*	*	*

* No stable conformation has been found during the molecular docking.

Analysis of the binding between PPT and tubulin revealed that the complex with the highest ligand affinity (−8.11491 kcal/mol and −61.9107 kcal/mol for GScore and MM/GBA, respectively; Figure 3) is obtained when the PPT is docked at the site known as the "PPT binding site" what is corroborated by the experimental data. We have also observed PPT binding at two other sites (1TVK, 4O2B). However, it had significantly lower affinities [X and Y, respectively]. In two complexes, formed with 1SA1 and 4O2B binding sites, respectively, the structure was stabilized by hydrogen bond formation between the hydroxyl group of PPT and Thr179 and Thr274 in case of 1SA1 and 4O2B binding sites, respectively. Interestingly, a complex of PPT bound to the 1TVK binding site (Figure 4) was characterized by lower affinity according to the Glide GScore and MM/GBSA values despite being stabilized by two H-bonds, formed with Asn101 and Cys24.

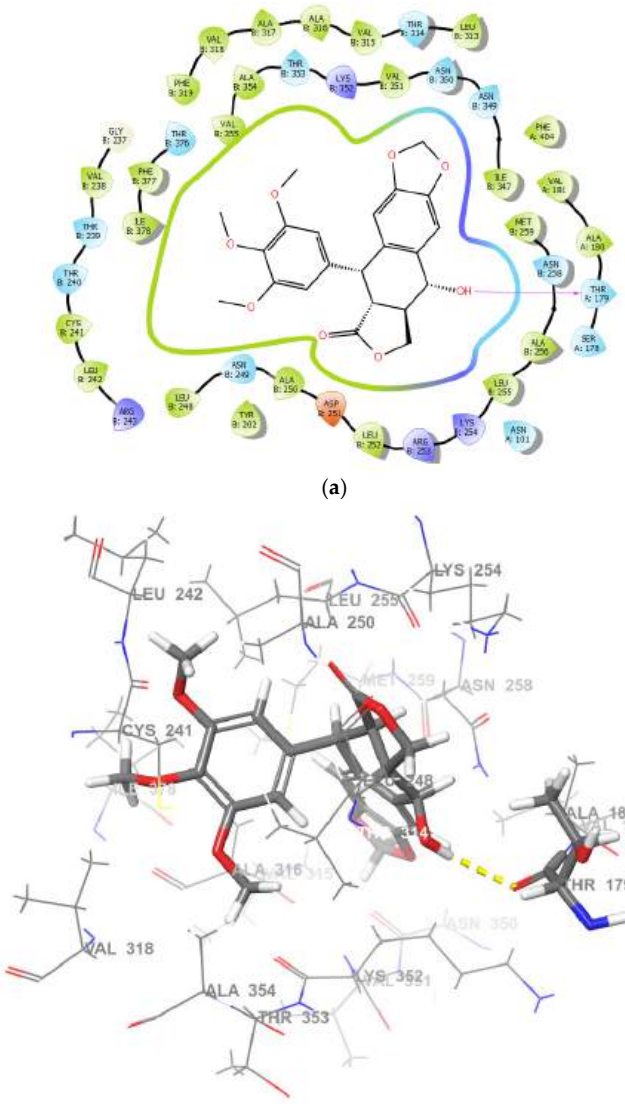
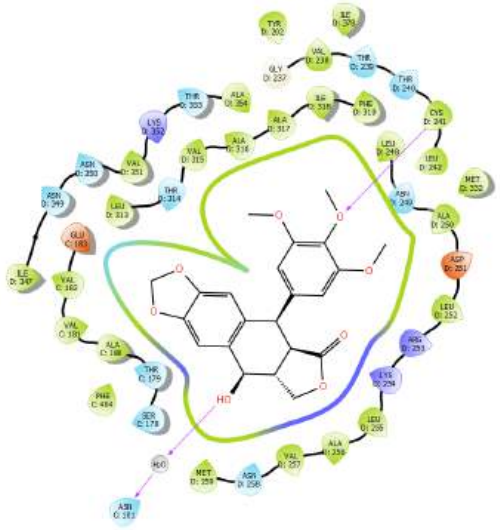
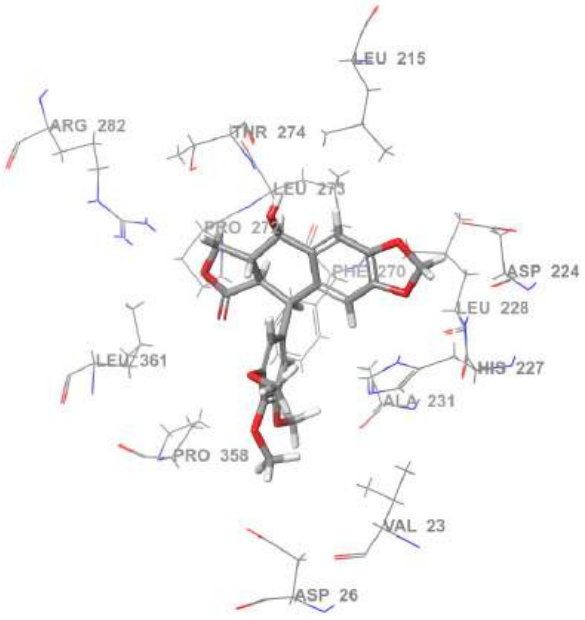


Figure 3. (**a**): Protein-ligand interactions between PPT and tubulin (1SA1). The violet line represents the H-bond. (**b**): Conformation of PPT in the active site of tubulin (1SA1). The yellow dashed line represents the H-bond.

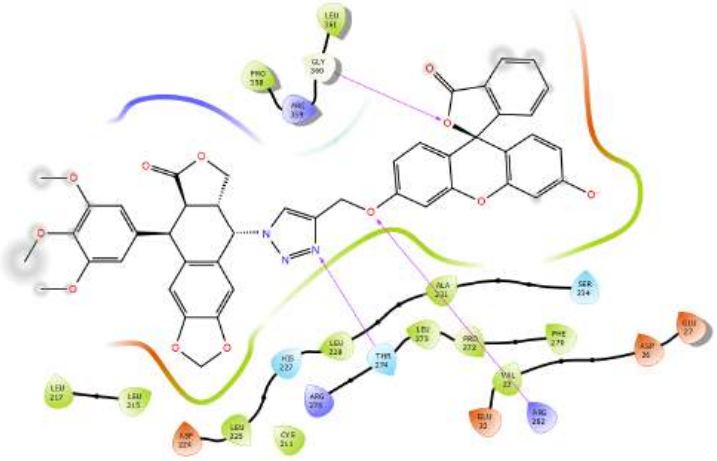


(**a**)



(**b**)

Figure 4. *Cont*.



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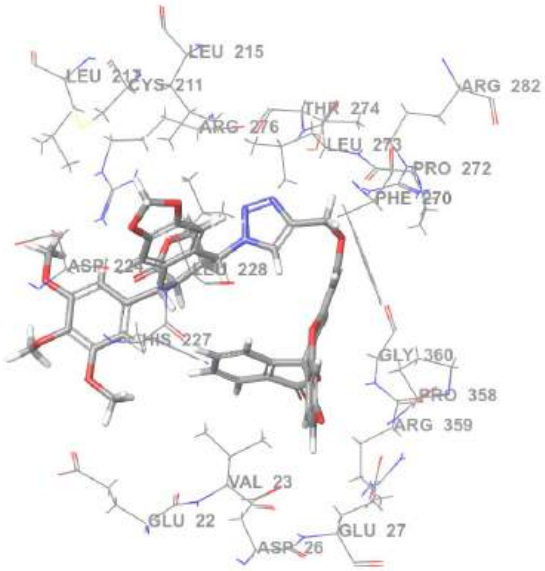
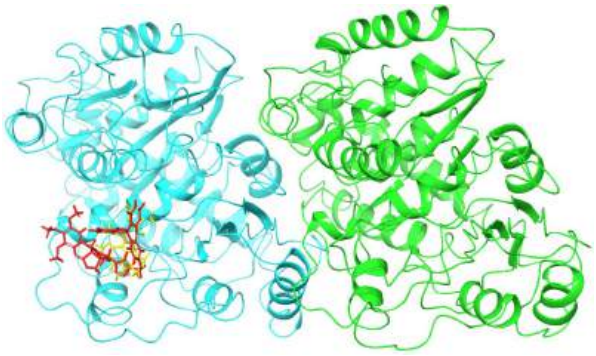


(**d**)

Figure 4. *Cont*.



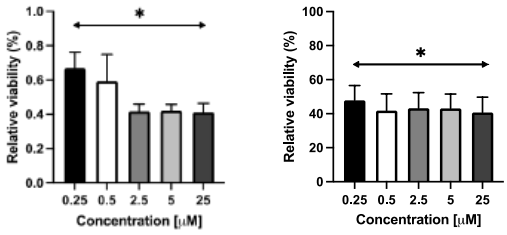
(**e**)

Figure 4. (**a**): Protein-ligand interactions between PPT and tubulin (1TVK). Violet lines represent the H-bonds. (**b**): Conformation of PPT in the active site of tubulin (1TVK). (**c**): Protein-ligand interactions between PPT-FL and tubulin (1TVK). Violet lines represent the H-bonds. (**d**): Conformation of PPT-FL in the active site of tubulin (1TVK). (**e**): Location of PPT (yellow) and PPT-FL (red) in the active site of tubulin (1TVK).

2.3. Cell-Based Assays

2.3.1. Viability Assay

HaCaT cells were treated with PPT at concentrations ranging from 0.25 to 25 μM for either 24 h or 48 h. After 24 h at 0.25 μM and 0.5 μM, PPT caused ~40% reduction in viability, leaving ~60% of live cells in comparison to controls treated with PPT solvent alone. After 48 h incubation, PPT reduced the HaCaT viability below 50% at both concentrations. This effect was dose-dependent at 0.25 μM and 0.5 μM. Above 0.5 μM, increasing concentrations demonstrated a plateau effect without further increase in cytotoxicity. Each of these concentrations reduced HaCaT viability to about 40% as early as after 24 h (Figure 5a,b). Ultrastructural changes were more pronounced with 0.5 μM treatment in comparison with 0.25 μM treatment for the same incubation time and for the longer incubation time versus the shorter incubation time for each given concentration. We calculated the IC_{50} value at both time points, and for PPT, it was 1 μM after 24 h and 0.4 μM after 48 h. In addition, the IC_{50} value for PPT-FL was 0.4 μM after 48 h of incubation.



(**a**) (**b**)

Figure 5. The viability of HaCaT cells is presented as a ratio percentage compared to the control group after incubation with increasing concentrations of PPT at 24 h (**a**) and 48 h (**b**). Stars: $p < 0.05$ at one-way ANOVA test. Compared to the control groups, for which the viability was 100% and is not shown in the figure, a statistically significant reduction in the viability of HaCaT cells was observed for each concentration. For concentrations up to 2.5 μM at 24 h, the viability-reduction effect was dose-dependent. Above this concentration, the graph flattens out, probably due to the saturation of the effects and the lack of linearity of the viability reduction effect for higher doses.

2.3.2. Intracellular Distribution of Fluorescent PPT Derivative (PPT-FL)

After docking experiments conformed binding to tubulin (albeit at different sites) of both PPT and PPT-FL, we used confocal microscopy to study intracellular localization of PPT-FL labeled structures in vivo. We have shown that PPT-FL enters HaCaT cells in a time-dependent manner. The bright intracellular fluorescence signal can be observed starting from 1 h after its addition to the cell culture media (Figure 6, middle and lower panel). Up to 10 min after PPT-FT addition, no detectable fluorescent signal was observed within cells. PPT-FL labeled structures appear to be intracytoplasmic inclusions or vesicles with speckled patterns in the cytoplasm of HaCaT cells. Formation of such inclusions appears to be a continuous process as their abundance and staining intensity increase with prolonged incubation times. After 24 h of incubation, a bright patched cytoplasmic signal was also visible. This suggests that PPT-FL is neither degraded nor excluded from the treated cells (Figure 6—lower right panel, 24 h).

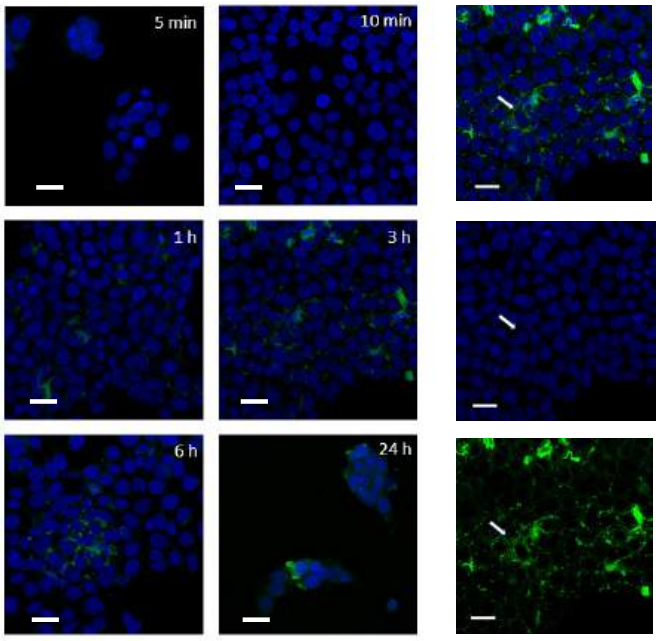


Figure 6. Time-dependent distribution of PPT-FL in HaCaT cells. Ha-CaT cells were incubated with PPT-FL (green), and nuclei were stained with HOECHST (blue). PPT-FL concentration 1 μL/mL—corresponding to 100 μM concentration of PPT. (**a**) Time-dependent analysis of PPT-FL distribution in HaCaT cells at 5 min, 10 min, 1 h, 3 h, 6 h and 24 h incubation with PPT-FL. (**b**)—Distribution of PPT-FL after 8 h of incubation—upper panel -merge, middle panel: HOECHST, lower panel: PPT-FL; speckled distribution of PPT-FL labeled structures in the cytoplasm of HaCaT cells. The white arrow shows the same cell in all three images. Scalebar: for (**a**,**b**): 20 μm.

2.3.3. Ultrastructural Analysis

To further characterize intracytoplasmic changes caused by PPT in HaCaT cells, we used transmission electron microscopy. Control HaCaT cells are displayed in Figure 7A,B. They contain a centrally located normally appearing cell nucleus with abundant heterochromatin, in one case with a nucleolus visible (B). Various cytoplasmic organelles are

localized in the peri-nucleolar region, with normally appearing mitochondria and ER. Cell membranes are continuous, and cellular protrusions are visible.

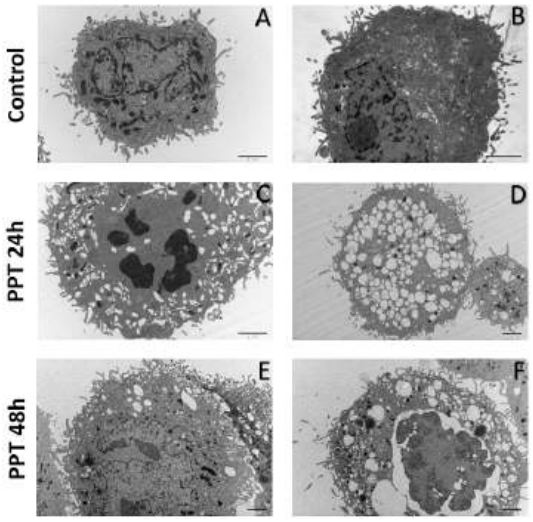
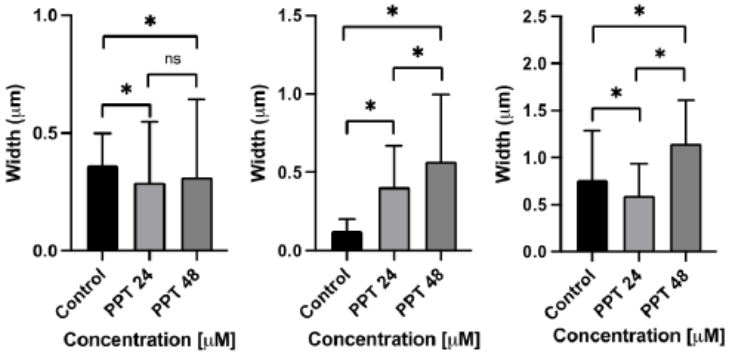


Figure 7. Ultrastructural changes of HaCaT cells treated with PTX for 24 h (**C**,**D**) and 48 h (**E**,**F**). In comparison to the control cells (**A**,**B**), PTX induces dilation of ER, suggesting presence of ER stress. All changes are more pronounced after a longer time of incubation. Scalebar 1 μm.

After treatment with PPT for 24 h, peripheral nuclear chromatin condensation is visible, while cytoplasm remains homogenous, with fewer mitochondria in comparison to controls and with intensively dilated ER. ER dilation is more pronounced in the peripheral region of the cytoplasm than in the perinuclear region (Figure 7C). The intensive vacuolization of ER is more advanced in Figure 7D—where the apical region of the cell is visualized.

Cells incubated for 48 h with PPT revealed more advanced vacuolization with sequestration of the cell nucleus (Figure 7E,F) and further dilation of ER cisternae in comparison with the shorter incubation time.

Morphometric analysis demonstrated that incubation of HaCaT cells with 10 μM PPT for 24 h led to significant morphological changes in ER, cellular processes, and mitochondria (Figure 8). The 24 h incubation with 10 μM PPT causes mitochondria shortening; however, after 48 h, mitochondria appear similar to the controls. After both 24 h and 48 h incubation, there appears to be no change in mitochondrial combs with similar width, length, and electron density. Nevertheless, despite the shortening of mitochondria, PPT does not appear to disrupt the integrity of either outer or inner mitochondrial membranes, which remain continuous and well delimited from the cytoplasm. At the same time, PPT causes progressive widening of ER cisternae, which is universally considered a hallmark of ER stress. Finally, PPT appears to induce an initial shortening of cytoplasmic processes. After an extended 48 h incubation, this shortening appears reversed, with cytoplasmic processes becoming more elongated than in control cells. We did not observe any changes in the ultrastructure of the cell nucleus, such as, e.g., chromatin condensation, pyknosis, or cell nucleus fragmentation after either 24 h or 48 h in most interphase cells, with exception of cells with massive vacuolization and disintegration of membranes where signs of apoptosis were detected (Figure 7F). In summary, 24 h treatment with 10 μM PPT-induced constant ER stress temporally shortens mitochondria as well as elongation of cytoplasmic processes.



(**a**) (**b**) (**c**)

Figure 8. Comparison of mitochondrial length (**a**), width of endoplasmic reticulum cisternae (**b**), and length of cellular protrusions (**c**) in HaCaT cells treated with PPT at 10 μM for 24 h and 48 h as compared with controls. Stars: $p < 0.05$ at one-way ANOVA test, ns: not significant.

Mitochondria showed a tendency towards reduction in mitochondrial length at both time points. The width of the ER cisternae increased in a time-dependent manner, achieving statistically significant ER widening at both time points. Cytoplasmic protrusions were shortened after 24 h, but after 28 h they were longer than in the control group.

3. Discussion

Despite advances in chemotherapy and immunotherapy, cancer remains one of the top killers, especially in developed countries, second only to cardiovascular disease. Its incidence and prevalence have increased worldwide. Therefore, the need arises for new therapeutic agents with greater precision and better safety than those currently available. This is especially true because as the disease progresses, cancer cells become drug-resistant.

The drug development process is long, arduous, and expensive. However, it can be accelerated and its costs reduced by using proven scaffold molecules with good bioavailability as a starting point for drug design.

PPT is an example of such a scaffold molecule, which has been used to obtain several derivatives [3,4]. The initial challenge was to improve PPT solubility in water to enable the systemic use of PPT derivatives [29,30]. Indeed, PPT derivatives such as etoposide and teniposide are water soluble and, therefore are used as part of systemic chemotherapy regimens against multiple cancer types [30,31]. Their antineoplastic activity is mediated via the inhibition of DNA-topoisomerase II, leading to cell cycle block and apoptosis [32]. Derivation of etoposide and teniposide was possible because of the thorough study of PPT itself [3]. We believe that additional in-depth PPT investigation will facilitate the development of further PPT derivatives with novel therapeutic applications.

The design and synthesis of the new fluorescent PPT derivative (PPT-FL) was undertaken in order to better understand the intracellular localization of PPT within normal human keratinocytes. PPT-FL turned out to be a stable compound, enabling its tracking for at least 24 h in vivo. Furthermore, this derivative was found to be stable after fixation with ethanol and did not separate from cells during washing or HOECHST staining, likely reflecting strong binding to cellular structures. As far as we know, this is the first report on the synthesis and in vivo use of a PPT fluorescein-labeled derivative.

Molecular docking analysis has confirmed that both native PPT and the PPT-FL derivative could bind to tubulin. However, it appeared that PPT-FL did not fit into the same site as PPT. Instead, PPT-FL formed a complex at another active site of the tubulin dimer, known as the "epothilone binding site" or "taxane binding pocket" [33]. Our

findings suggest that it is necessary to confirm the molecular fit of each new compound derived from a known parent molecule since spatial modification(s) can affect the docking position at the target protein complex. In addition, the inclusion of compound 2 may result in a new biological activity of PPT-FL.

Following a rapid uptake occurring within 60 min, likely via endocytosis, PPT-FL remains stable in vivo for at least 24 h. This contrasts with the short half-life of the parent PTT (1–4.5 h) and reported studies on the duration of PPT impact on the cytoskeleton [34]. PPT-FL demonstrates that it is possible to easily synthesize fluorescent PPT derivatives, which can be used to track PPT and its other derivatives within cells. This holds a promise for advancing our knowledge and potential therapeutic applications in the field of oncology [35].

Cell-based assays using HaCaT keratinocytes have confirmed the cytostatic/cytotoxic effects of PPT. This effect was dose-dependent until a plateau was reached without any further reduction of viability, with subsequently increasing doses suggesting a saturation of intracellular mechanism mediating this effect. Similar effects of PPT itself and its various derivatives have been reported using different cell culture systems [15,18]. It is important to note that even in clinical trials, PPT causes death and cytostatic effects in human keratinocytes [36].

We have focused our analysis on interphase cells, which were prevalent in our cell culture system.

Consistent with previous studies [15], PPT appeared to be highly cytotoxic. Even at low concentrations, it causes over 50% of the cells to die within 24 h. However, this effect is saturable as further increases in PPT concentrations do not result in increasing rates of cell death. Observed minor changes in mitochondrial morphology, suggests that PPT does not affect significantly mitochondrial structure or function. Initial shrinking in size followed by its recovery after a longer incubation suggests an adaptive mechanism, which requires further studies [15].

In contrast, following PPT incubation, the ER underwent significant changes, manifested as dilation of the ER cisternae, which was observed at 24 h and progressed further at 48 h. Vacuole formation likely represented extreme dilation of ER cisternae. Those morphological changes were consistent with ER stress, pointing towards the possible involvement of this mechanism in the initiation of programmed cell death in those cells, similar to previous reports [15,37,38]. ER stress is characterized by the accumulation of unfolded and/or misfolded proteins within the ER lumen, a process that can be triggered by the expression of abnormal proteins, proteasome inhibition, nutrient deficiency, hypoxia, calcium depletion, and other conditions. ER stress triggers a complex cellular reaction known as Unfolded Protein Response (UPR), which aims to restore ER homeostasis [39]. However, if UPR fails and/or ER stress is prolonged, it eventually leads to caspase activation and initiation of programmed cell death [40].

PPT caused an initial decrease in the length of cell protrusions (podia) after 24 h, which was followed by a relative increase after 48 h (albeit still below its original length). This observation indicates that PPT initially affects cytoskeletal components, resulting in a shortening of cellular protrusions. Further incubation likely initiates a compensatory response, which promotes protrusion regrowth, consistent with the known effects of PTT on filament dynamics [41,42]. This intriguing biphasic response suggests a complex interaction between PPT and cellular morphology that warrants further investigation.

This study provides valuable information on the effects of PPT on ultrastructure and viability of HaCaT cells. The observed cytotoxicity, morphological changes, and rapid cellular uptake of PPT highlight its potential as a backbone for new derivatives with better bioavailability and improved adverse effect profile. Our study has a few limitations. First, we have used only one cell line cultured in vitro, and extrapolation of these results to other cell types as well as to entire organisms should be approached with caution. Additionally, the ultrastructural changes observed in this study should be further correlated with mechanistic studies. More research is needed to investigate the

effectiveness, safety, and mechanisms of action of PPT and its derivatives in different types of cancer cells using various in vitro and in vivo models. Fluorescent labeling of PPT and its derivatives can prove a valuable tool in such an endeavor.

4. Materials and Methods

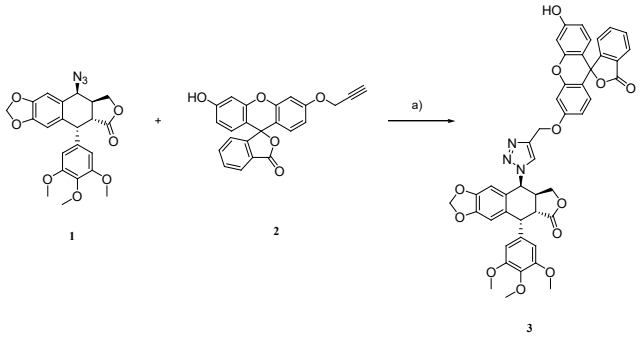
4.1. Chemicals

PPT was purchased from Sigma-Aldrich (Thermo Fisher Scientific, Inc., Waltham, MA, USA). It was dissolved in DMSO at 100 mM stock solution and kept at −20 °C until use.

4.2. Chemical Synthesis

A previously unknown fluorescently labeled PPT derivative, which can be used in numerous studies, was synthesized in a simple and efficient way (PPT-FL). First, building blocks for a "click" reaction were synthesized. Subsequently, PPT underwent an SN2 reaction, in which the hydroxyl group was exchanged with an excellent yield to an azide, giving compound **1**. In turn, fluorescein was transformed into an alkyne derivative (**2**) in one step, using an etherification reaction with propargyl bromide.

The fluorescein-labelled podophyllotoxin 3 (PPT-FL) was synthesized via a copper-catalyzed cycloaddition reaction between a PPT-derived azide (**1**) and an fluorescein-derived alkyne (**2**) (Scheme 1). The reaction was performed with acceptable yield, without generating side-products. PPT-FL isolation was straightforward and allowed us to obtain a pure compound, suitable for analytical characterization and biological testing.



Scheme 1. Synthesis of fluorescent-labeled podophyllotoxin. The following numbers in the diagram correspond to chemical formulas: (**1**) podophyllotoxin-derived azide; (**2**) fluorescein-derived alkyne; (**3**) fluorescent-labeled podophyllotoxin (PPT-FL); (a) $CuSO_4 \cdot 5H_2O$, sodium ascorbate, THF/H_2O, rt, 35%.

This method for synthesizing a fluorescent PPT derivative is simple, affordable, and can be performed even in non-specialized laboratories.

In particular, according to Scheme 1, PPT-derived azide **1** and fluorescein-derived alkyne **2** were prepared according to the procedures previously described [43,44]. A PPT-derived azide (0.05 mmol) and a fluorescein-derived alkyne (0.05 mmol) were dissolved in THF (4 mL). $CuSO_4 \cdot 5H_2O$ (0.15 eq). Then, sodium ascorbate (0.45 eq) dissolved in water (1 mL) was added to the solution, and the reaction mixture was stirred for 24 h. Subsequently, THF was removed under reduced pressure, brine (10 mL) was added, and the water solution was extracted three times with chloroform. The combined organic phases were washed with brine, dried over anhydrous $MgSO_4$, and filtered. The solvent was removed under reduced pressure, and the product was purified using column chromatography (silica gel, 2% methanol in chloroform). PPT-FL was obtained as a colorless oil with a 35% yield.

The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE apparatus. High-resolution mass spectra were recorded on the Shimadzu LC 9030 apparatus (Shimadzu, Kyoto, Japan) using the TOF MS ES+ method.

^{1}H NMR (300 MHz, $CDCl_3$), δ (ppm): 7.99 (d, J = 7.2 Hz, 1H, $H_{fluoresc}$), 7.63 (m, 2H, $2H_{fluoresc}$), 7.40 (d, J = 1.8 Hz, 1H, $H_{triazole}$), 7.14 (m, 1H, $H_{fluoresc}$), 6.82 (d, J = 2.1 Hz, 1H, $H_{fluoresc}$), 6.73 (d, J = 2.1 Hz, 1H, $H_{fluoresc}$), 6.68–6.52 (m, 4H, $2H_{fluoresc}$, H-5, H-8), 6.32 (s, 2H, H-2′, H-6′), 6.10 (d, J = 3.9 Hz, 1H, H-4), 5.97 (d, J = 13.2 Hz, 2H, O-CH_2-O), 5.14 (s, 2H, triazoleCH_2), 4.75 (d, J = 4.8 Hz, 1H, H-1), 4.37 (m, 1H, H-11), 3.81 (s, 3H, OCH3), 3.75 (s, 7H, H-11, 2OCH_3), 3.31–3.10 (m, 2H, H-2, H-3), 1.77 (br. S, 1H, OH).

^{13}C NMR (75 MHz, $CDCl_3$), δ (ppm): 173.4, 170.0, 159.8, 152.9, 152.6, 149.6, 148.3, 143.6, 137.7, 135.4, 134.4, 133.4, 129.9, 129.3, 126.8, 125.1, 124.5, 123.8, 112.8, 112.1, 110.7, 108.9, 108.4, 103.3, 102.2, 77.4, 62.0, 60.9, 59.1, 56.5, 43.8, 41.7, 37.2.

HR MS ES(+) (m/z) calculated for $C_{45}H_{36}N_3O_{12}$ ($[M + H]^+$) 810.2294, found 810.2297.

4.3. Molecular Docking

Schrödinger Maestro 12.8. version (Schrödinger, LLC, New York, NY, USA, 2023) was used for molecular docking and Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) computations.

4.3.1. Structural Modeling

Three-dimensional crystal structures of tubulin bound to the inhibitors of tubulin polymerization were retrieved from the RCBS PDB database [45]. A careful search of the RCBS database revealed four binding sites at which the ligands were located. For each of the sites, we have chosen the structure of the ligand: tubulin complex with the best resolution. Detailed information on the selected binding sites is presented in Table 2.

Table 2. Detailed information on the crystal structures of the complexes used for the molecular docking.

PDB Refcode	Resolution [Å]	Ligand Present at the Binding Site	Location of the Binding Site
1SA1	4.20	Podophyllotoxin	Between α and β subunits, closer to β
1TVK	2.89	Epothilone	β subunit, on the surface
4O2B	2.30	Colchicine	Between α and β subunits, closer to β
3HKD	3.70	(3Z,5S)-5-benzyl-3-[1-(phenylamino)ethylidene]pyrrolidine-2,4-dione	β subunit, inside

The Protein Preparation Wizard was used to prepare the structural models before docking in order to eliminate undesired metal ions and water molecules. In addition, this allows the formation of disulfide bonds, assignment of proper bond orders, adjustment of ionization states, simplification of multimeric complexes, and correction of the orientation of misoriented groups. The protein structure was further modified by adding hydrogen atoms, and standard protonation states at pH 7.0 were applied. Geometrically stable structural models were then produced by optimizing and minimizing the preprocessed models. After that, molecular docking was performed using the generated protein structure models.

4.3.2. Active Site Identification and Grid Generation

The grid center in each instance was the mass center of the co-crystallized ligand. The grid size of the cubic search box was chosen to a size that would allow the ligands to fit within completely. Using the OPLS 2005 force field, receptor grids were created using the default values for the charge cutoff (0.25) and van der Waals scaling factor (1.00).

4.3.3. Ligands Preparation

Using LigPrep from the Schrödinger Suite, the investigated ligands were made ready for docking: protonation states were produced at pH 7.4 ± 2.0 while maintaining the chiralities. Utilizing the OPLS 2005 forcefield, the geometry optimization was performed. The default values for LigPrep's other options were maintained.

4.3.4. Glide XP-Ligand Docking

The Grid-based Ligand Docking with Energetics (Glide) module and the extra precision (XP) approach were used to accomplish flexible protein-ligand docking. The first Glide docking stage's preset 20 postures were kept during the docking process. The glide score, or GScore, was determined as:

$$\text{GScore} = 0.065 \times \text{vdW} + 0.130 \times \text{Coul} + \text{Lipo} + \text{Hbond} + \text{Metal} + \text{BuryP} + \text{RotB} + \text{Site}$$

where vdW: van der Waals energy; Coul: Coulomb energy; Lipo: Lipophilic term; Hbond: Hydrogen-bonding; Metal: Metal-binding term; BuryP: Buried Polar groups' penalty; RotB: Penalty for rotatable bonds that have been frozen; Site: active site polar interactions.

4.3.5. Evaluation of Molecular Docking

Re-docking and cross-docking procedures were performed to assess molecular docking accuracy. Re-docking is the capacity to replicate the co-crystallized binding geometry and orientation of the related ligand in a rigid macromolecule form. Cross-docking is the process of comparing distinct ligand structures isolated from several crystal structure data of the same protein to a single rigid protein model structure. In all situations, success is measured as the root mean squared deviation (RMSD) between the docked position and the corresponding crystal conformer. RMSD levels below 2.0 Å are regarded desirable, although values near zero are optimal.

Glide's cross-docking script, xglide.py, was used to perform re-docking and cross-docking using the seven available crystal structures of tubulin stathmin-like domain complexes, refcodes 1SA1, 3HKC, 3HKD, 3HKE, 3N2G, 3N2K, 3UT5. Ligands from each structure were docked to the enzyme from each complex, using the Glide extra precision approach. The quality of fit was evaluated on the basis of ligand RMSD values with the following ranges: RMSD $\leq$ 1.0 Å for good pose, 1 Å < RMSD $\leq$ 2 Å for close pose, 2 Å < RMSD $\leq$ 3 Å for pose with errors and RMSD > 3 Å for bad pose. The calculated results are presented in Table 3.

Table 3. Matrix plot for root-mean-square deviation (RMSD, Å) analysis. Yellow color for values above 2.0 Å, dark green for values lower than 2.0 Å but higher than 1.0 Å, light green for values less than 1.0 Å.

	1SA1	**3HKC**	**3HKD**	**3HKE**	**3N2G**	**3N2K**	**3UT5**
1SA1	0.186	1.748	2.081	1.579	1.843	0.983	1.125
3HKC	0.874	0.218	1.482	2.210	1.645	0.598	0.874
3HKD	0.596	0.874	0.235	1.764	1.711	0.784	0.986
3HKE	1.417	1.742	1.784	0.342	1.832	0.899	0.763
3N2G	2.081	1.385	1.463	2.145	0.217	0.698	0.984
3N2K	1.573	1.428	1.748	1.125	0.874	0.213	0.892
3UT5	0.421	1.859	1.984	1.854	1.461	1.123	0.329

As expected, the lowest RMSD values were obtained for the re-docking. The lack of poses with errors and a significant majority of good poses indicate the good accuracy of the Glide docking approach and its applicability to perform the molecular docking for the studied compounds.

4.3.6. MM-GBSA Calculations

To evaluate ligand binding affinities in silico, binding free energy (ΔG_{bind}) values were calculated. The Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) rescoring approach was utilized to accurately calculate free energies of binding. The Prime MM/GBSA module was employed for this purpose.

For the first ligand-docked postures with the best scoring functions, MM/GBSA rescoring was conducted. Using the VSGB solvent model and the OPLS 2005 force field, the free energy changes that occur during protein-ligand interactions were computed.

The values of binding free energy were computed using the subsequent formula:

$$\text{MM/GBSA } \Delta G_{bind} = G_{complex(optimized)} - (G_{protein(optimized)} + G_{peptide(optimized)})$$

Free energy of each state, i.e., of complex, protein, and peptide, was estimated by accounting for molecular mechanics energies, solvation energies, and entropic terms as follows:

$$G = G_{int} + G_{Coulomb} + G_{vdW} + G_{GB} + G_{lipo} - TS$$

where G_{int}, $G_{Coulomb}$, and G_{vdW} are standard MM energy terms for bond (covalent, angle, and dihedral), Coulomb (electrostatic) and van der Waals interactions, G_{GB} and G_{lipo} are polar and non-polar (lipophilic) contributions to the solvation free energies, while T is an absolute temperature and S is an entropy value. Polar contribution (G_{GB}) was calculated using the Generalized Born model, while non-polar contribution (G_{lipo}) was estimated based on the solvent-accessible surface area (SASA).

4.4. Cell-Based Methods

4.4.1. Cell Culture

HaCaT cells (ATCC, Manassas, VA, USA), not exceeding 6 months of cell culture, were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing stable glutamine and supplemented with 10% FBS and a standard antibiotic antimycotic solution (Gibco). Cells were grown in a humidified atmosphere at 37 °C and passaged every three days with a standard Trypsin-EDTA solution (Gibco). Cells were tested for mycoplasma contamination by staining with HOECHST (Sigma-Aldrich, Saint Louis, MO, USA) and followed by confocal microscopy analysis using a Leica-SP4 (Leica, Wetzlar, Germany) microscope.

4.4.2. Viability Assays

To determine the cytotoxic/cytostatic effects of compounds involved in this study, we performed a Presto Blue assay (Thermo Fisher) as described in [46]. For this assay, cells were seeded in 96 well plates (Nest Biotechnology, Wuxi, China) at the density of 10^4 cells/well and treated in quadruplicates with increasing concentrations of PPT for 24 h or 48 h. After incubation, the Presto blue assay was performed according to the manufacturer's instructions. The absorbance of a particular probe was read in a Fluostar plate reader at 550 nM (BMG Labtech, Ortenberg, Germany). Cell viability was expressed as a percentage of the control (vehicle-treated) cells. All results in the study were based on at least three experiments. The experimental compounds were dissolved in DMSO, which could affect cell viability. Therefore, an initial experiment was performed to investigate the toxicity of DMSO. We tested DMSO in increasing concentrations, ranging from 0.1% to 5% for 48 h on HaCaT cells. The maximum concentration of DMSO, which did not decrease cell viability, was found to be 0.5%. We, therefore used 0.1% as the maximal DMSO concentration as a solvent, which constituted the control for cell-based assays. We determined viability curves from the viability test data, and from these curves, we calculated the concentration causing 50% of the effect compared to the controls, hereinafter referred to as IC_{50}. The IC_{50} was estimated for both PPT and PPT-FL.

4.4.3. Confocal Microscopy

Distribution of fluorescence associated with PPT-FL in HaCaT cells was performed using confocal microscopy (Zeiss LSM 510, Oberkochen, Germany). After incubation with PPT-FL in culture media—cells were washed 2 × 5 min with PBS, fixed for 2 min in cold, buffered 70% ethanol, washed twice with PBS, soaked in 0.9% NaCl, stained with Hoechst (Sigma Aldrich, Saint Louis, MO, USA) according to manufacturer's procedure, finally mounted in Vecta Shield mounting medium (Vector Laboratories, Zürich, Switzerland). An argon laser (488 nm) was used for the excitation of PTX-FL, and diode 405 nm was used for the excitation of Hoechst. Images were processed using the software package ZEN 2008.

4.4.4. Transmission Electron Microscopy (TEM)

HaCaT cells were plated on tissue culture dishes, treated with PPT for 24 h or 48 h, then washed in PBS, trypsinized, harvested, and centrifuged. The cell pellet was fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate (NaCac) buffer (pH 7.4), postfixed in 2% osmium tetroxide in NaCac, stained en bloc with 2% uranyl acetate, dehydrated with a graded ethanol series, and embedded in epon resin (all from Sigma Aldrich). Ultrathin sections were cut with a diamond knife on a Leica EM UC6 ultramicrotome (Leica Microsystems, Bannockburn, IL, USA), collected on copper grids, and stained with uranyl acetate and lead citrate. Cells were observed in a JEM 1400 transmission electron microscope (JEOL Co., Tokyo, Japan 2008) at 80 kV and imaged with an 11 Megapixel TEM Morada G2 (EMSIS GmbH, Munster, Germany) camera.

4.4.5. Morphometric Analysis of TEM Images

By comparing the scale bar with the length and width of cell processes, ER, and mitochondria, their median longitudinal (length) and transverse (width) dimensions were calculated by means of ImageJ 1.54i software (National Institutes of Health, Bethesda, MD, USA). We analyzed 10 representative images from one different HaCaT cell each. In each image, we labeled at least 15 cell processes, at least 10 mitochondria, and 10 ER cisternae. Due to their different cross-sections in space, we analyzed only changes in their width. In both cases, length and width was counted from the outermost layer of membranes starting at one pole of the organelle to the outer layer of the membrane at the other pole of the organelle. Length was defined as the dimension along longer axis and for width along the shorter axis. In the case of cellular processes, we considered their length, counting the distance from their origin on the cell membrane, ending at the most distal point of the process. In case of curved processes we have measured their actual length rather than the shortest straight line between two determined points.

4.4.6. Statistical Analysis

Statistical analyses have been performed using the GraphPad Prism version 9.0 for macOS (GraphPad Software, San Diego, CA, USA). The distribution was analyzed with the Kolmogorov–Smirnov test. Normally distributed data were presented with means and standard deviation (SD), whereas the non-parametric data were presented with median and percentiles 25th and 75th (interquartile range). Categorical variables were presented by percentages within each group. Between-group comparisons were carried out with one-way ANOVA test. The results were considered significant for $p < 0.05$.

5. Conclusions

PPT is toxic to healthy human keratinocytes even at low concentrations. As demonstrated with its fluorescent derivative (which forms complex with tubulin just like the parent compound), PPT promptly enters the cells, probably via endocytosis. PPT-FL is a useful tool to study intracellular changes associated with PPT treatment. At lower concentrations PPT causes swelling of the ER cisternae under ER- and mitochondrial stress. At higher concentration this leads to massive vacuolization of the cytoplasm with subsequent cell death occurring with longer incubation times. Human keratinocytes display

a biphasic response to PPT suggesting the presence of adaptive mechanism(s) requiring further studies.

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2.4. The Effects of Podophyllotoxin Derivatives on Noncancerous Diseases: A Systematic Review.

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Review

The Effects of Podophyllotoxin Derivatives on Noncancerous Diseases: A Systematic Review

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Abstract: Podophyllotoxin (PPT) is commonly used for genital warts due to its antimitotic properties and relatively good accessibility since it can be extracted from plants in low-economy countries. However, due to relatively high toxicity, it cannot be used in a systematic way (intravenously). Thus, there is a need to find or create an equally effective derivative of PPT that will be less toxic. Natural PPT is a suitable and promising scaffold for the synthesis of its derivatives. Many of them have been studied in clinical and preclinical models. In this systematic review, we comprehensively assess the medical applications of PPT derivatives, focusing on their advantages and limitations in non-cancerous diseases. Most of the existing research focuses on their applications in cancerous diseases, leaving non-cancerous uses underexplored. To do that, we systematically reviewed the literature using PubMed, Embase, and Cochrane databases from January 2013 to January 2025. In total, 5333 unique references were identified in the initial search, of which 44 were included in the quantitative synthesis. The assessment of the quality of eligible studies was undertaken using the PRISMA criteria. The risk of bias was assessed using a predefined checklist based on PRISMA guidelines. Each study was independently reviewed by two researchers to evaluate bias in study design, reporting, and outcomes. Our analysis highlights the broad therapeutic potential of PPT derivatives, particularly in antiviral applications, including HPV, Dengue, and SARS-CoV-2 infections. Apart from their well-known anti-genital warts activity, these compounds exhibit significant anti-inflammatory, antimitotic, analgesic, and radioprotective properties. For instance, derivatives such as cyclolignan SAU-22.107 show promise in antiviral therapies, while compounds like G-003M demonstrate radioprotective effects by mitigating radiation-induced damage. To build on this, our review highlights that PPT derivatives, apart from anti-genital warts potential, exhibit four key properties—anti-inflammatory, antimitotic, analgesic, and radioprotective—making them promising candidates not only for treating viral infections such as HPV, Dengue, and SARS-CoV-2 but also for expanding their therapeutic potential beyond cancerous diseases. In conclusion, while PPT derivatives hold great potential across various medical domains, their applications in non-cancerous diseases remain limited by the scarcity of dedicated research. Continued exploration of these compounds is essential to unlock their full therapeutic value.

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Keywords: podophyllotoxin; podophyllotoxin derivatives; noncancerous; genital warts; antimitotic; clinical studies

1. Introduction

For centuries, observations and research have been conducted on the medicinal properties of natural substances. These organic compounds have a wide variety of medical applications and attract the special attention of scientists due to their good availability and low acquisition cost. Moreover, such compounds, after simple purification, were accessible to people long before modern chemistry allowed large-scale synthesis of any compounds. Their high effectiveness in treating various types of diseases comes from their antiviral, antibacterial, and anticancer properties [1–4].

While the anticancer properties of PPT and its derivatives have been extensively studied, there is growing interest in their applications for non-cancerous diseases. These include their potential antiviral, analgesic, and radioprotective effects, which have been less explored in comparison to their oncological uses. Investigating these properties is essential, as they may provide new therapeutic options for viral infections, inflammatory conditions, and radiation-induced damage.

Podophyllum pellatum is an example of a plant-based substance. Native American tribes used it against helminths as a laxative and remedy for snake bites [5]. Its properties come from the fact that plants from the genus *Podophyllum*, belonging to the *Berberidaceae* family, are a natural source of a chemical substance called podophyllotoxin (PPT). It is a heterocyclic lignan that can be derived from its roots and rhizomes [6,7]. Podophyllotoxin's structure was first described in the 1930s [8]. The molecule has a five-ring arrangement, with four chiral centers, and contains functional groups: hydroxyl, lactone, acetal, and three methoxy groups, as shown in Figure 1 [8,9].

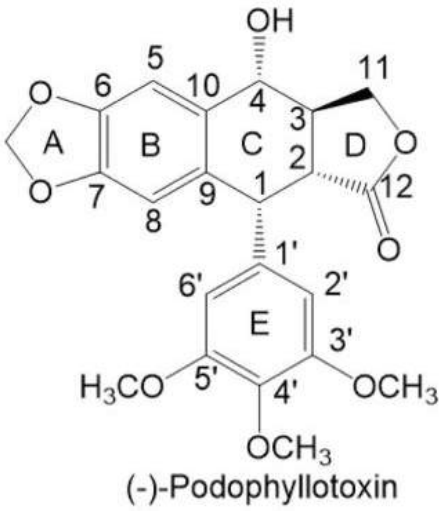

Figure 1. Podophyllotoxin's structure. Adopted from [8].

The specific mechanism of action of PPT is a frequently researched topic that still is only partially elucidated [10–12]. Analysis of the molecular structure suggests that the aromatic and dioxol rings can interfere with microtubules [9,13–15]. Scientific research proves that it

inhibits the polymerization of tubulin and, therefore, stops the production of microtubules. This leads to the impaired formation of the karyokinetic spindle, which is a crucial part of a cell division process, and cell cycle arrest, which gives the PPT antimitotic properties [16]. Moreover, since microtubules are necessary for the movement of intracellular vesicles by kinesin and dynein, interphasal cells are also affected by PPT. We have recently shown that PPT and its derivatives can induce an unfolded protein response and aggresome formation [17].

The 20th century saw a notable shift in the use of podophyllotoxin. The first reported application of PPT in genital warts treatment dates back to 1942 by Kaplan IW [18], and it is being used in this indication, nowadays commonly known as Wartec or Condyline [19,20].

Apart from its use in treating genital warts, PPT has also been explored for managing other dermatological conditions, such as psoriasis, molluscum contagiosum, and keratoacanthoma. These initial studies highlight its potential beyond oncology, particularly in viral and inflammatory skin conditions, although research in these areas remains limited.

With advances in pharmaceutical research, scientists began exploring its potential in cancer and non-cancerous treatments. As an advantage, PPT does not accumulate in the body—its half-life ranges from one to four and a half hours [21]. Like almost every medicine, it also has its drawbacks. Despite promising results from numerous studies demonstrating the high efficacy of PPT against cancerous cells [12,22,23], it turned out to be too toxic for systemic use (e.g., intravenous or intraperitoneally) [24,25]. Preclinical animal studies report that systemic application of PPT leads to its binding to muscle-free tubulin and causes changes in the metabolic pathways of skeletal muscle [26]. Additionally, scientific data is proving its local and systemic toxicity in humans. With its local use, side effects are observed, such as inflammation at the place of application (mostly glans and foreskin), itching, ulceration, pain, burning, dry skin, or bleeding [27–29]. PPT should not be used orally, and if accidentally swallowed, dangerous symptoms of poisoning may appear—diarrhea, abnormal heart rhythm, dizziness, bone marrow arrest, and respiratory failure [30,31]. Podophyllotoxin's toxicity can manifest itself even after topical application in high doses, resulting in coma with major neurologic, hematologic, and hepatic complications [32]. There are indications that podophyllotoxin can be used for pregnant women, but it may also affect fertility and be teratogenic [33]. Thus, Condyline is approved with the description: Do not use during pregnancy or breastfeeding [20].

Despite significant progress in understanding the therapeutic potential of podophyllotoxin and its derivatives, their applications in non-cancerous diseases remain underexplored. Existing reviews primarily focus on anticancer applications, leaving a knowledge gap in evaluating their broader pharmacological potential, particularly in the context of antiviral, analgesic, and radioprotective effects. Therefore, despite Podophyllotoxin being effective towards cancer cells [34], its antineoplastic use is limited to condyloma acuminata topical treatment [28]. This limitation arises from its selective cytotoxic effects on rapidly proliferating cells, such as those found in warts caused by the human papillomavirus (HPV), which parallels its potential antineoplastic mechanisms as discussed later in this manuscript. Other skin conditions, in which PPT use was introduced, include psoriasis [35], molluscum contagiosum [36], and keratoacanthoma [37].

To find better alternatives for PPT, scientists focused on the synthesis of its derivatives. Structural modifications of PPT led to the discovery of compounds such as teniposide and etoposide [38]. Contrary to the precursor compound, their antimitotic mechanism of action consists of the interaction with an enzyme—topoisomerase II, whose inhibition causes breaks in DNA [39].

Etoposide is a semi-synthetic derivative, a D-glucose PPT's glycoside, as shown in Figure 2. It is commonly used to treat retinoblastoma, ovarian cancer, testicular cancer, acute leukemia, malignant granuloma, and small-cell lung cancer [40–43]. Moreover, it

became the drug of first choice for the treatment of many malignancies such as testicular and small cell lung cancer [44]. Etoposide's systemic application was shown to induce a complete response in up to 25% of previously treated patients with acute nonlymphocytic leukemia [45].

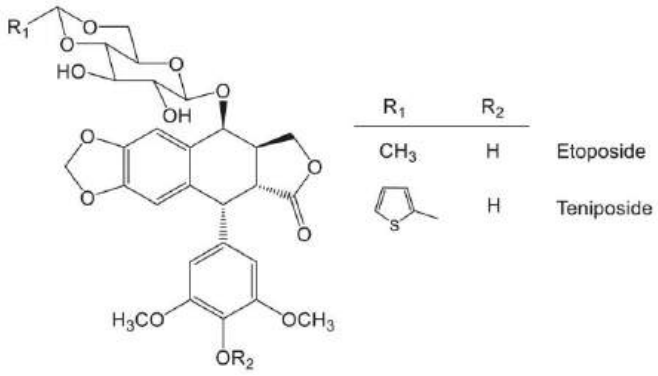

Figure 2. Etoposide and teniposide. Adopted form [17].

Another PPT derivative, teniposide, as shown in Figure 2, is chemically similar to etoposide. It is distinguished only by the thienyl group, whereas etoposide has a methyl group. It is mainly used to treat Hodgkin's lymphoma, acute lymphocytic leukemia, bladder cancer, and immature neuroblastoma [46].

Etoposide and teniposide are both derivatives of podophyllotoxin. The difference occurs in unmethylation along with an additional group of D-glucose derivatives (with methyl in the case of etoposide or thienyl in teniposide)—the differences are presented in Figures 1 and 2.

Even though PPT and its derivatives are widely researched as anticancer agents, we studied whether the derivatives are also used in non-cancerous diseases, choosing mainly clinical studies with such compounds.

We aimed to investigate the effects of PPT and its derivatives in the case of non-cancerous diseases [47].

Therefore, using database searches using specific keywords, we examined scientific data on this previously unexplored topic, formulated conclusions, and conducted an analysis regarding future applications of PPT and its derivatives in non-cancerous diseases. Only original research papers published within the last 10 years were qualified for further analysis.

This paper aims to systematically review and analyze the latest studies on PPT and its derivatives, focusing on their applications in non-cancerous diseases. By highlighting their therapeutic advantages, limitations, and potential, we seek to underscore the need for further research in this underexplored area to fully understand and expand their clinical utility.

2. Methods

This systematic review was conducted following the guidelines outlined by the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement [48]. On 15 January 2025, we searched the MEDLINE/PubMed, Embase, and Web of Science databases for papers about podophyllotoxin and its derivatives and their medical applications. Additionally, we consulted the reference lists of included studies to identify potentially relevant papers. No organizational registers or other external databases were

used. We selected papers that included "podophyllotoxin" or "PPT" and "non-cancerous" or "disease" or "antiviral" in their title or abstract. The combined search strategy is presented in Table 1.

Table 1. The combined search strategy for the systematic review.

Database	Number of Records	Search Strategy
PubMed/MEDLINE	408	((podophyllotoxin[Title/Abstract]) OR (ppt[Title/Abstract])) AND ((non-cancerous[Title/Abstract]) OR (antiviral[Title/Abstract]) OR (disease[Title/Abstract])) AND (("2013/01/01"[Date—Publication]: "3000"[Date—Publication]))
Embase	1104	('podophyllotoxin' OR 'ppt') AND ('non-cancerous' OR 'antiviral' OR 'disease') AND [2013–2025]/py AND 'article'/it
Web of Science	757	TS = (podophyllotoxin OR ppt) AND TS = (non-cancerous OR antiviral OR disease) AND PY = 2013–2025

We narrowed our search and added time restrictions to publications from January 2013 to January 2025. Using this algorithm, we identified 533 records after removing duplicates. All of the 533 papers eligible for abstract-based screening were randomly divided into two subgroups: Subgroup A, consisting of 266 records and Subgroup B, consisting of 267 records. We used EndNote Web to collect the data. Data collection from reports was conducted independently by two reviewers. No specific tools for data confirmation or investigator correspondence were utilized. Each subgroup was analyzed by a different pair of researchers. Two independent researchers reviewed every record and decided if it fulfilled all the inclusion criteria listed in Table 2. Based on this assessment, each of them made their own decision if the article should be included in our study. If they agreed, the article was included in this review. In case of any dispute, the third independent researcher made the final inclusion decision. The inter-rater agreement was assessed using Cohen's kappa coefficient. The reviewers evaluated the articles based on 'yes' or 'no' criteria. The calculated kappa values were 0.801 for Subgroup A and 0.816 for Subgroup B, indicating excellent agreement in both subgroups. No automation tools were used in the selection process; all records were screened manually. The exact exclusion criteria are listed in Table 2. We did not count how many articles were excluded by each of the criteria because numerous studies fulfilled two or more of them. Only original research papers were qualified for further analysis. Studies were grouped for synthesis based on their primary focus on the non-cancerous applications of podophyllotoxin and its derivatives, including antiviral, anti-inflammatory, analgesic, and radioprotective effects. Data items included outcomes related to antiviral, anti-inflammatory, analgesic, and radioprotective effects of PPT derivatives. Participant characteristics, intervention details, and funding sources were also collected when reported. We rejected records only about the anticancer activity of PPT and its derivatives, as well as articles without available abstracts or with abstracts written in a language other than English.

We created a table of evidence and assessed 63 full-text articles for eligibility. Furthermore, we excluded records without full text available in English, and unfinished articles. We only included adequate-quality articles with full text available in English. As an outcome of the selection process, there were 44 articles qualified for the final synthesis. No

specific examples of excluded studies are highlighted, as all exclusions were based on predefined eligibility criteria, such as language or focus on anticancer activity enlisted in Table 2. Selected articles were classified into folders based on the specific subtopics described. This systematic review focuses on identifying and categorizing the applications of podophyllotoxin and its derivatives in non-cancerous diseases. Given the heterogeneity of the included studies, effect measures such as mean differences or risk ratios were not uniformly applicable. Instead, the findings were synthesized narratively to provide a comprehensive overview of the therapeutic potential and limitations of these compounds. A tabular presentation of findings was used to summarize the results and highlight key trends. Due to heterogeneity in study designs, outcomes, and measures reported, narrative synthesis was employed to integrate findings. Subgroup analyses and sensitivity analyses were deemed inappropriate due to data limitations. Reporting bias was not systematically assessed, as the data were insufficient for funnel plot generation. Similarly, the certainty of evidence was not formally graded, given the exploratory nature of this review and the absence of standardized outcome measures across studies. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 flow diagram was used to illustrate the extraction of studies through the different phases of this review, Figure 3 [48].

Table 3. The flowchart of the systematic review methodology presenting the duplicate removal, abstract and full-text analysis of the records

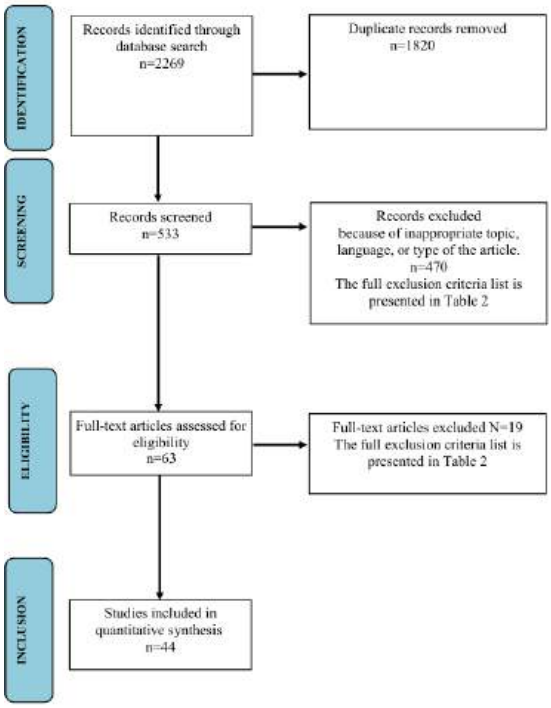

Figure 3. Flow diagram—the selection process of the articles. Adapted from "The PRISMA 2020 statement: an updated guideline for reporting systematic reviews" [48]. Copyright by the British Medical Association.

Table 2. The inclusion and exclusion criteria of the articles.

Inclusion Criteria	Exclusion Criteria
Original studies about Podophyllotoxin and its derivatives	Meta-analysis, systematic review, books, guidelines
Language of the article: English	Other unoriginal articles
Describing non-cancerous application and activity	Language other than English
Human, animal, cellular, and molecular studies	Not finished work
Good quality of the research	Articles about only anti-cancerous activity

3. Results and Discussion

3.1. Results

3.1.1. Toxic Effects of PPT and Its Derivatives

Toxicity of PPT to Non-Cancerous Cells

The first indication for the use of podophyllotoxin were genital warts, which are formed from keratinocytes (human skin cells). Studies in cellular models in the 1970s and 1980s focused on the characterization of the mechanisms of action, primarily in the human keratinocyte cell line HaCaT.

Podophyllotoxin (PPT) exhibits various mechanisms of inducing cell toxicity such as metabolic stress by mitochondrial oedema, and disruptions in protein metabolism in the endoplasmic reticulum and Golgi apparatus. Moreover, meiosis inhibition caused by destruction of chromosome alignment, spindle deformation, and DNA damage, especially in oocytes, were noticed. PPT is associated with epithelial cell proliferation and an increase in TNF-α, which causes general inflammation in the tissues. Subsequently, inflammation increases the overall expression of cytochrome c, caspase-8, caspase-9, and caspase-3, which contribute to caspase-induced apoptosis [10,49].

Similar conclusions were drawn when investigating the effects of feeding rats with PPT obtained from Juniperus sabina. Despite the noticeable effect of caspase activation, the TNF-α increase was correlated with the administered dose of podophyllotoxin, which also induced apoptosis. Adverse effects were observed when considering the rats' fertility, as sperm mobility was inhibited [50].

PPT has been investigated for its effects on animal oocyte maturation in multiple studies [10,51,52]. The exposure to PPT was significantly associated with inhibition of meiosis by spindle deformations. Blocked meiosis induces oxidative stress in the mechanism of DNA damage and severe chromosome arrangement disorders. Moreover, Annexin-V, which binds to phosphatidylserine as a signal for phagocytes, shows increased levels in podophyllotoxin-treated groups, indicating the early stages of apoptosis. Exposure to PPT is strongly correlated with the abnormal distribution rate of the Golgi apparatus and Endoplasmic reticulum [10,51,52].

Another major toxicity event, especially in the liver, was noticed when a study on Wistar rats was performed. Podophyllotoxin-4-O-D-glucoside, Podorhizol, PPT, Podophyllotoxin, and 3′,4′-O-O-Didemethylopodophyllotoxin were identified as the compounds essentially responsible for hepatotoxicity after using them on Wistar rats. This was manifested by particular changes in hepatic tissue: hepatocytes' edema, cytoplasmic vacuolization, and increased intrahepatic pigmentation. Aside from hepatotoxicity, other symptoms in rats, such as gastrointestinal irradiation with diarrhea were present. 3′,4′-O-O-Didemethylopodophyllotoxin was found to increase energy consumption, which is associated with a higher hepatotoxicity occurrence, which was behaviorally seen as different resting degrees and dyspnoea in rats [53].

More findings suggest that PPT mediates hepatotoxicity via the C5a/C5aR/ROS/NLRP3 axis and inhibition of autophagy through the cGMP/PKG/mTOR pathway. These mechanisms collectively lead to pyroptosis, a form of programmed cell death. Evidence from studies in rats

indicated that PPT administration increased serum markers such as AST/ALT and oxidative stress indicators while reducing antioxidant levels (e.g., SOD and T-AOC). Histopathological examination revealed liver damage characterized by congestion and inflammatory infiltration. Proteomic and phosphoproteomic analysis further highlighted a significant upregulation of inflammation-related proteins (e.g., NLRP3, caspase-1) and an inhibition of autophagy-related markers, suggesting a direct link between PPT toxicity and these pathways. These findings elucidate key molecular mechanisms underlying PPT-induced liver damage, providing critical insights for the safer use of PPT in clinical settings [54].

Comparative Analysis of PPT Derivatives: KL3

Our team has been studying PPT and its derivatives for years. We have synthesized PPT derivatives, one of which is KL3. As part of this study, KL3 and PPT's effect on non-cancerous cells were investigated. Potential PPT derivatives are expected to increase the expression of caspases to induce apoptosis.

Research comparing the podophyllotoxin derivative KL3 to PPT was conducted on the Human Keratinocyte cell line (HaCaT) to determine its effects. KL3, at concentrations greater than 10 μM, was found to be a better agent (less toxic and enabling recovery from metabolic stress with time) than PPT in this case by activating caspase-9 [17]. Nevertheless, KL3 did not cause necrosis of the cells in contrast to PPT [17].

Impact on Organelles

Previous studies examined the effect of PPT on individual organelles [51]. Particular attention was paid to the study of the organelles of mouse oocytes during meiosis. PPT, dissolved in DMSO, was administered at various concentrations to discern its effects comprehensively. The study found a notable reduction in the developmental competence of mouse oocytes post-PPT exposure [10]. Distinct alterations in organelle distribution were observed, reflecting compromised cellular functions.

Specifically, the endoplasmic reticulum (ER) failed to accumulate around the spindle periphery after PPT exposure, indicating a disruption in protein synthesis during oocyte meiotic maturation. This anomaly suggests potential interference with critical cellular processes, likely influencing subsequent developmental stages. Furthermore, abnormal distribution patterns of the Golgi apparatus were noted, accompanied by diminished localization of Rab11a-related vesicles, signifying compromised vesicle-based protein transport—a fundamental mechanism governing cellular homeostasis [51].

Moreover, aberrant lysosome accumulation within the cytoplasm of PPT-exposed oocytes was identified, suggesting disrupted protein degradation pathways [55]. This disruption could lead to intracellular accumulation of harmful substances, potentially exacerbating cellular stress responses. Additionally, perturbations in mitochondrial distribution were evident, indicative of mitochondrial dysfunction after PPT exposure. Mitochondrial dysfunction is particularly concerning, given its pivotal role in ATP production and cellular energy homeostasis.

PPT exposure induces significant disruptions in organelle dynamics during mouse oocyte meiotic maturation. These findings highlight the intricate interplay between PPT and cellular processes, emphasizing the necessity for further clarification of the underlying molecular mechanisms. Understanding the precise mechanisms through which PPT influences organelle dynamics is crucial for evaluating its safety profile and exploring potential therapeutic applications in clinical contexts [10].

Mitotic Spindle Inhibition

PPT and its derivatives have emerged as pivotal players in cellular dynamics, particularly in their remarkable ability to modulate the spindle apparatus during cell division.

The spindle, a microtubule-based structure, orchestrates the precise segregation of chromosomes, a process critical for cellular proliferation. Herein, we delve into these compounds' consequential cause-and-effect relationship of spindle inhibition, unraveling their profound impact on cellular events.

PPT, renowned for its antimitotic properties, disrupts the spindle's functionality, leading to the arrest of cell division [56]. This interference stems from the compound's potent interaction with tubulin, specifically targeting the colchicine domain located between α- and β-tubulin [9,57], precisely at the same site as colchicine. The colchicine site is mostly buried in the intermediate domain of the b subunit [9,58]. Tubulin is a key protein constituent of spindle microtubules. The binding alters the microtubule dynamics, inhibiting their polymerization and ultimately impeding spindle formation.

Remarkably, modifications of PPT, such as epimerization in specific positions, exhibit a nuanced influence on spindle inhibition. These alterations induce changes in the compound's spatial orientation, affecting its interaction with tubulin and subsequently modulating spindle dynamics. The consequential reduction in spindle activity is associated with a noteworthy decrease in cellular proliferation.

Emerging evidence suggests that PPT has potential therapeutic applications beyond its well-documented role in mitotic spindle inhibition, particularly as an anti-leishmaniasis agent. Leishmaniasis, a parasitic zoonotic disease, could benefit from PPT's ability to disrupt microtubule formation, presenting a novel mechanism of action when used alongside traditional anti-inflammatory topical treatments. This prospect is supported by promising findings from Ghayour et al., who demonstrated that PPT, at a concentration of 200 μg/mL, exhibited 83% lethality against Leishmania major promastigotes 48 h post-cultivation. In comparison, podophyllin achieved only 58% lethality under similar conditions, highlighting PPT's superior efficacy. Additionally, PPT has drawn significant attention for its antiparasitic activities against other pathogens, including Trypanosoma brucei and Giardia. Further investigations into PPT's toxicological and pharmacological profiles are warranted to advance its clinical utility in parasitic disease treatment [59].

In summary, PPT and its derivatives intricately intervene in the spindle apparatus, revealing a captivating tale of molecular interactions and their cascading effects on cellular processes [60]. Understanding the detailed mechanisms of spindle inhibition by these compounds not only enriches our knowledge of cell biology but also offers promising avenues for developing targeted antiproliferative therapies.

Embryology

PPT and its derivatives, recognized for their medicinal properties, notably impact embryonic development. Investigations into its effects on embryonic development reveal concerning outcomes, including disruptions in spindle organization, chromosome alignment, and DNA integrity during early embryo development [10]. In a study examining fertilized oocytes using a mouse model, exposure to PPT resulted in adverse effects on zygote cleavage, characterized by impaired spindle organization and chromosome alignment during the metaphase of the first cleavage. PPT exposure induced oxidative stress, as evidenced by elevated reactive oxygen species (ROS) levels. Remarkably, PPT-exposed embryos exhibited increased γH2A.X and Annexin-V signals, indicating embryonic DNA damage and early apoptosis [10]. These findings underscore the detrimental impact of PPT on spindle formation, chromosome alignment, and DNA integrity during early embryonic development.

3.1.2. Therapeutic Effects of PPT and Its Derivatives

Antiviral Properties

Podophyllotoxin derivatives are commonly used as anticancer chemotherapeutics but also in treating genital warts caused by Human papillomavirus (HPV). HPV infections are usually manifested in genital and plantar warts, which are transmitted by a sexual route [61]. Those appear when the virus infiltrates the epithelium in the basal and spinous layers, causing significant bulges in the form of warts. HPV types 1–4 replicate in epithelium cells, causing plantar warts. However, the other 6, 11 types are responsible for generating mucosal warts [62]. Most of the warts can disappear within the three years, but due to aesthetics and pain-free comfort, treatment is usually applied and recommended to prevent further spread. This includes mainly cryotherapy, electric coagulation, surgical removal, laser therapy, and podophyllotoxin derivative ointments [63].

Podophyllotoxin is used in cases of HPV infections due to its antiproliferative and proapoptotic properties. PPT might be used alone or in combination with other derivatives such as imiquimod. Both of these drugs are equally efficient [64,65]. Combination therapy using cryotherapy along with podophyllotoxin derivative ointments, such as imiquimod, is a standard practice of care for genital warts [66–68].

Nevertheless, some data imply that PPT might be more effective in clearing keratinized anogenital warts [69]. However, in some cases, PPT does not give positive results, therefore opting for imiquimod with cryotherapy might be considered a better option [66].

Moreover, Cantharidin–Podophyllotoxin–Salicylic acid (CPS) therapy was more effective than liquid nitrogen monotherapy and was associated with lower pain levels than Long-Pulsed Nd:YAG Laser [68–70]. High-intensity 20 MHz ultrasound following Imiquimod 5% cream was also reported as a good treatment option [71]. The abovementioned therapeutic methods are not always as effective as expected, therefore alternative options have to be considered. Tomić et al. presented a case of meatal intraurethral warts with unsuccessful treatment by cryotherapy, laser therapy, imiquimod, electrosurgical resection, and trichloroacetic acid, which was subsequently managed by 5-fluorouracil cream [72].

Podophyllotoxin and its derivatives are commonly used in HPV infections due to their antiproliferative and proapoptotic properties.

Recently, promising results have been reported for a new derivative, cyclolignan SAU-22.107, which decreased the viral yield of the dengue virus by altering the DENV genome replication or translation [73]. Additionally, an experimental study showed that podophyllotoxin exhibits neuroprotective activity in central nervous system (CNS) viral infections. It interacts with amino acid residues Gln189, Met49, Phe140, Glu166, and Asn142, allowing it to bind to the SARS-CoV-2 main protease. Podophyllotoxin also binds to the Cys480 residue of the SARS-CoV-2 spike's receptor-binding domain. These interactions may inhibit viral replication by modulating the virus's ability to bind to host cells, thus providing a potential therapeutic avenue for treating SARS-CoV-2 infections [74].

Analgesics and Anti-Inflammatory Properties

PPT and its derivatives, known for their diverse pharmacological properties, have recently gained attention for their intriguing anti-inflammatory and analgesic properties. The anti-inflammatory effects of these compounds are closely linked to their modulation of key molecular pathways involved in immune responses [75]. At the molecular level, PPT has been shown to inhibit pro-inflammatory mediators, such as cytokines and chemokines, thereby attenuating the inflammatory cascade. This inhibition correlates with a reduction in the activation of transcription factors like NF-κB [76], which are pivotal in orchestrating inflammatory responses. The consequential suppression of these pathways contributes to the observed anti-inflammatory efficacy of PPT and its derivatives.

Beyond their anti-inflammatory prowess, these compounds also exhibit noteworthy analgesic effects. The attenuation of inflammatory mediators intersects with the modulation of pain pathways, reducing pain perception. This dual action on both inflammation and pain highlights the potential of PPT and its derivatives in developing novel analgesic agents [75,77].

In summary, the anti-inflammatory and analgesic properties of PPT and its derivatives stem from their intricate molecular interactions, particularly in the regulation of key inflammatory pathways. These findings not only deepen our understanding of the pharmacological profile of these compounds but also offer promising avenues for their utilization in the development of therapeutics targeting inflammation and pain [75].

Radiation Protection

Radiation exposure, whether intentional or accidental, presents substantial risks to human health, including pulmonary inflammation, fibrosis, and hematopoietic suppression [78]. Developing effective radioprotective agents is crucial for the mitigation of these adverse effects. PPT and its derivatives, particularly formulations such as G-003M [77] and G-002M, have demonstrated promising radioprotective capabilities in preclinical investigations [55]. The radioprotective properties of PPT and its derivatives stem from their ability to scavenge reactive oxygen species (ROS) generated by ionizing radiation [79]. These compounds possess potent antioxidant activity, which helps mitigate oxidative stress-induced damage to cellular structures and DNA [77]. Additionally, PPT derivatives have been found to modulate various signaling pathways involved in the cellular response to radiation, including those regulating inflammation, apoptosis, and DNA repair processes. Furthermore, studies have elucidated the ability of PPT derivatives to enhance the activity of endogenous antioxidant enzymes, such as superoxide dismutase and catalase, thereby bolstering the cellular defense mechanisms against radiation-induced oxidative injury [77].

G-003M, a combination of PPT and rutin, exhibits notable radioprotective effects against gamma radiation-induced pulmonary damage [77]. Rutin, a flavonoid belonging to the flavonol group found in plants, demonstrates the ability to absorb UV radiation. It acts protectively on the skin by reducing DNA damage and the level of free radicals. Rutin's strong antioxidant properties [80] further enhance its potential as a protective ingredient against sunlight exposure [81] with promising applications in dermatology. Mice administered with G-003M before irradiation exhibited elevated survival rates and diminished pulmonary symptoms in contrast to irradiated individuals in the control group. In the context of G-003M dosage and radiation intensity, the observed outcomes delineate a complex cause-and-effect relationship, elucidating heterogeneous survival rates among mice. Notably, an individual intramuscular administration of G-003M, administered 60 min before exposure to a 9 Gy dose, yielded an 89% survival rate in C57BL/6J mice [76]. Further analysis revealed a remarkable 100% survival rate in mice pre-treated with G-003M, juxtaposed with a 66.50% survival rate in those exposed to 11 Gy thoracic gamma radiation (TGR) [77]. This disparity highlights the potential impact of G-003M on survivability under distinct radiation doses. Moreover, this study demonstrated effectiveness in preserving pulmonary vascular integrity while mitigating inflammation and fibrosis, as evidenced by [77]. These multifaceted observations underscore the promising prospects of G-003M as a radioprotective agent, suggesting its nuanced influence on survival rates and pulmonary health in the context of varying radiation exposures. Such insights contribute significantly to the evolving field of radioprotective research, offering valuable considerations for potential clinical applications.

G-002M, comprising PPT, podophyllotoxin-β-D glucoside, and rutin, demonstrated significant protection against radiation-induced hematopoietic suppression and cytogenetic

aberrations [55]. Investigations into comprehensive bodily shielding have revealed that a solitary preventative administration of G-002M ensured over 85% survival in mice subjected to a lethal dose of gamma radiation (9 Gy), notably safeguarding the radiosensitive hematopoietic and gastrointestinal organs [55]. Pre-treatment with G-002M preserved bone marrow progenitor cells, maintained myeloid/erythroid ratios, and reduced chromosomal aberrations [82]. Additionally, the formulation stimulated antioxidant responses, minimized DNA damage, and promoted lymphocyte proliferation, indicating its potential to safeguard hematopoietic function post-radiation exposure.

PPT derivatives, exemplified by G-003M and G-002M formulations, offer promising avenues for developing effective radioprotective strategies [55,76]. These compounds exhibit multifaceted mechanisms, including free radical scavenging, DNA protection, and modulation of apoptotic pathways and inflammatory responses. Further research and clinical trials are warranted to validate their safety and efficacy for human application in mitigating the adverse effects of radiation exposure. The summary of the use of PPT in preclinical and clinical studies is shown in Table 3.

Table 3. Summary of Podophyllotoxin and its derivatives: anticancer applications, non-anticancer applications, special features, and references.

Compound	Anticancer Applications	Non-Anticancer Applications	Special Features	Citation
Compounds Tested in Clinical Settings in Humans				
Podophyllotoxin (PPT)	Treatment of skin cancers (topical)	Treatment of genital warts, psoriasis, molluscum contagiosum, keratoacanthoma; antiviral properties (e.g., HPV, SARS-CoV-2)	Topical use only due to high systemic toxicity; interacts with microtubule spindle formation	[27–33]
Etoposide	Ovarian, testicular, and lung cancer; leukemia; Hodgkin's lymphoma; non-Hodgkin's lymphoma	Not currently used in non-cancer treatment.	Systemic application; a first-line treatment for testicular and small-cell lung cancers	[40–45]
Teniposide	Hodgkin's lymphoma, bladder cancer, acute lymphocytic leukemia, immature neuroblastoma	Not currently used in non-cancer treatment.	Systemic application, similar to etoposide but distinguished by its thienyl group	[46]
Compounds tested in preclinical settings in animals				
G-003M (PPT + rutin)	Not currently used in cancer treatment.	Radioprotection against gamma radiation-induced lung and tissue damage; reduces oxidative stress; enhances survival rates in preclinical models	Administered prophylactically; strong antioxidant properties; preserves pulmonary vascular integrity during radiation exposure	[77–81]
G-002M (PPT, rutin, derivatives)	Not currently used in cancer treatment.	Radioprotection against hematopoietic suppression and chromosomal aberrations; protects bone marrow and reduces DNA damage post-radiation	Single-dose preventative administration; protective effects for radiosensitive organs, including the gastrointestinal tract and bone marrow	[55,76–82]
Compounds tested in preclinical settings on cells				
KL3	Used in pre-clinical study as anticancer agent for Hela, MDA-MB, MCF7, PC3, DU-145, CFPAC cell lines	Reduces cytotoxicity in keratinocyte models compared to PPT; potential for less toxic therapeutic use	No necrotic effects; activates caspase-9 in keratinocytes	[17,34]
SAU-22.107	Not currently used in cancer treatment.	Decreases replication of Dengue virus; neuroprotective potential against SARS-CoV-2; modulates interferon-regulatory factors to prevent viral replication	Experimental compound; antiviral activity via modulation of viral binding to host cells	[73–75]

3.1.3. Graphical Summary

All the above-mentioned substances, which are derivatives of podophyllotoxin, are summarized in Figure 4. It categorizes them based on their activity: those with only anticancer effects, those with non-anticancer effects, and those exhibiting both types of activity.

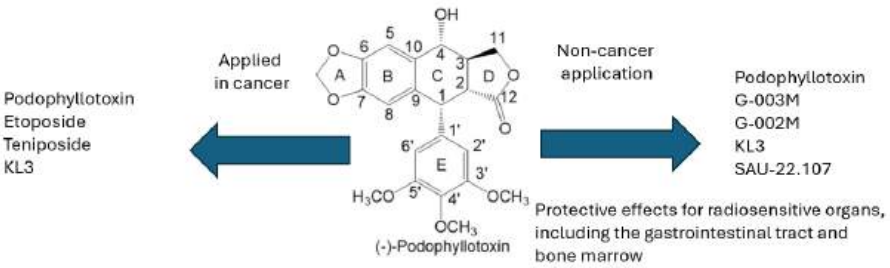

Figure 4. Summary of podophyllotoxin derivatives, categorizing them based on their activity: anticancer effects only, non-anticancer effects, and both anticancer and non-anticancer effects.

3.1.4. Geographical Distribution of Podophyllotoxin Research

Our review of the scientific literature indicates a regional interest in PPT research, particularly in areas where the Podophyllum plant is native. Notably, a significant number of studies from Asia and Latin America have explored the applications of PPT. This pattern suggests that local factors, such as geography, culture, or climate, might influence researchers' focus on PPT. However, it is critical to conduct further research to confirm these correlations and identify the specific influences on scientific inquiry into PPT and its derivatives. Such studies are vital to understanding the motivations behind PPT research and the role of local conditions in shaping scientific interests globally.

The geographical distribution of research on PPT, as illustrated in the map (Figure 5), further reinforces these regional trends. The map highlights the countries with the highest number of studies, revealing a concentration of research in regions where the Podophyllum plant is native. In particular, China and the United States stand out with 13 studies each, followed closely by India with 10 studies. This concentration may be attributed to the availability of the plant in these regions, as well as the local interest in exploring its applications. The pattern also suggests that environmental, cultural, and climatic factors could play a role in shaping the direction of PPT research in these countries.

While the map provides a general overview of the geographical distribution of PPT research, it is important to note that international collaborations, where authors come from multiple countries, may lead to a broader representation of global research efforts. Thus, the map should be viewed as a general reflection of global research activity rather than an exhaustive account.

Further research is necessary to confirm these correlations and delve deeper into the specific local conditions influencing the scientific interest in PPT. This will be crucial for understanding the broader motivations behind the growing body of PPT research and how local factors shape scientific inquiry in different regions.

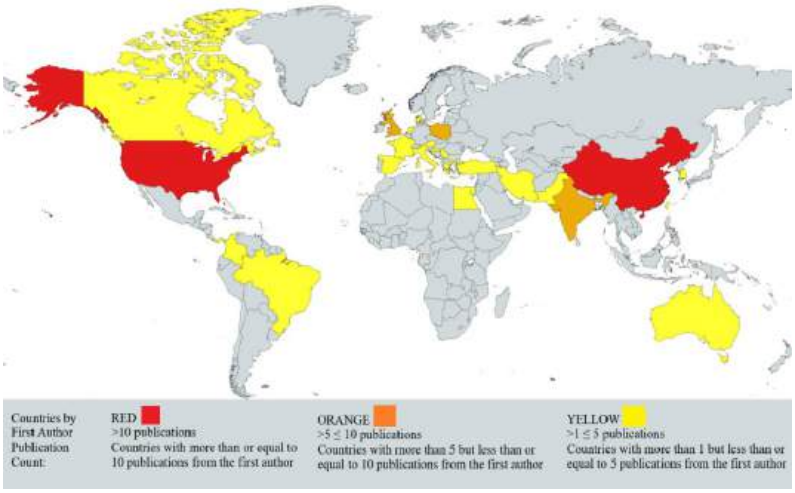

Figure 5. The map illustrates the geographical distribution of Podophyllotoxin (PPT) research based on the affiliation of the first author. The map was created using https://www.mapchart.net/ (accessed on 19 January 2025).

3.2. Discussion

The findings underscore the diverse effects of PPT and its derivatives, prompting discussions on their therapeutic potential and associated concerns.

A contentious issue arises from the observed reduction in mouse oocyte developmental competence post-PPT exposure, necessitating further research on its reproductive implications and impact on embryonic development. Alterations in organelle distribution and compromised cellular functions following PPT exposure raise safety concerns, particularly regarding its effects on critical cellular processes, such as protein synthesis and vesicle-based transport, during oocyte maturation.

PPT-induced dysfunctions in mitochondria and oxidative stress in embryonic development highlight potential teratogenic effects, demanding comprehensive safety assessments in clinical settings, especially in reproductive contexts.

Despite these concerns, PPT shows promise in treating various non-cancerous conditions, particularly dermatological disorders, and as a radioprotective agent against radiation exposure. These findings align with previous research demonstrating the antiviral and radioprotective properties of podophyllotoxin derivatives. The included studies varied significantly in terms of study design, population, and outcome measures, which limited the ability to perform a quantitative synthesis. Additionally, most studies lacked standardized reporting of effect sizes and confidence intervals, which hinders the direct comparability of results. Despite challenges, PPT remains a valuable therapeutic option, warranting continued investigation for optimized clinical application and patient safety. This review was limited by the exclusion of non-English studies, which may have omitted relevant evidence from other regions. Furthermore, the manual screening and data extraction processes, while thorough, could have introduced subjective biases.

Gaps and Future Perspectives

Despite the promising therapeutic potential of PPT and its derivatives, several challenges limit their clinical application. The broader therapeutic potential of these compounds in non-cancerous diseases, such as their applications in pain management and inflamma-

tion control, remains underexplored and warrants further investigation. Moving forward, rigorous research is needed to elucidate PPT's adverse effects and molecular mechanisms, ensuring safe clinical use. The systemic toxicity of PPT underscores the need for further research to establish safe dosage ranges and develop derivatives with improved safety profiles. While the antimitotic and cytotoxic mechanisms of PPT are well-documented, its antiviral, analgesic, anti-inflammatory, and radioprotective effects remain underexplored at the molecular level, limiting the rational design of targeted derivatives. The lack of clinical trials focused on non-cancerous applications, coupled with inconsistent outcome measures and small, homogenous study populations, hampers the development of evidence-based guidelines. Advances in the synthesis of derivatives, such as etoposide, demonstrate the potential for structural modifications to broaden therapeutic applications, yet efforts have largely focused on oncology, leaving non-cancerous uses insufficiently investigated. Furthermore, combination therapies, which show preliminary promise, require systematic exploration to optimize efficacy and minimize toxicity. Policymakers should consider supporting research into these compounds to address unmet medical needs, particularly in resource-limited settings where PPT is readily available. Future studies should aim to establish standardized outcome measures and explore novel derivatives with reduced toxicity profiles. Addressing these gaps through rigorous, inclusive, and interdisciplinary research is essential to fully realize the clinical utility of PPT in non-cancerous diseases.

4. Conclusions

Our systematic review explores the complex pharmacological effects of podophyllotoxin (PPT) and its derivatives across various medical domains. PPT demonstrates significant cytotoxic effects through mechanisms like mitochondrial dysfunction, protein metabolism disruption, and apoptosis induction. Its adverse impact on critical embryological processes also highlights the necessity for cautious application in reproductive health.

Additionally, our study reveals the potential of PPT derivatives in treating viral infections, notably their effectiveness against conditions such as genital warts caused by the Human papillomavirus (HPV). The development of novel derivatives, such as cyclolignan SAU-22.107, presents promising avenues in antiviral research, particularly when used in combination therapies with agents like imiquimod.

Furthermore, PPT and its derivatives have shown remarkable analgesic and anti-inflammatory properties, underscoring their potential utility in managing pain and inflammation. Their anti-inflammatory and immunomodulatory effects are particularly noteworthy, suggesting further exploration into autoimmune disorders, viral infections, and dermatological conditions.

The radioprotective properties of derivatives like G-003M and G-002M underscore their capacity to mitigate radiation-induced damage through mechanisms such as free radical scavenging and modulation of apoptotic pathways.

In conclusion, while current research has limitations, our findings emphasize the diverse pharmacological properties and therapeutic potential of PPT and its derivatives. To fully explore their clinical applicability, future research should focus on clinical trials targeting noncancerous applications, such as antiviral treatments and pain management. Additionally, efforts should be directed towards improving the synthesis methods of PPT derivatives to reduce their toxicity and enhance their therapeutic profiles. Continued investigation is essential to confirm their safety, efficacy, and long-term applicability in clinical settings. Nevertheless, our insights significantly contribute to the ongoing discussion on the therapeutic applications of these compounds, setting the stage for future research in this promising field.

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

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3. Podsumowanie i wnioski

3.1. Kluczowe informacje

- W 2018 roku przeprowadzono wstępne badania na sześciu liniach komórek nowotworowych. Wyniki wskazały, że nasz lek, KL3, wykazywał silny profil przeciwnowotworowy z mniejszą toksycznością wobec zdrowych komórek fibroblastów – NIH-3T3 w porównaniu do macierzystej PPT.
- W 2021 r. na dwóch dodatkowych nienowotworowych liniach komórkowych badania wykazały, że nasza pochodna KL3 jest mniej toksyczna niż PPT dla komórek keratynocytów HaCaT i jednojądrzastych komórek krwi obwodowej PBMC.
- Od początku projektu badane są mechanizmy działania macierzystej PPT i jej pochodnych. Wiadomo, że KL3 w większych dawkach i przy dłuższym czasie inkubacji (48 godzin) indukuje spadek ilości ATP, wzrost aktywności kaspazy 9, ale nie wywołuje nekrozy, w przeciwieństwie do PPT, która już przy stężeniach 10-krotnie niższych redukuje ilość ATP, aktywuje kaspazę 9, ale też indukuje nekrozę.
- W 2025 roku został opublikowany przegląd systematyczny, który miał na celu ocenę potencjalnych zastosowań pochodnych PPT w leczeniu chorób niezwiązanych z nowotworami.

3.2. Aktualny stan wiedzy i wyniki przeprowadzonych badań:

Postawione na początku hipotezy badawcze zostały wstępnie zweryfikowane w celu sprawdzenia zasadności dalszych badań.

W 2016 roku współpracujący z nami zespół chemików z Uniwersytetu Warszawskiego opracował metodologię stereoselektywnej syntezy cyklolignanów spokrewnionych z PPT. Na podstawie tego odkrycia przeprowadzono formalną syntezę PPT. Postawiliśmy sobie za cel, aby nasze nowe związki były rozpuszczalne i stabilne w roztworze wodnym, miały akceptowalną biodostępność, łatwo przenikały przez błonę komórek nowotworowych oraz powinny dostarczać dowodów na wysoką selektywność wobec komórek nowotworowych.

Potencjał terapeutyczny PPT i jego pochodnych często napotyka na ograniczenia wynikające z oporności na leki, hydrofobowości i niskiej selektywności. Pomimo istnienia wielu metod stereoselektywnej syntezy cyklolignanów, brak uniwersalności i często wysokie koszty syntezy pozostają istotnymi wadami.

Aby rozwiązać te problemy, opracowano nową metodologię, która wykorzystuje niedrogie i łatwo dostępne materiały wyjściowe. Kluczowym etapem syntezy jest fotocyklizacja. Strategia ta oferuje dodatkową korzyść: pozwala na nieograniczoną skalę syntezy, co jest istotnym czynnikiem w syntezach wieloetapowych. Co więcej, uniknięcie stosowania złożonych organokatalizatorów lub katalizatorów zawierających metale ciężkie ma kluczowe znaczenie dla wyeliminowania potencjalnego zanieczyszczenia metalami toksycznymi. W związku z tym nowa metoda opracowana we współpracy z Uniwersytetem Warszawskim oferuje znaczne korzyści w syntezie związków o potencjalnych zastosowaniach farmaceutycznych.

W 2018 roku opublikowano pracę Strus, P. et al. 2018 [31], w której zbadano właściwości przeciwnowotworowe trzech nowych pochodnych PPT - KL1, KL2, KL3. Związek KL1 jest pochodną 1-arylonaftalenu, wykazującą aktywność przeciwnowotworową. Pochodna KL2 zawiera w swojej strukturze cząsteczkę L-prolinolu, która działa jako donor wiązania H, a jej konformacyjnie ograniczony łańcuch główny może zwiększać wiązanie receptora. Związek KL3 jest koniugatem PPT i ugrupowania benzotiazolowego. W ostatnim czasie benzotiazole zostały uznane za obiecujące farmakofory posiadające różnorodne właściwości biologiczne, w tym aktywność przeciwnowotworową. Wszystkie te związki są zgodne z zasadą pięciu Lipińskiego, która zakłada mniej niż 5 donorów wiązań wodorowych, mniej niż 10 akceptorów wiązań wodorowych i masę cząsteczkową poniżej lub nieco powyżej 500 daltonów. Dużą zaletą tej metody jest wysoka wydajność syntezy związków i wykorzystanie niedrogich i łatwo dostępnych substratów.

W tym samym badaniu opisano szybką i tanią metodę badania przesiewowego pochodnych PPT jako środków przeciwnowotworowych przy użyciu testów cytotoksyczności, mikroskopii świetlnej z kontrastem fazowym i pomiaru wskaźnika selektywności. Zbadano wrażliwość komórek nowotworowych i nienowotworowych na nowe pochodne w porównaniu do macierzystej PPT. Przebadano linie komórek nowotworowych: HeLa (rak szyjki macicy), MDA-MB i MCF7 (rak piersi), PC3 i DU145 (rak prostaty) oraz CFPAC (rak trzustki). Jako linię nienowotworową dla porównania selektywności i cytotoksyczności przebadano również linię komórek fibroblastów NIH-3T3. Badanie to wykazało, że nowe pochodne PPT mają potencjał jako związki mniej toksyczne dla nienowotworowych komórek i bardziej skuteczne w liniach komórek nowotworowych niż macierzysta PPT.

Aby upewnić się, jak działają analizowane związki na ludzkie komórki nienowotworowe, przeprowadzono również serię eksperymentów na linii komórkowej ludzkich keratynocytów (HaCaT) oraz na komórkach jednojądrzastych krwi obwodowej (PBMC) opisanych przez Strus et al. 2021 w *Toxicology In Vitro* [37]. Przeprowadzono testy żywotności, testy wzrostu kolonii,

określono ilościowo ATP w komórkach aktywnych metabolicznie, zmierzono aktywność kaspazy 9, przeprowadzono kinetyczny test apoptozy i martwicy żywych komórek oraz test aneksyny V. Aby określić wpływ badanych substancji na ultrastrukturę komórek, wykorzystano transmisyjną mikroskopię elektronową TEM. Obrazy komórek zarejestrowane za pomocą mikroskopii elektronowej zostały poddane ocenie i analizie morfometrycznej. W rezultacie zbadano, że KL3 jest mniej toksyczny niż PPT dla komórek HaCaT i PBMC, oraz w niższych stężeniach (1μM) nie zaburza wytwarzania ATP, nie indukuje apoptozy ani nekrozy w komórkach nienowotworowych. Ponadto ludzkie keratynocyty po 24-godzinnej inkubacji z KL3 wykazują cechy stresu siateczki śródplazmatycznej (rozdęte cysterny ER) i zmiany w mitochondriach, które po 48 godzinach ulegają regresji z jednoczesnym wystąpieniem cech autofagocytozy, bez obecności nekrozy.

W 2024 roku opublikowany został trzeci artykuł oryginalny Strus et al. 2024 [36], w którym przeanalizowano ultrastrukturalne zmiany w ludzkich keratynocytach HaCaT wywołane przez macierzystą PPT. Ponadto na drodze unikatowej syntezy, dotąd nieopisywanej w literaturze, została wygenerowana pochodna fluorescencyjna PPT, zwana PPT-FL. Nowa pochodna PPT-FL wykazywała podobne do macierzystej PPT efekty cytostatyczne/cytotoksyczne w testach żywotności. Następnie za pomocą mikroskopii konfokalnej zbadano dystrybucję i farmakokinetykę nowo zaprojektowanej fluorescencyjnej pochodnej PPT (PPT-FL). Związek PPT-FL był stabilny i gromadził się w komórkach w cytoplazmie już po 3 godzinach inkubacji z PPT-FL. Sygnał fluorescencyjny był obserwowany do 48 godzin inkubacji. Przeprowadzono także badania dokowania molekularnego w celu porównania powinowactwa obu związków do tubuliny. Okazało się, że obecność dużej fluoresceiny związanej kowalencyjnie z PPT zmienia gęstość elektronową wokół miejsca pierwotnego wiązania do β-tubuliny. PPT-FL wykazuje powinowactwo do β -tubuliny tylko w nieznacznie zmienionej pozycji zmieniając orientację przestrzenną całej cząsteczki wobec kieszonki i szkieletu białkowego β-tubuliny.

Dalsze wnioski z badania Strus et al. 2024:

Toksyczność i zmiany ultrastrukturalne wywołane PPT

- PPT wykazuje silne działanie cytotoksyczne wobec keratynocytów HaCaT, prowadząc do śmierci komórek nawet przy niskich stężeniach. Ponadto indukuje śmierć ludzkich mononuklearów krwi obwodowej.
- Po 24 godzinach ekspozycji zaobserwowano w komórkach linii HaCaT:
    - Wydłużenie wypustek cytoplazmatycznych.
    - Tworzenie się wakuol w cytoplazmie.

    - Stres retikulum endoplazmatycznego (ER).
    - Skrócenie osi długiej mitochondriów.
- Po 48 godzinach ekspozycji:
    - Dochodziło do dezintegracji błony komórkowej.
    - Obserwowano postępującą wakuolizację.
    - Pojawiały się pęcherzyki apoptotyczne i nekrotyczne.
    - Struktura jądra komórkowego ulegała zmianom wstecznym, w tym obserwowano mniejszą gęstość elektronową chromatyny.

Badania dystrybucji PPT-FL

- PPT-FL został wykryty w komórkach HaCaT w ciągu godziny od rozpoczęcia inkubacji.
- Związek pozostawał w komórkach przez cały okres obserwacji, co sugeruje, że nie ulegał szybkiemu rozkładowi ani usunięciu.
- Za pomocą mikroskopii konfokalnej wykazano, że PPT-FL lokalizował się głównie w cytoplazmie, tworząc punktowe struktury.

Wyniki dokowania molekularnego

- PPT i PPT-FL tworzyły stabilne kompleksy z tubuliną, ale w różnych miejscach wiązania:
    - PPT wiązał się głównie w klasycznym miejscu wiążącym PPT na tubulinie.
    - PPT-FL nie pasował do tego samego miejsca, lecz wiązał się w tzw. miejscu wiążącym epotilon.
- Analiza MM/GBSA wykazała, że oba związki miały wysokie powinowactwo do tubuliny, ale różniły się specyficznością wiązania.

Końcowe wnioski z badania

- **Wysoka cytotoksyczność PPT**: związek jest wysoce toksyczny dla ludzkich keratynocytów, nawet w niskich stężeniach, co ogranicza jego potencjalne zastosowanie terapeutyczne.
- **Mechanizm działania PPT**: prawdopodobnie dostaje się do komórek przez endocytozę i powoduje uszkodzenia ER oraz mitochondriów, prowadząc do śmierci komórkowej.
- **Nowe możliwości badawcze**: PPT-FL może służyć jako narzędzie do śledzenia dystrybucji PPT w komórkach, co może być przydatne w dalszych badaniach farmakologicznych.
- **Kierunki przyszłych badań**: Wskazane są dalsze badania nad mniej toksycznymi pochodnymi PPT oraz ich wpływem na różne typy komórek, szczególnie nowotworowych.

W 2025 roku został opublikowany przegląd systematyczny Strus et al. 2025 [35], który miał na celu ocenę potencjalnych zastosowań pochodnych PPT w leczeniu chorób niezwiązanych z nowotworami. Opisano w nim właściwości przeciwwirusowe, w tym silne działanie przeciw HPV, co uzasadnia ich stosowanie w leczeniu brodawek narządów płciowych. Obiecujące wyniki uzyskano też w kontekście terapii przeciwko wirusowi dengi i SARS-CoV-2. Nowa pochodna PPT, cyclolignan SAU-22.107, zmniejsza replikację wirusa dengi, co sugeruje jej potencjał w terapii zakażeń wirusowych. Opisano również działanie przeciwbólowe i przeciwzapalne poprzez hamowanie aktywacji szlaków zapalnych, w tym NF-κB, co skutkuje zmniejszeniem produkcji cytokin prozapalnych. Kolejną cechą były właściwości radioprotekcyjne. Badanie wskazują więc na możliwość wykorzystania pochodnych PPT w leczeniu infekcji wirusowych, bólu, stanów zapalnych i ochronie przed promieniowaniem.

Podsumowując badania, otrzymano pochodne PPT, które są mniej toksyczne dla normalnych komórek i bardziej skuteczne niż macierzysta PPT wobec wybranych linii komórek nowotworowych. Wynaleziono nowatorską, zieloną i tanią syntezę potencjalnych leków o działaniu przeciwnowotworowym w modelu komórkowym. Przeanalizowano także dostępną literaturę o innych niż przeciwnowotworowe wskazania pochodnych PPT, w ramach przeglądu systematycznego, zaopatrując lukę w aktualnej wiedzy. Wykazano, że dalsze badania dokładnego mechanizmu działania PPT i nowych pochodnych są konieczne.

3.3. Podsumowanie znaczenia badań

Znaczenie podjętych przeze mnie badań polega na interdyscyplinarnym ujęciu tematu z charakterystyką badanych związków pod kątem ich stabilności, biodystrybucji w komórkach, efektywności i selektywności przeciwnowotworowej w modelu komórkowym. Ponadto opisaliśmy wpływ badanych związków na ultrastrukturę ludzkich keratynocytów, pokazując nieopisywany dotąd ciekawy mechanizm regeneracji prawidłowych komórek i wyjścia ze stresu poprzez indukcję autofagocytozy. Zobrazowaliśmy tym samym, jak mogą regenerować się komórki prawidłowe.

Istniejące terapie przeciwnowotworowe, w tym PPT, często nie zapewniają równowagi między skutecznością terapeutyczną a bezpieczeństwem. Działania niepożądane mogą być przyczyną przerwania leczenia i niepowodzeniem terapii. Nasze badanie, poprzez syntezę bezpieczniejszej i skuteczniejszej pochodnej PPT, może być rozwiązaniem tych trudności. Potrzebne są jednak pogłębione badania przedkliniczne, między innymi na modelu zwierzęcym oraz w kolejnym etapie badania kliniczne.

Opracowane i wdrożone zasady „zielonej syntezy" polegające na wykorzystaniu ekologicznych, czasochłonnych i niedrogich metod syntezy może zapewnić przewagę ekonomiczną i środowiskową nad tradycyjnymi technikami wytwarzania leków. W związku z tym możliwe jest, że innowacyjne podejście do syntezy przyczyni się do nowych wdrożeń albo będzie inspiracją dla innych w dążeniu do zrównoważonych metod produkcji leków w przemyśle farmaceutycznym.

Ponadto, niektóre pochodne PPT mają ugruntowaną rolę w medycynie, należą do nich takie leki jak etopozyd, etopofos i tenipozyd. W tym nurcie w laboratoriach na całym świecie wciąż badane są nowe pochodne PPT. Opisywane w niniejszej rozprawie pochodne zaprojektowane i opisane przez nasz zespół, są unikalne, stabilne, mają celowany mechanizm działania oraz okazały się mało toksyczne wobec komórek nienowotworowych. Nasz projekt, poprzez odkrycie nowych, bezpieczniejszych i skuteczniejszych pochodnych PPT, może przyspieszyć dalsze badania nad tą klasą leków, a tym samym w przyszłości poszerzyć zakres terapeutyczny istniejących terapii przeciwnowotworowych.

Wreszcie, spodziewamy się, że kontynuacja niniejszych badań wniesie znaczący wkład w zrozumienie mechanizmu działania PPT i jej pochodnych. Pomimo ich terapeutycznego znaczenia, dokładny mechanizm, poprzez który leki te wywierają swoje działanie, pozostaje nie do końca zbadany. Sama PPT blokuje polimeryzację mikrotubul w komórce, ale jej pochodna – etopozyd jest inhibitorem topoizomerazy istotnej w procesie replikacji DNA, pokazując, że różne pochodne mogą mieć odmienne mechanizmy działania. Wykorzystując zaawansowane technologie, takie jak mikroskopia elektronowa i sekwencjonowanie RNA, nasz projekt ma na celu pogłębione badanie efektów nowych pochodnych na ekspresję genów i ludzki transkryptom, a tym samym może przyczynić się do rozwoju bardziej ukierunkowanych i skutecznych terapii przeciwnowotworowych w przyszłości.

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Oświadczenia określające indywidualny wkład (udział merytoryczny i procentowy) każdego z autorów w powstanie publikacji.

Warszawa 10.02.2025

Kamil Lisiecki
(imię i nazwisko)

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Jako współautor pracy pt. "Novel podophyllotoxin derivatives as Anticancer Agents: Design, Synthesis, and Biological Screening" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: Synteza związków chemicznych, powiązanie ich struktury z aktywnością, przygotowanie schematu nr 1.

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Mój udział procentowy w przygotowaniu publikacji określam jako 5 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 50 %,

obejmował on: pomysł pracy, hodowle komórkowe, testy żywotności, mikrofotografie hodowli komórkowych, przeprowadzenie eksperymentów, analizę uzyskanych wyników, opracowanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

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Zbigniew Czarnocki
(podpis oświadczającego)

Warszawa 10.02.2025

Izabela Młynarczuk-Biały
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Novel podophyllotoxin derivatives as Anticancer Agents: Design, Synthesis, and Biological Screening" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: nadzór merytoryczny nad całością pracy, oznaczenia indeksu selektywności, interpretację wyników, dyskusję, autor korespondencyjny.

Mój udział procentowy w przygotowaniu publikacji określam jako 15 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 50 %,

obejmował on: pomysł pracy, hodowle komórkowe, testy żywotności, mikrofotografie hodowli komórkowych, przeprowadzenie eksperymentów, analizę uzyskanych wyników, opracowanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

dr hab. n. med. Izabela Młynarczuk-Biały
ADIUNKT
LEKARZ SPECJALISTA MEDYCYNY RODZINNEJ
NPWZ 2459102

Izabela Młynarczuk-Biały
(podpis oświadczającego)

Warszawa 10.02.2025

Łukasz P. Biały
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Novel podophyllotoxin derivatives as Anticancer Agents: Design, Synthesis, and Biological Screening" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: nadzór merytoryczny nad całością pracy, ostateczna rewizja manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 10 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 50 %,

obejmował on: pomysł pracy, hodowle komórkowe, testy żywotności, mikrofotografie hodowli komórkowych, przeprowadzenie eksperymentów, analizę uzyskanych wyników, opracowanie manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

Łukasz P. Biały
(podpis oświadczającego)

Warszawa 11.02.2025

Karol Borensztejn
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Novel podophyllotoxin and benzothiazole derivative induces transitional morphological and functional changes in HaCaT cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: praca nad wstępną wersją manuskryptu, hodowla komórkowa, analiza danych

Mój udział procentowy w przygotowaniu publikacji określam jako 20 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 30 %,

obejmował on: pomysł, pracę nad wstępną wersją manuskryptu, hodowle komórkowe i eksperymenty, analizę danych.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

Karol Borensztejn

Karol Borensztejn
(podpis oświadczającego)

Warszawa, 10.02.2025

Andrzej Antoni Szczepankiewicz
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Novel podophyllotoxin and benzothiazole derivative induces transitional morphological and functional changes in HaCaT cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: mikroskopia elektonowa- opracowanie materiału, przeprowadzenie analizy, wytworzenie elektronogramów.

Mój udział procentowy w przygotowaniu publikacji określam jako 8 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 30 %,

obejmował on: pomysł, pracę nad wstępną wersją manuskryptu, hodowle komórkowe i eksperymenty, analizę danych.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

Andrzej Szczepankiewicz

Andrzej Antoni Szczepankiewicz
(podpis oświadczającego)

LABORATORIUM MIKROSKOPII ELEKTRONOWEJ
Instytut Biologii Doświadczalnej
im. M. Nenckiego PAN
02-093 Warszawa, ul. Pasteura 3
Tel. (22) 58 92 374 (474)
Fax (22) 822 53 42

Warszawa 10.02.2025

Kamil Lisiecki
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Novel podophyllotoxin and benzothiazole derivative induces transitional morphological and functional changes in HaCaT cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: synteza związków chemicznych,

Mój udział procentowy w przygotowaniu publikacji określam jako 8 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 30 %,

obejmował on: pomysł, pracę nad wstępną wersją manuskryptu, hodowle komórkowe i eksperymenty, analizę danych.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

Kamil Lisiecki
(podpis oświadczającego)

Warszawa 10.02.2025

Zbigniew Czarnocki
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Novel podophyllotoxin and benzothiazole derivative induces transitional morphological and functional changes in HaCaT cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: Nadzór nad częścią chemiczną publikacji.

Mój udział procentowy w przygotowaniu publikacji określam jako 2 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 30 %,

obejmował on: pomysł, pracę nad wstępną wersją manuskryptu, hodowle komórkowe i eksperymenty, analizę danych.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

.....

Zbigniew Czarnocki
(podpis oświadczającego)

Warszawa 10.02.2025

Hanna Nieznańska
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Novel podophyllotoxin and benzothiazole derivative induces transitional morphological and functional changes in HaCaT cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: mikroskopia elektronowa - nadzór nad merytoryczną częścią i ekspertyza rycin oraz interpretacji uzyskanych wyników.

Mój udział procentowy w przygotowaniu publikacji określam jako 2 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 30 %,

obejmował on: pomysł, pracę nad wstępną wersją manuskryptu, hodowle komórkowe i eksperymenty, analizę danych

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

Hanna Nieznańska
(podpis oświadczającego)

Portland USA, 10.02.2025

Cezary Wojcik
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Novel podophyllotoxin and benzothiazole derivative induces transitional morphological and functional changes in HaCaT cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: praca nad rewizją manuskryptu, pomoc w przygotowaniu ostatecznej części pracy.

Mój udział procentowy w przygotowaniu publikacji określam jako 10 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 30 %,

obejmował on: pomysł, pracę nad wstępną wersją manuskryptu, hodowle komórkowe i eksperymenty, analizę danych.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

dr hab. n. med. Cezary Wójcik
Amgen Inc., Thousand Oaks, CA, USA
Department of Undergraduate Medical Education, OHSU School of Medicine, Portland, OR
(podpis oświadczającego)

Warszawa 10.02.2025

Łukasz P. Biały
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Novel podophyllotoxin and benzothiazole derivative induces transitional morphological and functional changes in HaCaT cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: Opis i interpretacja wyników elektronogramów, nadzór nad analizą morfometryczną, rewizja ostatecznej wersji manuskryptu.

Mój udział procentowy w przygotowaniu publikacji określam jako 5 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 30 %,

obejmował on: pomysł, pracę nad wstępną wersją manuskryptu, hodowle komórkowe i eksperymenty, analizę danych.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

.....

Łukasz P. Biały
(podpis oświadczającego)

Warszawa 10.02.2025

Izabela Młynarczuk-Biały
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Novel podophyllotoxin and benzothiazole derivative induces transitional morphological and functional changes in HaCaT cells" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: nadzór nad całością badań, nad pisaniem manuskryptu, jego rewizją, wykonanie analizy cyklu komórkowego, oznaczenia aktywności kaspaz, testy apoptozy i nekrozy, autor korespondencyjny.

Mój udział procentowy w przygotowaniu publikacji określam jako 15 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 30 %,

obejmował on: pomysł, pracę nad wstępną wersją manuskryptu, hodowle komórkowe i eksperymenty, analizę danych.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

Izabela M-Biały

dr hab. n. med. Izabela Młynarczuk-Biały
ADIUNKT
LEKARZ SPECJALISTA MEDYCYNY RODZINNEJ
NPWZ: 2459102

Izabela Młynarczuk-Biały
(podpis oświadczającego)

Warszawa 10.02.2025

Karol Sadowski
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Cellular Distribution and Ultrastructural Changes in HaCaT Cells, Induced by Podophyllotoxin and Its Novel Fluorescent Derivative, Supported by the Molecular Docking Studies" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: metodologia, analiza wyników, badania, pisanie artykułu, wizualizację wyników.

Mój udział procentowy w przygotowaniu publikacji określam jako 8 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 25 %,

obejmował on: konceptualizację pracy, przeprowadzenie eksperymentów, walidację, analizę danych, dostarczenie zasobów, pisanie artykułu, wizualizację wyników, zarządzanie projektem, zapewnienie finansowania.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

Sadowski Karol

Karol Sadowski
(podpis oświadczającego)

Warszawa 10.02.2025

Julia Kostro
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Cellular Distribution and Ultrastructural Changes in HaCaT Cells, Induced by Podophyllotoxin and Its Novel Fluorescent Derivative, Supported by the Molecular Docking Studies" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: metodologia, analiza, badania, pisanie artykułu,

Mój udział procentowy w przygotowaniu publikacji określam jako 5 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 25 %,

obejmował on: konceptualizację pracy, przeprowadzenie eksperymentów, walidację, analizę danych, dostarczenie zasobów, pisanie artykułu, wizualizację wyników, zarządzanie projektem, zapewnienie finansowania.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

Julia Kostro

Julia Kostro
(podpis oświadczającego)

Warszawa 10.02.2025

Andrzej Antoni Szczepankiewicz
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Cellular Distribution and Ultrastructural Changes in HaCaT Cells, Induced by Podophyllotoxin and Its Novel Fluorescent Derivative, Supported by the Molecular Docking Studies" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: metodologia, software, analiza wyników, badania, materiały, wizualizacja.

Mój udział procentowy w przygotowaniu publikacji określam jako 8 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 25 %,

obejmował on: konceptualizację pracy, przeprowadzenie eksperymentów, walidację, analizę danych, dostarczenie zasobów, pisanie artykułu, wizualizację wyników, zarządzanie projektem, zapewnienie finansowania.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

Andrzej A. Szczepankiewicz

Andrzej Antoni Szczepankiewicz
(podpis oświadczającego)

Warszawa 10.02.2025

Hanna Nieznańska
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Cellular Distribution and Ultrastructural Changes in HaCaT Cells, Induced by Podophyllotoxin and Its Novel Fluorescent Derivative, Supported by the Molecular Docking Studies" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: walidacja.

Mój udział procentowy w przygotowaniu publikacji określam jako 2 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 25 %,

obejmował on: konceptualizację pracy, przeprowadzenie eksperymentów, walidację, analizę danych, dostarczenie zasobów, pisanie artykułu, wizualizację wyników, zarządzanie projektem, zapewnienie finansowania.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

.....

Hanna Nieznańska
(podpis oświadczającego)

Warszawa 10.02.2025

Magdalena Niedzielska
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Cellular Distribution and Ultrastructural Changes in HaCaT Cells, Induced by Podophyllotoxin and Its Novel Fluorescent Derivative, Supported by the Molecular Docking Studies" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: metodologia, walidacja,

Mój udział procentowy w przygotowaniu publikacji określam jako 5 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 25 %,

obejmował on: konceptualizację pracy, przeprowadzenie eksperymentów, walidację, analizę danych, dostarczenie zasobów, pisanie artykułu, wizualizację wyników, zarządzanie projektem, zapewnienie finansowania.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

Magdalena Niedzielska
(podpis oświadczającego)

Warszawa 10.02.2025

Andrei Zlobin
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Cellular Distribution and Ultrastructural Changes in HaCaT Cells, Induced by Podophyllotoxin and Its Novel Fluorescent Derivative, Supported by the Molecular Docking Studies" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: metodologia.

Mój udział procentowy w przygotowaniu publikacji określam jako 3 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 25 %,

obejmował on: konceptualizację pracy, przeprowadzenie eksperymentów, walidację, analizę danych, dostarczenie zasobów, pisanie artykułu, wizualizację wyników, zarządzanie projektem, zapewnienie finansowania.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

Andrei Zlobin
(podpis oświadczającego)

Warszawa 10.02.2025

Pramukti Nawar Ra'idah
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Cellular Distribution and Ultrastructural Changes in HaCaT Cells, Induced by Podophyllotoxin and Its Novel Fluorescent Derivative, Supported by the Molecular Docking Studies" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: metodologia.

Mój udział procentowy w przygotowaniu publikacji określam jako 2 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 25 %,

obejmował on: konceptualizację pracy, przeprowadzenie eksperymentów, walidację, analizę danych, dostarczenie zasobów, pisanie artykułu, wizualizację wyników, zarządzanie projektem, zapewnienie finansowania.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

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Pramukti Nawar Ra'idah
(podpis oświadczającego)

Warszawa 10.02.2025

Zuzanna Molęda
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Cellular Distribution and Ultrastructural Changes in HaCaT Cells, Induced by Podophyllotoxin and Its Novel Fluorescent Derivative, Supported by the Molecular Docking Studies" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: software, badania, nadzór.

Mój udział procentowy w przygotowaniu publikacji określam jako 12 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 25 %,

obejmował on: konceptualizację pracy, przeprowadzenie eksperymentów, walidację, analizę danych, dostarczenie zasobów, pisanie artykułu, wizualizację wyników, zarządzanie projektem, zapewnienie finansowania.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

Zuzanna Molęda

Zuzanna Molęda
(podpis oświadczającego)

Warszawa 10.02.2025

Joanna Szawkało
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Cellular Distribution and Ultrastructural Changes in HaCaT Cells, Induced by Podophyllotoxin and Its Novel Fluorescent Derivative, Supported by the Molecular Docking Studies" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: metodologia, badania,

Mój udział procentowy w przygotowaniu publikacji określam jako 1 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 25 %,

obejmował on: konceptualizację pracy, przeprowadzenie eksperymentów, walidację, analizę danych, dostarczenie zasobów, pisanie artykułu, wizualizację wyników, zarządzanie projektem, zapewnienie finansowania.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

J. Szawkało
.....
Joanna Szawkało
(podpis oświadczającego)

Warszawa 10.02.2025

Zbigniew Czarnocki
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Cellular Distribution and Ultrastructural Changes in HaCaT Cells, Induced by Podophyllotoxin and Its Novel Fluorescent Derivative, Supported by the Molecular Docking Studies" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: konceptualizacja pracy, nadzór.

Mój udział procentowy w przygotowaniu publikacji określam jako 2 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 25 %,

obejmował on: konceptualizację pracy, przeprowadzenie eksperymentów, walidację, analizę danych, dostarczenie zasobów, pisanie artykułu, wizualizację wyników, zarządzanie projektem, zapewnienie finansowania.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

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Zbigniew Czarnocki
(podpis oświadczającego)

Portland USA, 10.02.2025

Cezary Wójcik
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Cellular Distribution and Ultrastructural Changes in HaCaT Cells, Induced by Podophyllotoxin and Its Novel Fluorescent Derivative, Supported by the Molecular Docking Studies" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: pisanie, przegląd i ostateczna edycja artykułu.

Mój udział procentowy w przygotowaniu publikacji określam jako 10 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 25 %,

obejmował on: konceptualizację pracy, przeprowadzenie eksperymentów, walidację, analizę danych, dostarczenie zasobów, pisanie artykułu, wizualizację wyników, zarządzanie projektem, zapewnienie finansowania.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

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Warszawa 10.02.2025

Łukasz Szeleszczuk

(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Cellular Distribution and Ultrastructural Changes in HaCaT Cells, Induced by Podophyllotoxin and Its Novel Fluorescent Derivative, Supported by the Molecular Docking Studies" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: konceptualizację pracy, software, zasoby, pisanie artykułu, nadzór.

Mój udział procentowy w przygotowaniu publikacji określam jako 5 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 25 %,

obejmował on: konceptualizację pracy, przeprowadzenie eksperymentów, walidację, analizę danych, dostarczenie zasobów, pisanie artykułu, wizualizację wyników, zarządzanie projektem, zapewnienie finansowania.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

Łukasz Szeleszczuk

Łukasz Szeleszczuk

(podpis oświadczającego)

Warszawa 10.02.2025

Izabela Młynarczuk-Biały
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "Cellular Distribution and Ultrastructural Changes in HaCaT Cells, Induced by Podophyllotoxin and Its Novel Fluorescent Derivative, Supported by the Molecular Docking Studies" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: konceptualizacja pracy, walidacja, badania, zasoby, ostateczne zredagowanie artykułu, wizualizacja wyników, nadzór nad projektem.

Mój udział procentowy w przygotowaniu publikacji określam jako 12 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 25 %,

obejmował on: konceptualizację pracy, przeprowadzenie eksperymentów, walidację, analizę danych, dostarczenie zasobów, pisanie artykułu, wizualizację wyników, zarządzanie projektem, zapewnienie finansowania.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

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Izabela Młynarczuk-Biały
(podpis oświadczającego)

Warszawa 10.02.2025

Karol Sadowski
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "The Effects of Podophyllotoxin Derivatives on Noncancerous Diseases: A Systematic Review" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: zbieranie i analiza danych, pisanie artykułu.

Mój udział procentowy w przygotowaniu publikacji określam jako 10 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 40 %,

obejmował on: konceptualizację pracy, analizę uzyskanych wyników, pisanie, opracowanie i redakcję manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

Sadowski Karol

Karol Sadowski
(podpis oświadczającego)

Warszawa 10.02.2025

Weronika Ploch
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "The Effects of Podophyllotoxin Derivatives on Noncancerous Diseases: A Systematic Review" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: zbieranie i analiza danych, pisanie artykułu.

Mój udział procentowy w przygotowaniu publikacji określam jako 10 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 40 %,

obejmował on: konceptualizację pracy, analizę uzyskanych wyników, pisanie, opracowanie i redakcję manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

W. Ploch
.....
Weronika Ploch
(podpis oświadczającego)

Warszawa 10.02.2025

Adrianna Jażdżewska
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "The Effects of Podophyllotoxin Derivatives on Noncancerous Diseases: A Systematic Review" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: zbieranie danych, pisanie artykułu.

Mój udział procentowy w przygotowaniu publikacji określam jako 8 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 40 %,

obejmował on: konceptualizację pracy, analizę uzyskanych wyników, pisanie, opracowanie i redakcję manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

Adrianna Jażdżewska

Adrianna Jażdżewska
(podpis oświadczającego)

Warszawa 10.02.2025

Paulina Okniańska
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "The Effects of Podophyllotoxin Derivatives on Noncancerous Diseases: A Systematic Review" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: zbieranie danych, pisanie artykułu.

Mój udział procentowy w przygotowaniu publikacji określam jako 9 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 40 %,

obejmował on: konceptualizację pracy, analizę uzyskanych wyników, pisanie, opracowanie i redakcję manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

P. Okniańska
.....
Paulina Okniańska
(podpis oświadczającego)

Warszawa 10.02.2025

Oliwia Raniszewska
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "The Effects of Podophyllotoxin Derivatives on Noncancerous Diseases: A Systematic Review" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: zbieranie danych, pisanie artykułu.

Mój udział procentowy w przygotowaniu publikacji określam jako 8 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 40 %,

obejmował on: konceptualizację pracy, analizę uzyskanych wyników, pisanie, opracowanie i redakcję manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

Oliwia Raniszewska

Oliwia Raniszewska
(podpis oświadczającego)

Warszawa 10.02.2025

Izabela Młynarczuk-Biały
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. "The Effects of Podophyllotoxin Derivatives on Noncancerous Diseases: A Systematic Review" oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: rewizja, nadzór merytoryczny.

Mój udział procentowy w przygotowaniu publikacji określam jako 15 %.
Wkład Piotra Strusa w powstawanie publikacji określam jako 40 %,

obejmował on: konceptualizację pracy, analizę uzyskanych wyników, pisanie, opracowanie i redakcję manuskryptu.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Piotra Strusa

Izabela M-Biały

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Izabela Młynarczuk-Biały
(podpis oświadczającego)