

POLITECHNIKA WARSZAWSKA

DYSCYPLINA NAUKOWA – NAUKI CHEMICZNE
DZIEDZINA NAUK ŚCISŁYCH I PRZYRODNICZYCH

Rozprawa doktorska

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**Synteza i badanie właściwości spektroskopowych i biologicznych
pochodnych stilbenu i izosterów stilbenu- potencjalnych leków w
terapii nowotworów.**

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WARSZAWA 2024

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Projekt Interdyscyplinarne Studia Doktoranckie "Od chemii do bioinnowacji" TRI-BIO-CHEM jest realizowany w ramach Programu Operacyjnego Wiedza Edukacja Rozwój 2014-2020 współfinansowanego ze środków Europejskiego Funduszu Społecznego



Rzeczpospolita
Polska

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Europejski Fundusz Społeczny



Serdecznie dziękuję moim promotorkom – dr hab. inż., prof. uczelni Hannie Krawczyk oraz dr hab. Ewie Joachimiak za propozycję ciekawej tematyki badawczej, okazane wsparcie, cierpliwość oraz serdeczność.

Szczególne podziękować pragnę złożyć mojej rodzinie w szczególności babci, która to zaszczerpiła we mnie głód chemicznej wiedzy. Narzeczonej oraz przyjaciółom za ich nieustającą wiarę we mnie.

Streszczenie w języku polskim

Mikrotubule są to wysoce dynamiczne i powszechnie występujące struktury komórkowe zbudowane z heterodimerów α - i β -tubuliny. Stanowią istotny składnik cytoszkieletu komórkowego oraz pełnią różnorodne role w komórce, takie jak utrzymanie struktury komórki, wewnątrzkomórkowy transport białek czy segregacja chromosomów potomnych podczas podziału komórkowego. Polimery tubuliny pełnią kluczową rolę w mitozie i są uznawane za doskonały cel dla środków chemioterapeutycznych stosowanych w leczeniu nowotworów. Niniejsza praca doktorska przedstawia zarys syntezy i mechanizmu działania nowych związków celujących w dynamikę polimeryzacji/depolimeryzacji mikrotubul. Rozprawa doktorska została oparta na dwóch artykułach przeglądowych, które posłużyły za podstawę do napisania wstępu poniższej rozprawy oraz dwóch artykułach badawczych, które zostały opisane w autoreferacie poniższej rozprawy. We wstępie omówiono budowę oraz dynamiczną niestabilność mikrotubul oraz czynniki biologiczne regulujące stabilność oraz dynamikę mikrotubul takie jak modyfikacje potranslacyjne tubuliny oraz ekspresja różnych izotypów tubuliny. Następnie skupiono się na czynnikach chemicznych regulujących stabilność oraz dynamikę mikrotubul, w szczególności związkach będących pochodnymi (izomerami oraz izosterami) stilbenu. Głównie skupiono się na opisie związków oddziałujących z domeną kolchicynową. W następnej części rozprawy doktorskiej zaprezentowano koncepcję fotofarmakologii oraz fotoprzełączalne inhibitory/stabilizatory dynamiki mikrotubul z szczególnym uwzględnieniem związków zawierających fragment azobenzenu w swojej strukturze. Pierwsza część autoreferatu omawia systematyczne badania nad oceną aktywności przeciwnowotworowej wybranych pochodnych stilbenu oraz dibenzo[*b,f*]oksepy. W badaniu tym syntezowano dziewięć pochodnych stilbenu oraz dziewiętnaście pochodnych dibenzo[*b,f*]oksepy oraz określono ich aktywność cytotoksyczną. Dla najbardziej obiecujących molekuł przeprowadzono badania dokowania molekularnego oraz określono wpływ na cytoszkielet mikrotubularny za pomocą immunofluorescencji. Przeprowadzone badania potwierdziły, że cytotoksyczność tych związków jest związana z ich zdolnością do oddziaływania z tubuliną. W kolejnym etapie badań przeprowadzono syntezę oraz badanie właściwości spektroskopowych i biologicznych fotochromowych pochodnych kolchicyny. Opracowane przełączniki molekularne ulegają fotoizomeryzacji pod wpływem światła widzialnego, zamiast promieniowania UV, które jest stosowane w klasycznych przełącznikach molekularnych. Jest to kluczowe ze względu na niestabilność kolchicyny pod wpływem promieniowania UV. Dla związku wiodącego wykazano zależną od światła cytotoksyczność wobec linii komórek nowotworowych HCT116 i MCF-7. Hamowanie polimeryzacji

oczyszczonej tubuliny oraz zaburzenie organizacji mikrotubul stanowią dowód wykorzystania fotochromowych pochodnych kolchicyny jako przełączników molekularnych zakłócających dynamikę mikrotubul. Podsumowując, udowodniono, że fotochromowe przełączniki molekularne oparte na strukturze kolchicyny stanowią podstawę do dalszych badań i rozwoju nowych klasy światłoczułych inhibitorów polimeryzacji tubuliny, które mogą znaleźć zastosowanie w przyszłych badaniach i aplikacjach klinicznych

Słowa kluczowe: fotofarmakologia, przełączniki molekularne, azobenzeny, izostery stilbenu, dibenzo[*b,f*]oksepy

Streszczenie w języku angielskim

Microtubules are highly dynamic and ubiquitous cellular structures composed of α - and β -tubulin heterodimers. They are a crucial component of the cellular cytoskeleton and play diverse roles within the cell, such as maintaining cell structure, intracellular protein transport, and segregation of daughter chromosomes during cell division. Tubulin polymers play a pivotal role in mitosis and are considered excellent targets for chemotherapeutic agents used in cancer treatment. This doctoral dissertation presents an outline of the synthesis and mechanism of action of new compounds targeting the dynamics of microtubule polymerization/depolymerization. The dissertation is based on two review articles, which served as the foundation for writing the introduction, and two research articles, described in the self-referential section of the dissertation. The introduction discusses the structure and dynamic instability of microtubules, as well as biological factors regulating their stability and dynamics, such as post-translational modifications of tubulin and the expression of different tubulin isotypes. The focus then shifts to chemical factors regulating microtubule stability and dynamics, particularly compounds that are derivatives (isomers and isosteres) of stilbene. Special emphasis is placed on compounds interacting with the colchicine-binding domain. The next section of the dissertation introduces the concept of photopharmacology and photoswitchable inhibitors/stabilizers of microtubule dynamics, with particular attention to compounds containing an azobenzene fragment in their structure. The first part of the self-referential section discusses systematic studies evaluating the anticancer activity of selected derivatives of stilbene and dibenzo[*b,f*]oxepine. In this study, nine stilbene derivatives and nineteen dibenzo[*b,f*]oxepine derivatives were synthesized, and their cytotoxic activity was determined. For the most promising molecules, molecular docking studies were conducted, and their effects on the microtubular cytoskeleton were assessed using immunofluorescence. The studies confirmed that the cytotoxicity of these compounds is related to their ability to interact

with tubulin. In the next stage of the research, photochromic derivatives of colchicine were synthesized and their spectroscopic and biological properties were studied. The developed molecular switches undergo photoisomerization under visible light instead of UV radiation, which is typically used in classical molecular switches. This is critical due to the instability of colchicine under UV irradiation. For the lead compound, light-dependent cytotoxicity was demonstrated in the HCT116 and MCF-7 cancer cell lines. The inhibition of purified tubulin polymerization and the disruption of microtubule organization provide evidence for the use of photochromic colchicine derivatives as molecular switches disrupting microtubule dynamics. In summary, it was demonstrated that photochromic molecular switches based on the structure of colchicine form the foundation for further research and development of a new class of light-sensitive tubulin polymerization inhibitors that may find applications in future studies and clinical applications.

Keyword: Photopharmacology, molecular switches, azobenzenes, stilbene isosteres, dibenzo[b,f]oxepines

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ŻYCIORYS NAUKOWY

EDUKACJA:

Studia I stopnia: Biotechnologia, Wydział Chemiczny, Politechnika Warszawska, 2014 rok.

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Studia II stopnia: Biotechnologia, Wydział Chemiczny, Politechnika Warszawska, 2016 rok.

Uzyskanie tytułu zawodowego magistra inżyniera na podstawie pracy dyplomowej pt. ”Studies on a new mechanism of rearrangement of selected unsaturated carboxylic acids”

ARTYKUŁY Z LISTY FILADELFIJSKEJ:

ARTYKUŁY STANOWIĄCE PODSTAWĘ ROZPRAWY DOKTORSIEJ:

[P1] **F. Borys**, E. Joachimiak, H. Krawczyk, H. Fabczak, *Review article*: Intrinsic and Extrinsic Factors Affecting Microtubule Dynamics in Normal and Cancer Cells. *Molecules* 25, 2020, 3705; doi:10.3390/molecules25163705. IF = 4.2

[P2] **F. Borys**, P. Tobiasz, M. Poterała, H. Krawczyk *Review article*: Development of novel derivatives of stilbene and macrocyclic compounds as potent of anti-microtubule factors. *Biomed. Pharmacother.* 133, 2021, 110973, doi:10.1016/j.biopha.2020.110973. IF = 6.9

[P3] **F. Borys**, P. Tobiasz, E. Joachimiak, H. Fabczak, H. Krawczyk, Systematic studies on anti-cancer evaluation of stilbene and dibenzo[*b,f*]oxepine derivatives. *Molecules* 28, 2023, 3558, doi:10.3390/molecules28083558. IF = 4.2

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- [P14]** P. Tobiasz, **F. Borys**, M. Borecka, H. Krawczyk Synthesis of building blocks with dibenzo[*b,f*] oxepin for use in photopharmacology. *Int. J. Mol. Sci.* 22, 2021, 11033. doi:10.3390/ijms222011033. IF = 4.9
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- [P16]** E. Mironiuk-Puchalska, O. Karatsai, A. Żuchowska, W. Wróblewski, **F. Borys**, L. Lehka, M.J Rędownicz, M. Koszytkowska-Stawińska, Development of 5-fluorouracil-dichloroacetate mutual prodrugs as anticancer agents. *Bioorg. Chem.* 140, 2023, 106784, doi: 10.1016/j.bioorg.2023.106784 IF = 4.5
- [P17]** P. Tobiasz, **F. Borys**, M. Kucharska, M. Poterała, H. Krawczyk, Synthesis and Study of Building Blocks with Dibenzo[*b,f*]oxepine: Potential Microtubule Inhibitors *Int. J. Mol. Sci.* 25, 2024, 6155, doi: 10.3390/ijms25116155 IF = 4.9

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1. **Filip Borys**, Hanna Krawczyk, pt. "Diazowe pochodne kolchicyny oraz sposób ich otrzymywania." Nr P.442759, data zgłoszenia 08.11.2022
2. **Filip Borys**, Piotr Tobiasz, Jakub Sobel, Hanna Krawczyk, pt." Fluorowe pochodne metoksydibenzo[b,f]oksepiny oraz sposób ich otrzymywania." Nr P.441238, data zgłoszenia 20.05.2022
3. **Filip Borys**, Piotr Tobiasz, Anna Arbaszewska, Hanna Krawczyk, pt." Sposób selektywnej redukcji grup nitrowych w pochodnych dibenzo[b,f]oksepiny ." Nr P.429386, data zgłoszenia 25.03.2019

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1. **Filip Borys**, Piotr Tobiasz, Jakub Sobel, Hanna Krawczyk, pt." Fluorinated derivatives of methoxydibenzo[b,f]oxepin and method for obtaining thereof." Nr PCT/PL2023/050036, data zgłoszenia 19.05.2023

GRANTY:

1. Uczestnictwo w grantach -Rady Naukowej Dyscypliny Nauki Chemiczne NChem 1 i NChem 3.
2. Interdyscyplinarne studia doktoranckie TRI-BIO-CHEM” praca doktorska na Wydziale Chemicznym PW i w Instytucie Nenckiego, w ramach wspólnego projektu- POWR.03.02.00-00-I007/16-00, pt."Od chemii do bioinnowacji dla lepszego życia”.

STAŻE ZAGRANICZNE:

1. COST Training School, Italy, Siena, 27.04.-01.05.2016.
2. COST Short term scientific mission (STSM), Groningen, Netherlands, 20.03.2017–13.04.2017 Topic: "Studies toward combination of biocatalytic Baeyer–Villiger oxidation of $\alpha\beta$ -unsaturated ketones and their subsequent biohydrolysis in tandem process."

NAGRODY: Laureat stypendium FEBS dla młodych naukowców-prezentacja wyników na konferencji międzynarodowej w Krakowie- FEEBS, 44th FEBS Congress, From Molecules to Living Systems, Krakow, July 6-11, 2019.

Sumaryczny Impact Factor: 68,1

Indeks Hirsha: 7

Sumaryczna Punktacja MNiSW/MEN: 1 840

CELE I ZAKRES BADAŃ

Pomimo wybuchu ogólnoświatowej pandemii Covid-19, spowodowanej przez nowego koronawirusa SARS-CoV-2 choroby nowotworowe pozostają drugą najczęstszą przyczyną zgonów na świecie ustępując tylko chorobom układu krążenia. Przedmiotem poniższej rozprawy doktorskiej jest projektowanie, synteza oraz określenie właściwości spektroskopowych jak i biologicznych pochodnych stilbenu i izosterów stilbenu – potencjalnych leków w terapii nowotworów. Do głównych celów prezentowanej rozprawy należą:

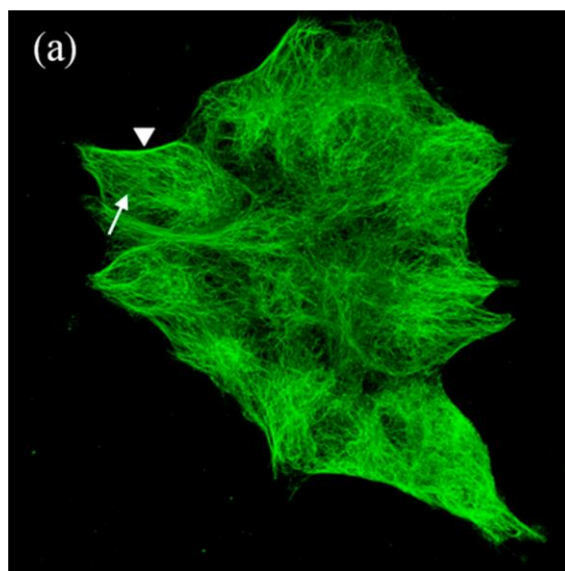
1. Opracowanie i krytyczna analiza literatury naukowej na temat czynników wewnętrznych oraz zewnętrznych wpływających na dynamikę mikrotubul w komórkach nowotworowych oraz komórkach nienowotworowych (publikacja **[P1]**).
2. Opracowanie i krytyczna analiza literatury naukowej przedstawiającej syntezę oraz mechanizm działania związków zaburzających dynamikę polimeryzacji tubuliny. W szczególności skupiono się na pochodnych stilbenu, związkach makrocyclicznych jak i również fotochromowych przełącznikach molekularnych hamujących polimeryzację tubuliny (publikacja **[P2]**).
3. Systematyczne badania na temat syntezy, charakterystyki oraz określenia właściwości przeciwnowotworowych na wybranych liniach komórkowych nowych podstawionych stilbenów oraz pochodnych stilbenu – dibenzo[*b,f*]oksepin (publikacja **[P3]**).
4. Synteza i badanie właściwości spektroskopowych oraz biologicznych izosterów stilbenu – azobenzenów o właściwościach fotochromowych jako potencjalnych leków w terapii fotofarmakologicznej nowotworów (publikacja **[P4]**).

Publikacje przeglądowe **[P1]** oraz **[P2]** zostały omówione we wstępie niniejszej rozprawy. Natomiast publikacje eksperymentalne **[P3]** oraz **[P4]** zostały przybliżone w autoreferacie niniejszej rozprawy.

WSTĘP

1. Budowa cytoszkieletu eukariotycznego

W komórkach eukariotycznych cytoszkielet jest to sieć włóknistych białek, które rozciągają się poprzez cytoplazmę. Aby wykonywać szeroki zakres funkcji takich jak utrzymanie kształtu komórki, jej ruch, udział w transporcie wewnątrzkomórkowym jak i również podziale komórkowym musi posiadać elementy strukturalnie stabilne, jak i dynamiczne zdolne do dynamicznej reorganizacji. Obecnie pod terminem „cytoszkielet” opisuje się trzy elementy struktur w komórce eukariotycznej: filamenty aktynowe, filamenty pośrednie oraz mikrotubule (Rysunek 1.). Niedawno zaproponowano septyny – konserwowane białka wiążące GTP jako kolejny element cytoszkieletu [1,2].



Rysunek 1. Cytoszkielet mikrotubularny w komórkach HCT116 zwizualizowany za pomocą immunofluorescencji z użyciem przeciwciała przeciwko- α -tubulinie, 12G10 – strzałka: mikrotubule rozmieszczone wzdłuż długiej osi komórki; grot: mikrotubule układające się na granicy komórki..

Wszystkie białka cytoszkieletu posiadają wspólne cechy, takie jak występowanie w postaci liniowych polimerów w komórkach eukariotycznych oraz zdolność do samoorganizacji w polimery *in vitro*. Złożona sieć cytoszkieletu jest regulowana przez kilka klas białek między innymi czynniki promujące nukleację do inicjacji wzrostu włókien, czynniki terminujące wzrost włókien, polimerazy, białka depolimeryzujące oraz białka tnące rozcinające włókna jak

i również białka sieciujące i stabilizujące, które współuczestniczą w budowaniu struktur wyższego rzędu [3].

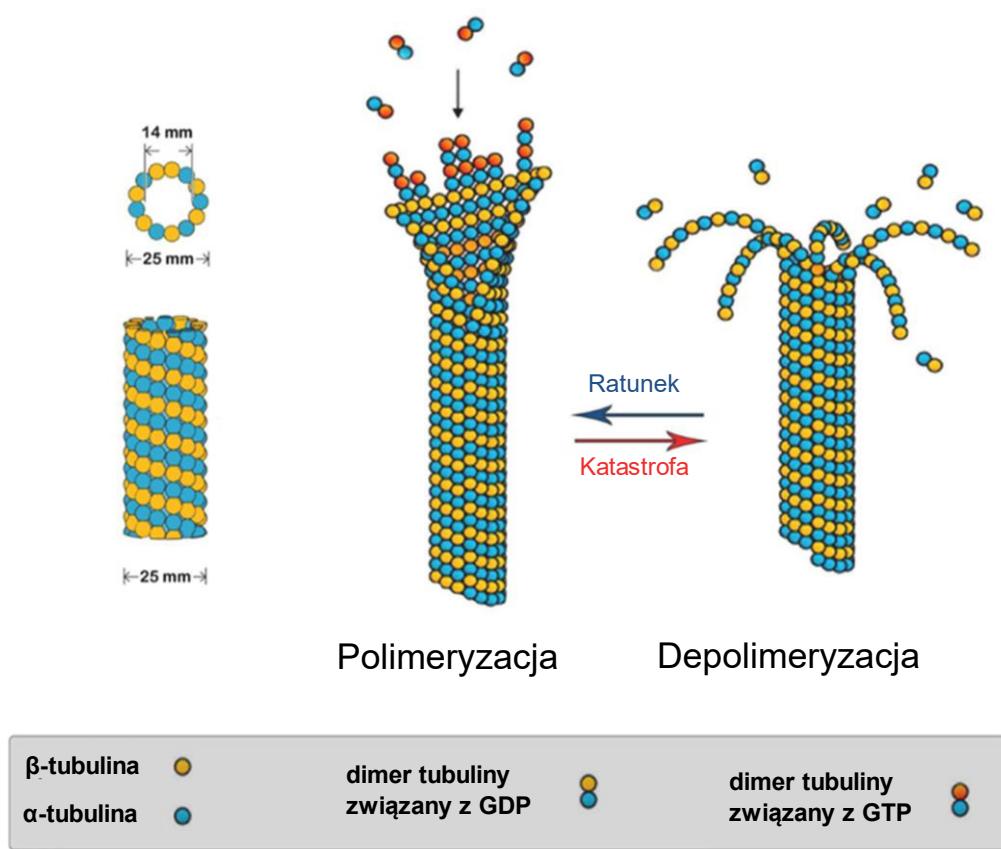
Badania nad wyrafinowanymi narzędziami badawczymi służącymi do zbadania struktury oraz dynamiki cytoszkieletu w środowisku komórkowym jak i *in vivo* są wysoce pożądane, ze względu na wiele funkcji cytoszkieletu w niemal wszystkich procesach komórkowych, które pomimo intensywnych badań nie zostały wystarczająco poznane do dnia dzisiejszego. Wyjaśnienie roli oraz funkcji dynamiki cytoszkieletu w kluczowych procesach komórkowych takich jak: cykl komórkowy, utrzymanie kształtu komórki, wewnątrzkomórkowy transport oraz segregacja chromosomów potomnych podczas mitozy jest kluczowe dla opracowania nowych leków na wiele chorób, takich jak na przykład choroby nowotworowe [4]. Dlatego małe związki organiczne, których celem molekularnym jest cytoszkielet okazały się niezbędne do wyjaśnienia procesów komórkowych, w których on uczestniczy. Ich wykorzystanie w badaniach podstawowych doprowadziło do opracowania leków zatwierdzonych klinicznie do stosowania podczas wystąpienia dny moczanej oraz w leczeniu niektórych nowotworów. Obecnie dostępne środki działają poprzez bezpośrednią cytotoksyczność prowadząc do zakłócenia dynamiki tubulina-mikrotubule, powodując depolimeryzację mikrotubul np. winkrystyna (alkaloid wyodrębniony z roślin gatunku *Catharanthus roseus*, wcześniej *Vinca rosea* stąd tzn. alkaloidy *Vinca*) bądź stabilizacje mikrotubul np. paklitaksel (alkaloid wyodrębniony z cisa krótkolistnego). Wśród środków zaburzających waskulaturę (sieć naczyń krwionośnych) towarzyszącą nowotworom (VDA, vascular disrupting agents) kombretastatyna A-4 „wkroczyła” w fazę badań klinicznych [5].

2. Mikrotubule

2.1.Struktura i nukleacja mikrotubul

Mikrotubule – wszechobecne komórkowe biopolimery są ważnym składnikami cytoszkieletu oraz pełnią wiele istotnych funkcji komórkowych między innymi utrzymują kształt komórki, uczestniczą w procesie transportu wewnątrzkomórkowego, a także tworzą rzęski oraz wici, które umożliwiają ruch komórki. Jednak ze względu na zastosowanie w chemioterapii najistotniejszą funkcją mikrotubul jest ich udział w procesie mitozy, gdzie tworzą wrzeciono kariokinetyczne i odpowiedzialne są za rozdział oraz prawidłową segregację chromosomów potomnych. Mikrotubule zbudowane są z heterodimerów $\alpha\beta$ -tubuliny. Podjednostki α - oraz β -tubuliny wykazują 40% identyczności sekwencji aminokwasów, z rdzeniem składającym się z dwóch harmonijek- β otoczonych α -helisą, co potwierdzono za pomocą mikroskopii

elektronowej z rozdzielczością 3.7 Å [6]. Podjednostki α - oraz β -tubuliny łączą się w heterodimery $\alpha\beta$ -tubuliny („monomery tubuliny”), cząsteczki o niesymetrycznej strukturze, w których wyróżnia się cztery powierzchnie: koniec +, koniec -, powierzchnię H3 i powierzchnię ML. Koniec - zlokalizowany jest w „dolnej” części podjednostki α , natomiast koniec + w „górnej” części podjednostki β . Powierzchnie końców + i - uczestniczą w oddziaływaniach wzdłużnych pomiędzy heterodimerami, podczas gdy powierzchnie H3 i ML w oddziaływaniach bocznych. Każdy monomer tubuliny wiąże dwie cząsteczki guanozyny-5'-trifosforanu (GTP). GTP związane z podjednostką β może ulegać rozpadowi do GDP i wymianie, natomiast cząsteczka GTP związana z podjednostką α nie podlega tym procesom. W procesie polimeryzacji, heterodimery $\alpha\beta$ -tubuliny łączą się wiązaniami niekowalencyjnymi wzdłużnie tworząc protofilament, a protofilamenty łączą się poprzecznie tworząc mikrotubule [7]. W wyniku tego procesu powstaje mikrotubula budową przypominająca pusty w środku cylinder, której długość może sięgać kilku mikrometrów. W komórkach eukariotycznych właściwości mikrotubul regulowane są głównie przez regulację dynamiki ich polimeryzacji i



Rysunek 2. Struktura oraz dynamiczna niestabilność mikrotubul

depolimeryzacji. *In vivo* mikrotubule polimeryzują i depolimeryzują z większą prędkością na końcu +. Z kolei koniec - najczęściej zakotwiczony jest w centrosomach i nie podlega dynamicznym zmianom. Wzrost mikrotubuli następuje w dwóch etapach: nukleacji oraz elongacji. W komórkach eukariotycznych inicjacja polimeryzacji tubuliny (nukleacja) jest związana z obecnością matrycy – tak zwanych pierścieni nukleacji (γ -TuRC, ang. γ -tubulin ring complex) czyli kompleksów złożonych m.in. z trzeciego typu tubuliny – γ -tubulin lub nowo utworzonych końców mikrotubuli powstałych w wyniku przerwania ciągłości już istniejącej mikrotubuli [8].

2.2.Dynamika utworzonej mikrotubuli

Proces powstawania mikrotubul jest niezwykle dynamiczny ponieważ mikrotubule przechodzą cykle polimeryzacji i depolimeryzacji, znane jako dynamiczna niestabilność mikrotubul [9]. Szybka depolimeryzacja mikrotubuli nazywa się „katastrofą”, natomiast przejście z fazy skracania się mikrotubuli do ponownego wzrostu „ratunkiem”. Podczas wzrostu mikrotubuli do jej dodatniego końca dodawane są monomery tubuliny związane z cząsteczką guanozyno-5'-trifosforanu (GTP), który po jakimś czasie jest hydrolizowany do guanozyno-5'-difosforanu (GDP), co skutkuje obniżeniem powinowactwa monomeru do otaczających podjednostek i wymusza zakrzywienie mikrotubuli [10]. Tak długo jak dodatni koniec zawiera „czapeczkę GTP” mikrotubula wydłuża się. Jeśli jednak hydroliza GTP jest szybsza niż dołączanie nowych monomerów mikrotubula ulega gwałtownemu skróceniu (Rysunek 2).

2.3.Czynniki biologiczne regulujące stabilność oraz dynamikę mikrotubul

Biorąc pod uwagę szeroki zakres funkcji pełnionych przez mikrotubule w komórce eukariotycznej, kluczowa jest precyzyjna biologiczna regulacja stabilności oraz dynamiki mikrotubul, która odbywa się za pomocą kilku mechanizmów. Mechanizmy te obejmują oddziaływania z różnymi typami białek, modyfikacje potranslacyjne tubuliny oraz ekspresję różnych izotypów tubuliny. Do modyfikacji potranslacyjnych zaliczane są: acetylacja, metylacja, poliaminacja, glutamylacja oraz poliglutamylacja, glicylacja oraz poliglicylacja, tyrozynacja, fosforylacja. Mogą one prowadzić do znaczącej zmiany właściwości monomerów tubuliny jak i samych mikrotubuli [11-14]. Na przykład acetylacja lizyny 40 w α -tubulinie przez N-acetyl transferazę 1 (ATAT1, ang. α -tubulin N-acetyltransferase 1) prowadzi do powstania stabilniejszych mikrotubul, poprzez osłabienie kontaktów bocznych między protofilamentami, w efekcie zwiększając elastyczność mikrotubul [15-17]. Ludzki genom koduje siedem izotypów α -tubuliny oraz dziewięć izotypów β -tubuliny[18]. W konsekwencji mikrotubule

Tabela 1. Cechy izotypów tubuliny występujących u człowieka.

Izotyp tubuliny	Nazwa genu kodującego	Występowanie	Komórkowo specyficzne funkcje	Sekwencja C-terminalna
α Ia	TUBA1A	powszechnie	migracja neuronów	VEGEGEEEEGEEY
α Ib	TUBA1B	powszechnie		VEGEGEEEEGEEY
α Ic	TUBA1C	powszechnie		ADGEDEGEEY
α IIIc	TUBA3C	komórki rozrodcze		VEAEAEEGEEY
α IIIe	TUBA3E	komórki rozrodcze		VEAEAEEGEAY
α IVa	TUBA4A	powszechnie	biogeneza płytek krwi	YEDEDEGEE
		wysokie: serce i mięśnie szkieletowe		
α VIII	TUBA8	umiarkowane: mózg, jądra i tarczyca	spermatogeneza	FEEENEKEEF
		bardzo niskie: wszystkie inne tkanki		
β I	TUBB	powszechnie	przeżycie zróżnicowanych komórek nerwiaka zarodkowego	EEEEDFGEEAEEEE
β IIa	TUBB2A	wysokie: mózg		DEQGEFEEEEGEDEA
β IIb	TUBB2B	bardzo niskie: wszystkie inne tkanki	wzrost wypustek neuronalnych	DEQGEFEEEEGEDEA
β III	TUBB3	umiarkowane: mózg niskie: jądra	stres oksydacyjny, regeneracja nerwów i aksonów	EEEGEMYEDDEEESEAQGPK
β IVa	TUBB4A	wysokie: mózg umiarkowane/niskie: inne tkanki	Funkcjonowanie neuronów i oligodendrocytów	EEGEFEEEEAEVEA
β IVb	TUBB4B/ TUBB2C	powszechnie		EEGEFEEEEAEVEA
β V	TUBB6	powszechnie w małym stopniu	regeneracja mięśni szkieletowych	NDGEEAFEDDEEEIDG
β VI	TUBB1	bardzo niskie we wszystkich tkankach poza szpikiem kostnym oraz śledzioną	cytoskielet płytek krwi	VLEEDDEVTAAEMEPEDKGH
β VIII	TUBB8	komórki rozrodcze	dojrzewanie oocytów	EEEEDEEYAEVEA

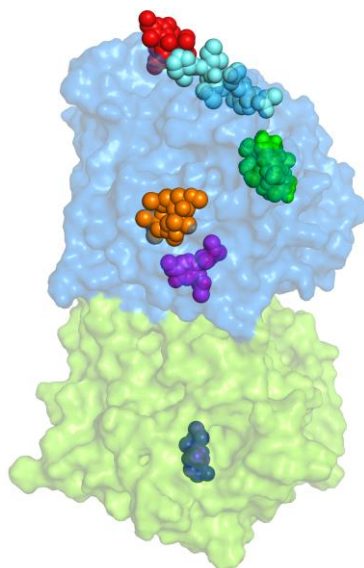
złożone z kombinacji różnych izotypów α - i β -tubuliny występują specyficznie w zależności od rodzaju tkanki oraz stadium rozwojowego, znacząco różniąc się od siebie właściwościami [19]. Ludzka α -tubulina należy do czterech klas (z dziewięciu opisanych u ssaków) α I, α III, α IV oraz α VIII (Tabela 1). Warto zaznaczyć, że dwie klasy wykazują tkankowo specyficzną dystrybucję, z klasą α III występującą głównie w męskich komórkach rozrodczych oraz klasą α VIII obecną głównie w komórkach mięśniowych, sercowych oraz szkieletowych. Pozostałe dwie klasy są szeroko rozpowszechnione. Chociaż funkcje izotypów α -tubuliny specyficzne dla niektórych tkanek lub komórek zostały ostatnio opisane w literaturze naukowej, to ich dokładna rola w dynamice mikrotubul wciąż pozostaje nie do końca poznana [20-23]. Ludzka β -tubulina należy do siedmiu klas (z dziewięciu występujących u ssaków): β I – β VI oraz β VIII. Z dostępnych danych naukowych wydaje się, że najszerszej rozpowszechnione są izotypy β I, β IVb, β V oraz β VIII, podczas gdy pozostałe izotypy β -tubuliny wykazują bardziej tkankowo specyficzne występowanie [24]. Izotyp β VIII jest niezbędny do prawidłowego rozwoju oocytu oraz wczesnego rozwoju zarodka człowieka [25]. Występowanie tubuliny β II, β III oraz β IVa głównie w mózgu sugeruje istotność tych izoform w procesach związanych z układem nerwowym [26]. Postuluje się wpływ obecności izotypu β III w ochronie przed stresem oksydacyjnym oraz wskazuje jego funkcje w procesie wzrostu aksonów i regeneracji nerwów [27, 28]. Od ponad dwóch dekad wiadomo, że mikrotubule złożone z tubuliny β III są bardziej dynamiczne niż, mikrotubule złożone z tubuliny β II lub β IV [29]. Na podstawie niedawno opublikowanych danych wykazano, że główna różnica jest spowodowana znacznym wzrostem częstotliwości „katastrofy” (depolimeryzacji) mikrotubul składających się z tubuliny β III, podczas gdy tempo wzrostu mikrotubuli pozostaje na podobnym poziomie [30,31]. Białka związane z mikrotubulami (MAPs, ang. microtubule-associated proteins) są kolejnym biologicznym czynnikiem wpływający na dynamikę mikrotubul w komórce eukariotycznej. Ogólnie białka związane z tubuliną można podzielić na stabilizujące bądź destabilizujące mikrotubule, jednak stabilizacja lub destabilizacja mikrotubul może zachodzić poprzez wpływ na jeden z kilku procesów takich jak: nukleacja mikrotubuli, stabilizacja lub destabilizacja matrycy lub końca mikrotubuli. Dlatego białka związane z mikrotubulami podzielono na kilka funkcjonalnych kategorii: (i) nukleatory mikrotubul, (ii) białka wiążące się do końców mikrotubuli, (iii) białka wiążące się z powierzchnią mikrotubuli, (iv) enzymy tnące mikrotubule (katanina, spastyna), (v) białka motoryczne (kinezy, dyneiny) [32]. Nukleatory mikrotubul zwiększają częstość inicjacji powstania nowej mikrotubuli zarówno na kompleksie γ -TuRC jak i na „zarodkach” mikrotubul oraz stabilizują wzrost mikrotubul. Białka wiążące się z końcami mikrotubuli są specyficzne do dodatniego (+TIPs, ang. microtubule-plus-end tracking

proteins) lub ujemnego (-TIPs, ang. microtubule-minus-end tracking proteins) końca mikrotubuli. +TIPs należą do rodziny około dwudziestu różnych rodzin białek. Mogą wykazywać właściwości zarówno stabilizujące jak i destabilizujące mikrotubule. Wśród białek wiążących się z matrycą mikrotubul należy wyróżnić siedem klasycznych rodzin tych białek: MAPs, MAP1, MAP2, MAP4, MAP6, MAP7 oraz białko tau, które promują polimeryzację tubuliny a tym samym stabilizację oraz wzrost mikrotubul. Regulują one również asocjację mikrotubul z innymi elementami cytoszkieletu, organellami oraz membraną komórkową. Wpływają również na transport wzdłuż mikrotubul oraz rozcinanie mikrotubul poprzez kompetencyjne wiązanie się na powierzchni mikrotubul w miejscach wiązania enzymów tnących mikrotubule oraz białek motorycznych [33]. Organizacja mikrotubul jest również regulowana poprzez enzymy tnące mikrotubule takie jak: katanina i spastyna, których aktywność może prowadzi do skracania się mikrotubul lub nawet ich depolimeryzacji, ale zarazem do powstania wielu „zarodków” mikrotubul, które służą jako nukleatory mikrotubul oraz wolnych heterodimerów $\alpha\beta$ -tubuliny, które mogą być następnie wbudowane w strukturę nowej mikrotubuli. Tak więc, enzymy tnące mikrotubule mogą wykazywać w stosunku do dynamiki oraz masy polimeru mikrotubul zarówno pozytywny jak i negatywny efekt [34].

2.4.Czynniki chemiczne regulujące stabilność oraz dynamikę mikrotubul

Badania nad mikrotubulami były ściśle połączone z wykorzystaniem małych organicznych cząsteczek oddziałujących z tubuliną. W ciągu ostatnich lat prowadzone były intensywne badania nad syntezą oraz określeniem właściwości biologicznych i spektroskopowych związków oddziałujących z mikrotubulami (ang. microtubule-targeting agents), nie tylko ze względu na wyjaśnienie procesów komórkowych w których uczestniczą mikrotubule, ale również w celu zastosowania klinicznego. Wiele związków oddziałujących z mikrotubulami syntezowane jest przez rośliny, grzyby oraz bezkręgowce jako naturalny środek obrony przeciwko pasożytom lub innym konkurencyjnym gatunkom [35]. Biorąc pod uwagę mechanizm działania możemy wyróżnić dwa główne typy związków oddziałujących z mikrotubulami: związki stabilizujące mikrotubule (inhibitory depolimeryzacji tubuliny) oraz związki destabilizujące mikrotubule (inhibitory polimeryzacji tubuliny). W globularnej części heterodimeru $\alpha\beta$ -tubuliny możemy wyróżnić przynajmniej sześć miejsc (tzw. kieszeni) wiązania związków organicznych (które nazywane są od leków wiążących się z tymi miejscami), które wiążąc cząsteczkę ligandu indukują zmianę struktury tubuliny i w konsekwencji zmianę dynamiki mikrotubul (Rysunek 3) [36]. Cecha ta wykorzystywana jest w chemioterapii nowotworów [37]. Spośród czterech miejsc wiązania zlokalizowanych na

podjednostce β -tubuliny najobszerniej opisane są miejsce wiązanie alkaloidów *Vinca* oraz miejsce wiązania taksanów. Miejsce wiązania kolchicyny zlokalizowane jest na powierzchni pomiędzy podjednostkami α - i β -tubuliny. Natomiast miejsce wiązania związku o angielskiej nazwie „pironetin” jako jedyne zlokalizowane jest na podjednostce α -tubuliny [36]. W niniejszej pracy doktorskiej skupiono się na związkach, które oddziałują z miejscem wiązania kolchicyny.

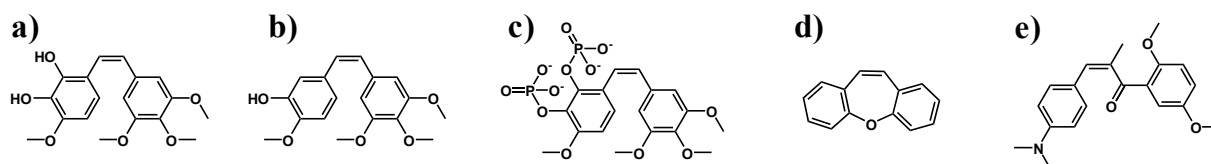


Rysunek 3. Lokalizacja miejsc wiązania związków oddziałujących z mikrotubulami w strukturze heterodimeru $\alpha\beta$ -tubuliny (PDB: 1SA0). Kolchicyna zwizualizowana w kolorze fioletowym.

2.5. Związki oddziałujące z domeną kolchicynową

Kolchicyna jest to związek pochodzenia naturalnego wyizolowany z nasion zimowita jesiennego (*Colchicum autumnale*) – rośliny występującej na terenie Europy. Alkaloid ten zawiera w swojej strukturze trzy pierścienie, z których dwa pierścienie oddziałują z podjednostką β -tubuliny, natomiast pierścień aromatyczny oddziałuje z podjednostką α -tubuliny (Rysunek 4). Wiązanie się kolchicyny do tubuliny jest wolnym i praktycznie nieodwracalnym procesem silnie zależnym od temperatury [38]. Związanie liganda w miejscu wiązania kolchicyny powoduje stabilizację zakrzywionej konformacji heterodimeru $\alpha\beta$ -tubuliny. Podczas polimeryzacji mikrotubuli nowo dołączone heterodimery $\alpha\beta$ -tubuliny ulegają przejściu z konformacji zakrzywionej do konformacji prostej, co wymaga zmiany ułożenia kilku struktur drugorzędowych β -tubuliny bliżej siebie. W rezultacie miejsce wiązania

kolchicyny ulega skurczeniu. Gdy miejsce to jest związane z ligandem takie zmiany konformacji nie mogą wystąpić. W związku z tym kompleks ligand – heterodimer $\alpha\beta$ -tubuliny nie może być monomerem podczas polimeryzacji mikrotubuli [54,55]. Pomimo iż, kolchicina znalazła zastosowanie kliniczne w leczeniu chorób nienowotworowych (np. dna moczanowa, rodzinna gorączka śródziemnomorska, choroba Behçeta [39-41]) to ani kolchicina, ani inne pokrewne związki nie znalazły zastosowania w chemioterapii nowotworów ze względu na ich wysoką toksyczność dla zdrowych tkanek w stężeniach potrzebnych do wywołania efektu przeciwnowotworowego [42]. W ciągu ostatnich kilkudziesięciu lat opisano oraz zbadano aktywność biologiczną wielu cząsteczek o mniejszej toksyczności dla zdrowych komórek między innymi: pochodne stilbenu – kombretastatyny (kombretastatyna A-1, kombretastatyna A-4, difosforan kombretastatyny A-1(Oxi4500)), pochodne dibenzooksepiny lub pochodne chalkonu (MDL 27048) [43-47]. Dibenzo[*b,f*]oksepiny posiadają dwa pierścienie aromatyczne połączone z jednej strony mostkiem tlenowym, z drugiej mostkiem winylowym, co warunkuje ich ułożenie w konfiguracji (*Z*)-stilbenu (Rysunek 4). Ponadto dibenzo[*b,f*]oksepiny poza aktywnością przeciwnowotworową wykazują szereg istotnych właściwości biologicznych takich jak: właściwości przeciwzapalne, przeciwdepresyjne oraz przeciwpsychotyczne [48-52]. Pomimo intensywnych badań do dnia dzisiejszego nie znaleziono związku oddziałującego z domeną kolchicynową, który zostałby zatwierdzony w klinicznym leczeniu nowotworów, kilka molekuł znajduje się w fazie badań klinicznych [53].

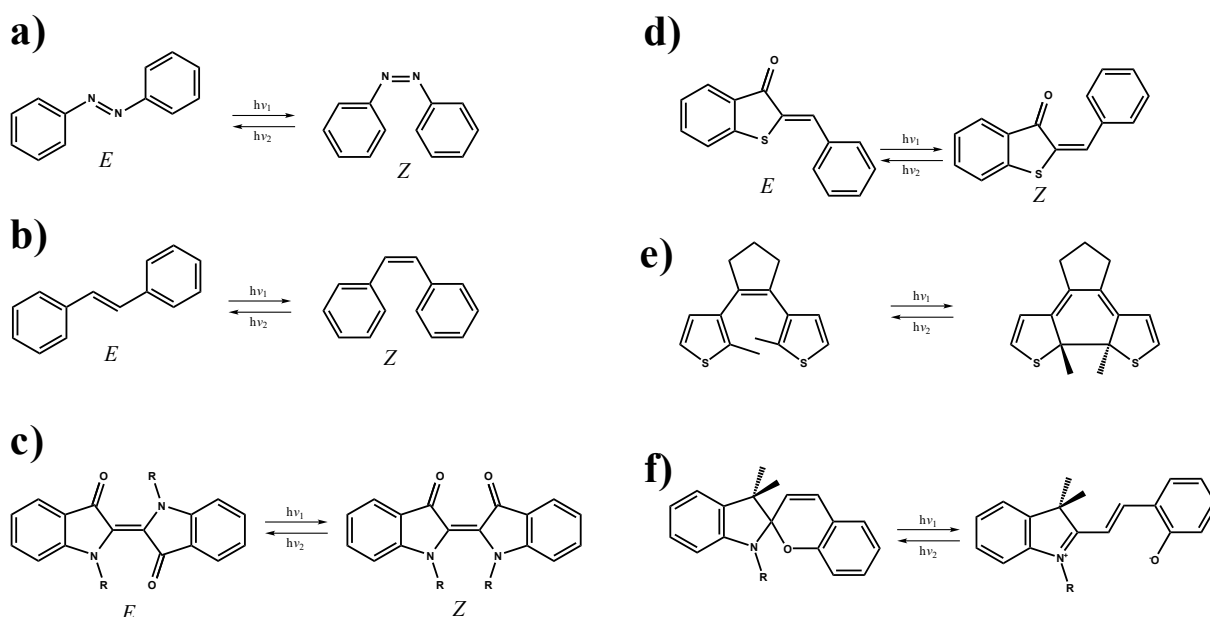


Rysunek 4. Struktura wybranych związków oddziałujących z domeną kolchicynową tubuliny: a) kombretastatyna A-1, b) kombretastatyna A-4, c) difosforan kombretastatyny A-1, d) dibenzo[*b,f*]oksepina, e) MDL 27048 ((*E*)-1-(2,5-dimetoksyfenyl)-3-[4-(dimetyloamino)fenyl]-2-metylprop-2-en-1-on).

3. Fotofarmakologia

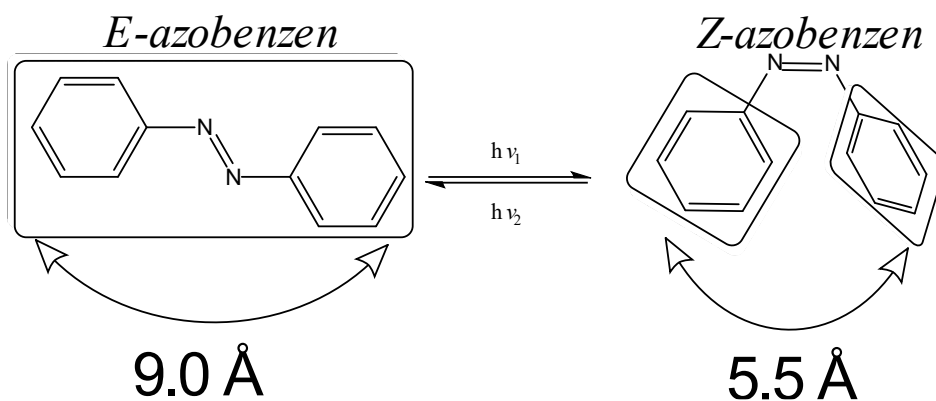
Fotofarmakologia jest to stosunkowo nowa technika do zastosowania w medycynie polegająca na wbudowaniu elementu fotochromowego – podlegającego odwracalnej zmianie struktury pod wpływem naświetlania promieniowaniem elektromagnetycznym (tzw. przełącznika molekularnego) w strukturę związku wyjściowego o znanej lub oczekiwanej aktywności

biologicznej. Celem fotofarmakologii jest zmniejszenie skutków działania substancji poza celem komórkowym i poważnymi ogólnoustrojowymi lub środowiskowymi skutkami ubocznymi. Można to zrealizować poprzez ustanowienie zewnętrznych i selektywnych metod kontrolowania aktywności związków aktywnych z precyzją czasową oraz przestrzenną. Metoda ta obejmuje projektowanie, syntezę oraz zastosowanie leków w postaci fotochromowych przełączników molekularnych, których strukturę, a w konsekwencji aktywność biologiczną można kontrolować za pomocą światła o specyficznej długości fali [56,57]. Choć fotofarmakologia jest stosunkowo nową techniką, która nie znalazła jeszcze zastosowania klinicznego, ostatnie lata „obfitowały” w badania mające na celu opracowanie nowych fotochromowych bioaktywnych związków między innymi: agonistów receptorów sprzężonych z białkami G, modulatorów kanałów jonowych oraz inhibitorów enzymów [58-60]. Fotochromowy przełącznik molekularny jest to cząsteczka chemiczna, która posiada dwa lub więcej stanów stabilnych termodynamicznie, w które może się przekształcać pod wpływem światła. Fotoizomeryzacja przełączników molekularnych zachodzi poprzez dwa główne mechanizmy, są to: izomeryzacja izomerów (*E*) – (*Z*) oraz 6π elektrocyclizacja trienów. Pochodne azobenzenów, indygo, hemitioindygo oraz stilbenów ulegają izomeryzacji wiązania podwójnego, podczas gdy ditienyloeteny zmieniają swoją strukturę poprzez 6π elektrocyclizację. Istnieją również związki które ulegają mieszanemu mechanizmowi jak np. spiropirany. W pierwszym etapie ulegają 6π elektrocyclizacji, a następnie izomeryzacji wiązania podwójnego (Rysunek 5) [61]. W poniższej pracy skupiono się na syntezie i określeniu właściwości spektroskopowych oraz biologicznych przełączników molekularnych opartych na strukturze azobenzenu – izostere stilbenu, dlatego w dalszej części zostaną one opisane bardziej szczegółowo. Jak już wspomniano, azobenzeny mogą ulegać fotoizomeryzacji pomiędzy izomerami (*E*) – (*Z*) pod wpływem światła, która prowadzi do znacznych zmian w właściwościach fizycznych takich jak geometria cząsteczki, moment dipolowy czy widmo absorpcyjne. Izomer (*E*) azobenzenu jest stabilniejszy o około 12 kcal/mol od izomeru (*Z*). Izomer ten pod wpływem naświetlania światłem z zakresu 320–350 nm izomeryzuje do izomeru (*Z*). Reakcja ta jest odwracalna i izomer (*E*) powstaje ponownie pod wpływem naświetlania światłem z zakresu 400–450 nm lub



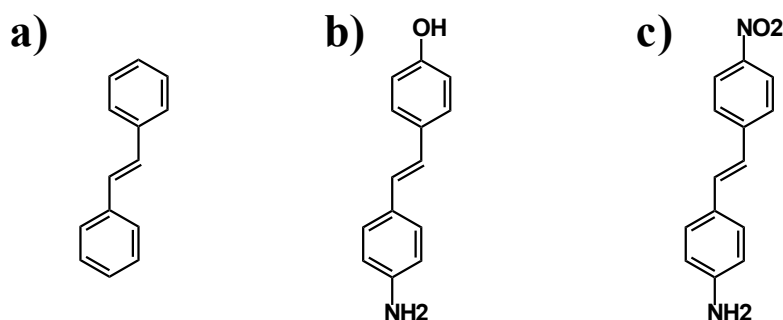
Rysunek 5. Przykłady przełączników molekularnych ulegających izomeryzacji *E/Z* – a) azobenzeny, b) stilbeny, c) pochodne indygo, d) pochodne hemitioindygo; 6 π elektrocyklizacji e) ditynyloeten lub mieszanemu mechanizmowi f) spiropirany.

w wyniku spontanicznej relaksacji układu do stanu równowagi termodynamicznej, który przeważnie składa się wyłącznie z izomeru (*E*) [62]. Mechanizm fotoizomeryzacji azobenzenów nie jest do końca poznany, postuluje się jednak dwa główne prawdopodobne mechanizmy: rotację wokół wiązania podwójnego oraz płaskiej inwersji. Wprowadzenie podstawników w pierścieniach aromatycznych skutkuje znacznymi zmianami we właściwościach spektroskopowych oraz fotochemicznych pochodnych azobenzenu. Odpowiednio dobrane mogą nawet spowodować, że izomer (*Z*) będzie termodynamicznie trwalszy od izomeru (*E*) [63]. Proces fotoizomeryzacji azobenzenu powoduje zmniejszenie się odległości pomiędzy dwoma przeciwległymi atomami węgla w pozycji 4 w pierścieniach aromatycznych z 9.0 Å w formie (*E*) do 5.5 Å w formie (*Z*). Izomer (*E*) azobenzenu jest praktycznie płaski i nie posiada momentu dipolowego, natomiast izomer (*Z*) posiada zakrzywioną geometrię cząsteczki oraz wykazuje moment dipolowy równy 3.0 D. W formie (*Z*) jeden z pierścieni aromatycznych ulega rotacji co spowodowane jest względami sterycznymi i bliskością chmury elektronowej drugiego pierścienia aromatycznego (Rysunek 6) [64,65]. Układ pierścieni aromatycznych ma również wpływ na strukturę widma



Rysunek 6. Proces fotoizomeryzacji azobenzenu prowadzi do zmian w geometrii cząsteczki.

protonowego magnetycznego rezonansu jądrowego (^1H NMR, ang. nuclear magnetic resonance). Sygnały pochodzące od izomeru (Z) pojawiają się przy niższych częstościach w porównaniu do sygnałów pochodzących od izomeru (E) ze względu na diamagnetyczne przesunięcie sygnałów. Pasma w widmach absorpcyjnych UV-Vis izomerów (E) oraz (Z) azobenzenu są odmienne jednak nakładające się. W widmie tym możemy wyróżnić dwa charakterystyczne maksima absorpcji odpowiadające przejściom elektronowym $\pi \rightarrow \pi^*$ oraz $n \rightarrow \pi^*$. W widmie (E) azobenzenu obserwuje się pasmo o mniejszej intensywności ok. 440 nm ($n \rightarrow \pi^*$) oraz o większej intensywności ok. 320 nm ($\pi \rightarrow \pi^*$). W widmie izomer (Z) azobenzenu również występuje maksimum absorpcji około wartości 440 nm ($n \rightarrow \pi^*$ o większej intensywności w porównaniu z izomerm (E)), natomiast pasmo odpowiadające przejściu $\pi \rightarrow \pi^*$ ulega przesunięciu hipsochromowemu z maksimami absorpcji przy długościach fali 250 oraz 280 nm. Naświetlanie azobenzenu światłem o długości fali wynoszącym 313 nm lub 436 nm skutkuje ustaleniem się stanu równowagi (stan fotostacjonarny) złożonym w przybliżeniu odpowiednio z 20% lub 90% izomeru (Z) [66]. Proces izomeryzacji może być badany zarówno za pomocą magnetycznego rezonansu jądrowego jak i za pomocą spektroskopii UV-Vis. Ze względu na charakter widma UV-Vis pochodne azobenzenu można podzielić na trzy kategorie: związki typu azobenzenu, związki typu aminoazobenzenu oraz związki typu pseudostilbenów (Rysunek 7) [67]. Związki typu azobenzenu wykazują podobną strukturę widma do niepodstawionego azobenzenu. Związki typu aminoazobenzenu posiadające podstawniki w pozycjach *orto*- lub *para*- z grupą elektronodonorową wykazują w swoim widmie batochromowe przesunięcie pasm oraz skrócenie czasu półtrwania w stosunku do związków typu azobenzenu. Natomiast związki typu pseudostilbenów wykazują jeszcze większy efekt batochromowego przesunięcia pasm i charakteryzują się jeszcze większym skrócenie czasu półtrwania [65].

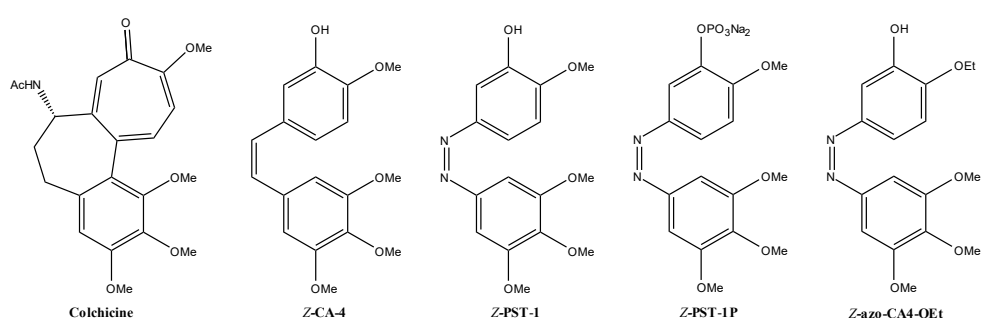


Rysunek 7. Przykłady związków należących do związków typu azobenzenu (niepodstawiony azobenzen), typu aminoazobenzenu (4'-hydroksy-4-aminoazobenzen) oraz typu pseudostilbenów (4'-nitro-4-aminoazobenzen).

4. Przełączniki molekularne oparte o strukturę azobenzenu zaburzające stabilność oraz dynamikę mikrotubul

Przełączniki molekularne zaburzające stabilność oraz dynamikę mikrotubul możemy podzielić podobnie jak typowe związki oddziałujące z tubuliną na przełączniki molekularne destabilizujące mikrotubule np. poprzez inhibicję polimeryzacji tubuliny oraz przełączniki molekularne stabilizujące mikrotubule np. poprzez aktywację polimeryzacji tubuliny oraz zapobieganie depolimeryzacji już powstałej mikrotubuli. Wiodącym przykładem przełączników molekularnych opartych o strukturę azobenzenu, które powodują destabilizację mikrotubul są analogi kombretastatyny A-4, w której strukturze wiązanie podwójne węgiel-węgiel zostało zastąpione przez izosteryczne wiązanie podwójne azot-azot (tzw. fotostatyny **PTS-1** bądź azokombretastatyna **azo-CA-4**, Rysunek 8). Zostały po raz pierwszy syntezowane oraz opisane równolegle przez trzy zespoły badawcze [68-70]. Na podstawie przeprowadzonych badań zidentyfikowano związek **PTS-1** oraz prolek tego związku **PTS-1P** zawierający grupę fosforanową, jako najbardziej obiecujące analogi do dalszych badań. Naświetlanie biologicznie nieaktywnego izomeru *E* tych pochodnych azobenzenu prowadzi do powstawania izomeru *Z*, który wykazuje silne właściwości destabilizujące mikrotubule *in vitro*. W eksperymentach przeprowadzonych na hodowlach komórkowych linii raka szyjki macicy (HeLa) fotostatyny wykazywały nawet 250-krotnie wyższą cytotoksyczność po aktywacji światłem ultrafioletowym lub niebieskim ($\lambda=380-410$ nm) w porównaniu do warunków bez obecności światła. We wszystkich trzech grupach badawczych wykazano spadek cytotoksyczności fotostatyn w porównaniu do kombretastatyny A-4 oraz istotny wzrost aktywności cytotoksycznej po naświetleniu tych związków promieniowaniem

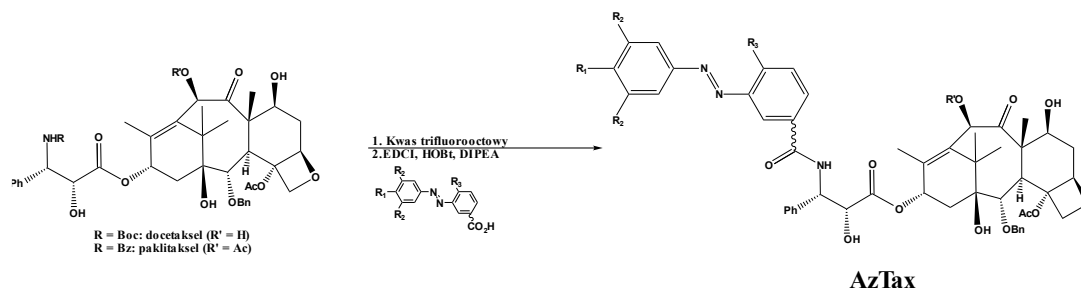
elektromagnetycznym z zakresu 370–410 nm. Poza wspomnianą wyżej linią komórkową HeLa, badacze zaobserwowali ten efekt stosując linie komórkowe komórek nabłonkowych gruczołakoraka piersi (MDA-MB-231) oraz linii komórkowej śródbłónka żyły pępowinowej człowieka (HUVECs) [68–70]. W badaniach polimeryzacji tubuliny z użyciem oczyszczonej tubuliny zaobserwowano, że po naświetleniu światłem związek **PTS-1** wykazywał silną inhibicję polimeryzacji tubuliny, podczas gdy nienaświetlony związek nie miał wpływu na cytoskielet mikrotubularny, podobnie jak obserwowano w kontroli negatywnej. Co więcej, przeprowadzono analizę cyklu komórkowego (komórki linii HeLa, MDA-MB-231 oraz HEK293T – ludzkie komórki embrionalne nerki) i dowiedziono, że fotostatyny indukują



Rysunek 8. Struktura kolchicyny i kombretastatyny A-4 oraz przełączników molekularnych opartych o strukturę kombretastatyny A-4.

zatrzymania cyklu komórkowego w fazie G₂/M i w konsekwencji śmierć komórki w sposób zależny od światła. W 2017 roku opublikowane zostało badanie opisujące syntezę związku **azo-CA4-OEt**, analogu kombretastatyny A-4 zawierającego strukturę azobenzeny, charakteryzującego się ulepszonymi właściwościami fotofarmakologicznymi w porównaniu do fotostatyny **PST-1**. W związku tym jedna z grup metoksyłowych została zastąpiona grupą etoksyłową (Rysunek 9) [71]. Częsteczka ta wykazywała ponad 500-krotnie większą aktywność cytotoksyczną wobec komórek linii HeLa oraz H157 (linia komórkowa ludzkiego płaskonabłonkowego raka płuc) w obecności światła ultrafioletowego ($\lambda=390\text{--}400\text{nm}$), niż w ciemności. Przeprowadzone badania dokowania molekularnego wykazały, że konformacja cząsteczek **PST-1** oraz **azo-CA4-OEt** podczas oddziaływania z tubuliną jest niemal identyczna oraz pokrywająca się ze strukturą kombretastatyny A-4 związanej z tubuliną uzyskanej za pomocą krystalografii rentgenowskiej. Na podstawie uzyskanych wyników stwierdzono, że zarówno cząsteczka **PST-1** oraz **azo-CA4-OEt** oddziałują z domeną kolchicynową w cząsteczce heterodimeru $\alpha\beta$ -tubuliny. W związku z tym fotostatyny stanowią cenne narzędzie w

badaniach nad biologią komórki oraz obiecującą klasę precyzyjnych środków terapeutycznych, których toksyczność można precyzyjnie kontrolować w czasie i przestrzeni za pomocą światła.



Rysunek 9. Schemat syntezy związków **AzTax**. (DIPEA - *N,N*-diizopropyletyloamina, EDCI – 1-etylo-3-(3-dimetyloaminopropyl)karbodiimid, HOBt – hydroksybenzotriazol)

Jak już wspomniano przełączniki molekularne stabilizujące mikrotubule, w przeciwieństwie do związków destabilizujących mikrotubule, aktywują polimeryzację tubuliny oraz zapobiegają demontażowi już powstałych mikrotubul. Wiodącym przykładem przełączników molekularnych opartych o strukturę azobenenu o właściwościach stabilizujących mikrotubule są pochodne paklitakselu oraz docetakselu, alkaloidów terpenowych z grupy taksanów stosowanych klinicznie jako leki przeciwnowotworowe. Zostały one po raz pierwszy otrzymane oraz opisane w badaniu z 2020 roku [72]. Otrzymane związki nazwano „azotaksanami” **AzTax**. Ich synteza została przeprowadzona dwu etapowo. W pierwszym etapie usunięto grupę *tert*-butoksykarbonylową z handlowo dostępnego docetakselu w reakcji z kwasem trifluorooctowym. Następnie przeprowadzono reakcję sprzęgania otrzymanej w pierwszym etapie aminy z pochodnymi kwasu *orto*-, *para*- oraz *meta*-(fenyloazo)benzoesowego (Rysunek 9). Wykazano, że położenie grupy azowej w pozycji *meta*-względem grupy amidowej powodowało najmniejszy spadek aktywności cytotoksycznej oraz prowadziło do uzyskania największej różnicy w cytotoksyczności pomiędzy izomerami *E* oraz *Z*. Właściwości fotochemiczne były zbliżone w obrębie danego typu wprowadzonych podstawników. Związki niepodstawione żadnymi podstawnikami wykazywały 3-krotny większy procent izomeru *Z* w stanie fotostacjonarnym dla długości fali $\lambda=375\text{nm}$ (~80% izomeru *Z*) niż w stanie fotostacjonarnym dla długości fali $\lambda=410\text{nm}$ (~26% izomeru *Z*) oraz posiadały znacząco wolniejszy czas relaksacji termicznej, niż wynosi skala czasowa eksperymentów biologicznych (czas półtrwania ok. 50 dni). Związki podstawione grupami metoksyłowymi wykazywały 9-krotny większy procent izomeru *Z* w stanie fotostacjonarnym

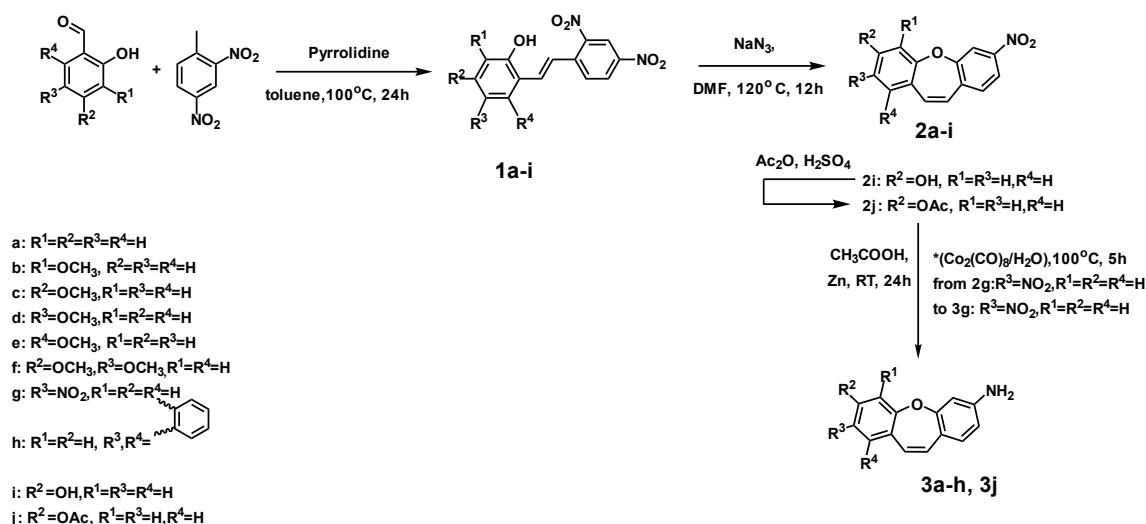
dla długości fali $\lambda=375\text{nm}$ (~96% izomeru *Z*) niż w stanie fotostacjonarnym dla długości fali $\lambda=535\text{nm}$ (~11% izomeru *Z*) oraz również posiadały znacząco wolniejszy czas relaksacji termicznej, niż wynosi skala czasowa eksperymentów biologicznych (czas półtrwania ok. 24 godziny). Natomiast okres półtrwania pochodnych podstawionych grupą dimetyloaminową w pozycji *para*- był zbyt krótki, aby go dokładnie wyznaczyć. Testy żywotności komórek przeprowadzone na linii komórkowej HeLa pozwoliły zidentyfikować **AzTax3MP** jako związek wiodący, o profilu bioaktywności porównywalnym z docetakselem, lecz o mniejszej sile działania. Działa on jako związek stabilizujący mikrotubule, a jego aktywność wzrasta po napromieniowaniu światłem, co potwierdzono w testach polimeryzacji tubuliny *in vitro* oraz w obrazowaniu immunofluorescencyjnym. W badaniu cytometrii przepływowej wykazano dwukrotny wzrost liczby komórek w fazie G2/M po ekspozycji **AzTax3MP** na światło w stosunku do kontroli. Dodatkowo wykazano, że **AzTax3MP** może być stosowany do odwracalnej fotomodulacji sieci mikrotubul oraz funkcji związanych z mikrotubulami w komórkach nowotworowych (HeLa) i neuronach (pierwotne neurony hipokampalne szczura) z wysoką rozdzielczością czasową oraz precyzją na poziomie pojedynczych komórek [72].

AUTOREFERAT

1. Systematyczne badania nad oceną aktywności przeciwnowotworowej wybranych pochodnych stilbenu oraz dibenzo[*b,f*]oksepin [P3]

1.1. Synteza wybranych pochodnych stilbenu oraz dibenzo[*b,f*]oksepin

Po przeprowadzeniu krytycznej analizy literaturowej dotyczącej związków oddziaływujących z tubuliną doszedłem do wniosku, że pochodne oraz izostery stilbenu są istotną klasą związków bioaktywnych o potencjalnych właściwościach przeciwnowotworowych. Dlatego też, podczas przeprowadzonych prac badawczych syntezowano 28 pochodnych stilbenu oraz dibenzo[*b,f*]oksepin realizując trzy etapy syntetyczne. Zrealizowana ścieżka syntezy przedstawiona została na Rysunku 10. W pierwszym etapie przeprowadzona została reakcja kondensacji pomiędzy 2,4-dinitrotoluenem a odpowiednią pochodną 2-hydroksybenzaldehydu w obecności pirolidyny jako katalizatora reakcji. Wydzielono 8 pochodnych **1a-h** za pomocą krystalizacji z dodatkiem węgla aktywnego. Jako rozpuszczalnika w procesie krystalizacji użyto metanol lub etanol. Pochodnej **1h** nie wydzielono, a surowa mieszanina poreakcyjna została użyta w następnej reakcji. W kolejnym etapie syntetycznym była reakcja cyklizacji otrzymanych pochodnych 2,4-dinitrostilbenu **1a-i** do pochodnych dibenzo[*b,f*]oksepin w obecności azydru sodu. W tym etapie wydzielono 9 pochodnych nitrodibenzo[*b,f*]oksepin **2a-i** stosując metodę chromatografii kolumnowej. Pochodną dibenzo[*b,f*]oksepiny zawierającą podstawnik hydroksylowy **2i** poddano reakcji acetylowania wobec bezwodnika kwasu octowego oraz katalitycznej ilości kwasu siarkowego (VI). Otrzymaną pochodną **2j** oczyszczono za pomocą chromatografii kolumnowej. W ostatnim etapie syntetycznym nitrodibenzo[*b,f*]oksepiny **2a-f**, **2h**, **2i** poddane zostały reakcji redukcji grupy nitrowej do grupy aminowej z zastosowaniem aktywowanego pyłu cynkowego w kwasie octowym. Pochodna dibenzo[*b,f*]oksepiny zawierająca dwie grupy nitrowe **2g** została selektywnie zredukowana do pochodnej **3g** z wykorzystaniem oktakarbonylku dikobaltu w wodzie jako rozpuszczalniku. W celu określenia struktury produktów reakcji pochodnych stilbenów **1a-i** oraz dibenzo[*b,f*]oksepin **2a-j**, **3a-h**, **3j** wykonano widma ^1H i ^{13}C NMR wszystkich produktów w roztworach deuterowanych rozpuszczalników. Wartości stałych sprzężenia $^3J_{\text{H-H}}$ dla protonów olefinowych w związkach **1a-i** wynosiły w przybliżeniu 16 Hz co odpowiada konfiguracji *E* badanych izomerów. Podczas gdy, dla związków **2a-j**, **3a-h**, **3j** wartości odpowiednich stałych sprzężenia $^3J_{\text{H-H}}$ wynosiły około 11 Hz co potwierdza konfigurację *Z* otrzymanych izomerów.

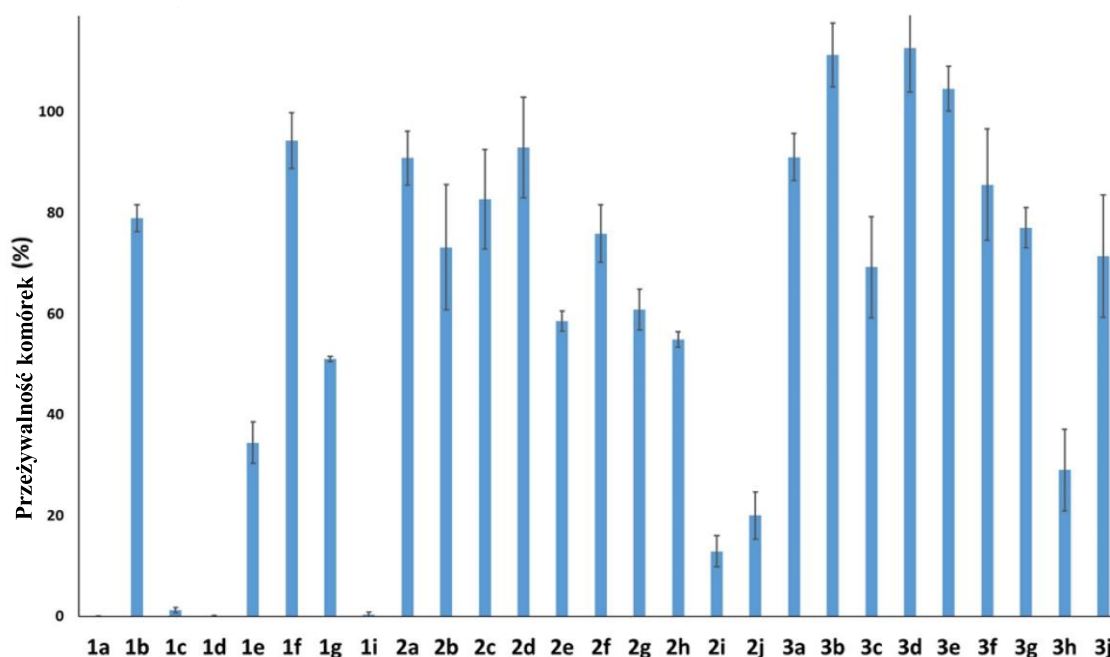


Rysunek 10. Schemat przedstawiający przeprowadzoną ścieżkę syntetyczną celem otrzymania pochodnych stilbenów oraz dibenzo[*b,f*]oksepin.

1.2. Badania aktywności cytotoksycznej otrzymanych pochodnych stilbenu oraz dibenzo[*b,f*]oksepin [P3]

W celu zweryfikowania czy syntezowane pochodne stilbenu oraz dibenzo[*b,f*]oksepin wykazują aktywność cytotoksyczną *in vitro* wyznaczono przeżywalność/żywołność komórek określoną jako procent ilości żywych komórek w badanej próbce do ilości żywych komórek w próbce kontrolnej. Badania przeprowadzono z użyciem linii komórek ludzkiego gruczolakoraka jelita grubego – HTC116. Do określenia poziomu przeżywalności komórek zastosowano test kolorymetryczny MTT. Oparty jest on na enzymatycznej redukcji rozpuszczalnego w wodzie bromku 3-(4,5-dimetylotiazol-2-yl)-2,5-difenylo-tetrazolowego (MTT) o barwie żółtej do nierozpuszczalnego w wodzie formazanu o ciemnoniebieskiej barwie, która zachodzi jedynie wewnątrz komórek aktywnych metabolicznie [73]. Na Rysunku 11. przedstawiono przeżywalność komórek linii HTC116 po 48 godzinach inkubacji w pożywce zawierającej 100 μ M roztwór badanych pochodnych stilbenu oraz dibenzo[*b,f*]oksepin **1a–g**, **1i**, **2a–2j**, **3a–3h**, **3j**. Spośród testowanych związków, cztery pochodne stilbenu (**1a**, **1c**, **1d**, **1i**) oraz trzy pochodne dibenzo[*b,f*]oksepin (**2i**, **2j**, **3h**) wykazały wysoką aktywność cytotoksyczną (żywołność komórek < 30%), dlatego związki te zostały wybrane do kolejnej części badania, w której wyznaczono wartość parametru IC_{50} (ang. *half maximal inhibitory concentration*) – określającego stężenie potrzebne do zahamowania *in vitro* danego procesu biologicznego (przeżywalności komórek) o 50%. W badaniach użyto dwóch linii komórek nowotworowych (ludzkiego gruczolakoraka jelita grubego – HCT116 oraz ludzkiego gruczolakoraka piersi – MCF-7) oraz dwóch linii komórek nienowotworowych (ludzkie

komórki embrionalne nerki – HEK293 oraz ludzkie fibroblasty skórne – HDF-A). Na tej podstawie wyliczony został indeks selektywności SI (ang. *selectivity index*) definiowany jako stosunek wartości IC_{50} linii nienowotworowej do wartości IC_{50} linii nowotworowej. Wartość indeksu selektywności powyżej 1 oznacza wyższą cytotoksyczność badanego związku wobec komórek nowotworowych niż cytotoksyczność wobec komórek normalnych. Otrzymane wyniki zebrano w Tabeli 2.



Rysunek 11. Wpływ związków **1a–g**, **1i**, **2a–2j**, **3a–3h**, **3j** na przeżywalność komórek linii HTC116 na podstawie testu MTT. (Roztwór badanej substancji w pożywce 100 μ M, czas inkubacji 48 godzin.)

Badane związki hamowały wzrost komórek w sposób zależny od dawki i najczęściej silniejszy efekt był obserwowany dla komórek linii HTC166 niż dla komórek linii MCF-7 oraz dla komórek linii HEK293 niż dla komórek linii HDF-A.

Tabela 2. Obliczone wartości parametru IC₅₀ oraz indeksu selektywności dla związków **1a**, **1c**, **1d**, **1i**, **2i**, **2j**, **3h**.

Związek	HEK293	HDF-A	HCT116	SI ₁	SI ₂	MCF-7	SI ₃	SI ₄
	IC ₅₀ (μM)					IC ₅₀ (μM)		
1a	18 ± 1	38 ± 1	23 ± 1	0.8	0.8	36 ± 2	0.5	1.1
1c	34 ± 4	40 ± 4	38 ± 5	0.9	1.1	43 ± 2	0.7	1.0
1d	33 ± 2	40 ± 2	18 ± 3	1.8	2.2	47 ± 1	0.7	0.9
1i	54 ± 2	41 ± 2	52 ± 2	1.0	0.8	69 ± 3	0.8	0.6
2i	88 ± 5	93 ± 7	55 ± 4	1.6	1.7	67 ± 6	1.2	1.4
2j	85 ± 4	ND	53 ± 3	1.6	Nd	36 ± 3	2.4	ND
3h	48 ± 3	127*	66 ± 2	0.7	1.9	ND	ND	ND

SI₁= IC₅₀(HEK293)/ IC₅₀(HCT116); SI₂= IC₅₀(HDF-A)/ IC₅₀(HCT116); SI₃=

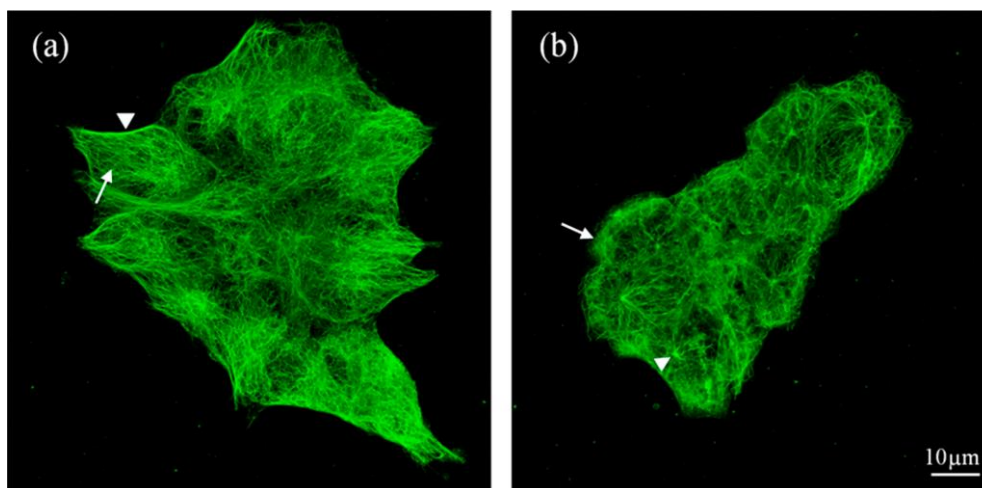
IC₅₀(HEK293)/ IC₅₀(MCF-7); SI₄= IC₅₀(HDF-A)/ IC₅₀(MCF-7); *-przewidziana wartość

IC₅₀ (powyżej maksymalnego testowanego stężenia – 100μM); ND – nie obliczono

1.3. Wpływ wybranej pochodnej stilbenu na strukturę mikrotubul w komórkach nowotworowych ludzkiego gruczolaka rakowego jelita grubego.

Wartość IC₅₀ stilbenu **1d** uzyskana dla komórek linii HTC116 (18 μM) była najniższą uzyskaną wartością spośród wszystkich badanych związków, podczas gdy indeks selektywności (SI) należał do najwyższych w porównaniu z obydwoma typami komórek kontrolnych. Dlatego też postanowiono zwizualizować cytoszkielet mikrotubularny za pomocą metody immunofluorescencji. Na podstawie analiz immunobarwienia α-tubuliny wykazano, że komórki kontrolne zawierały dobrze rozwinięty cytoszkielet mikrotubularny z gęstą siecią mikrotubul ułożonych wzdłuż długiej osi oraz na brzegach komórek (odpowiednio strzałka i grot strzałki na Rysunku 12a). Natomiast komórki HTC116 inkubowane z 60 μM roztworem związku **1d** były zaokrąglone, a gęstość sieci mikrotubul zmniejszyła się (Rysunek 12b). Co więcej w wielu komórkach obserwowano amorficzny materiał, którym być może była zdepolimeryzowana tubulina (strzałka na Rysunku 12b). W przeciwieństwie do komórek kontrolnych, w przypadku komórek inkubowanych z 60 μM roztworem związku **1d** zaobserwowano wyraźne punkty organizacji mikrotubul (przypuszczalnie centrosomy) z kilkoma prawdopodobnie najbardziej stabilnymi mikrotubulami (grot strzałki na Rysunku 12b).

Powyższe obserwacje potwierdzają, że związek **1d** wpływa na cytoszkielet mikrotubularny, co z kolei może prowadzić do śmierci komórek.

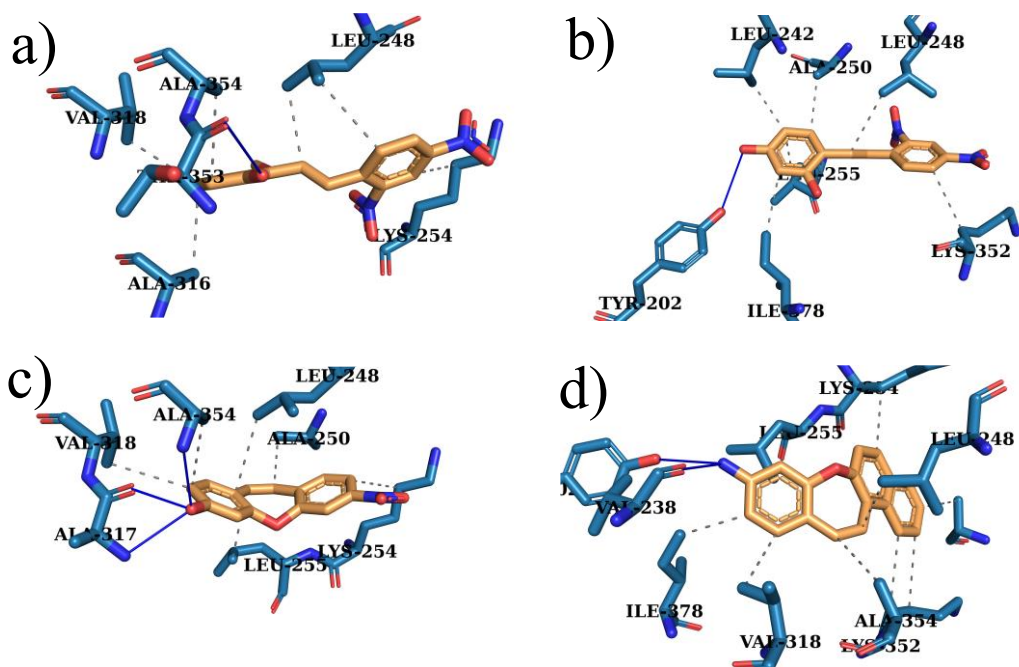


Rysunek 12. Sieć mikrotubularna w komórkach linii HTC116. a) Próba kontrolna – strzałka: mikrotubule rozmieszczone wzdłuż długiej osi komórki; grot: mikrotubule układające się na granicy komórki. b) Komórki inkubowane z związkiem **1d** – strzałka: amorficzna struktura mikrotubul; grot: centrum organizacji mikrotubul z powstałymi mikrotubulami.

1.4. Analiza obliczeniowa i dokowanie molekularne

Połączenie metod eksperymentalnych i komputerowych odgrywa kluczową rolę w odkrywaniu i ulepszaniu nowych, skutecznych związków przeciwnowotworowych [74]. Dokowanie molekularne jest to metoda komputerowa polegająca na badaniu, jak konformacja ligandu dopasowuje się do miejsca aktywnego w białku lub makrocząsteczce, aż do osiągnięcia minimalnej energii wiązania. Co istotne, technika ta umożliwia obliczanie energii wiązania, uwzględniając procesy odpowiedzialne za rozpoznanie molekularne [75]. Na podstawie dostępnej literatury oraz otrzymanych wyników dotyczących cytotoksyczności, spodziewano się, że wybrane pochodne stilbenu oraz dibenzo[*b,f*]oksepiny mogą być inhibitorami polimeryzacji tubuliny, wiążącymi się w miejscu wiązania kolchicyny. Dlatego też, interakcje pomiędzy najbardziej aktywnymi związkami **1a**, **1c**, **1d**, **1i**, **2i**, **2j**, **3h** a heterodimerem $\alpha\beta$ -tubuliny (kod PDB: 1SA0) analizowano za pomocą modelowania molekularnego. Strukturę związków optymalizowano metodą teorii funkcjonału gęstości (DFT, ang. density functional theory, (funkcjonał B3LYP w bazie 6-311++g (2d,p), z zastosowaniem metody PCM – the polarizable continuum model – w celu modelowania efektów solwatacji) przy użyciu programu Gaussian 03W [76]. Dokowanie molekularne przeprowadzono z wykorzystaniem

oprogramowania AutoDock Vina [77]. Protokół dokowania został zweryfikowany przez zadokowanie natywnego liganda – DAMA-kolchicyny, która została wyekstrahowana z struktury krystalicznej zdeponowanej w bazie danych PDB. Zastosowany algorytm poprawnie odtworzył sposób wiązania natywnego liganda. W krystalicznej strukturze pierścień tropolonowy kolchicyny jest stabilizowany przez kontakty van der Waalsa z Val181, Ser178 i Val315. Grupa karbonylowa jest stabilizowana przez wiązanie wodorowe z Val181. Natomiast pierścień z zawierający trzy podstawniki metoksyłowe jest stabilizowany oddziaływaniami hydrofobowymi z Lys352, Asn350, Leu378, Ala316, Leu255, Lys254, Ala250 oraz Leu242. Grupa metoksy w pozycji 3 uczestniczy w wiązaniu wodorowym z grupą cysteinową Cys241. Obliczone najniższe wartości energii wiązania badanych związków **1a**, **1c**, **1d**, **1i**, **2i**, **2j**, **3h** wahały się od -7,7 do -9,3 kcal/mol, w porównaniu z -8,9 kcal/mol dla ponownie zadokowanej DAMA-kolchicyny. Analiza modelu wiązania najbardziej aktywnych związków do tubuliny pozwoliła na przewidzenie kilku interakcji między badanymi związkami, a resztami aminokwasowymi w miejscu wiązania kolchicyny. Związki **1a**, **1c**, **1d**, **1i**, **2i**, **2j** i **3h** były stabilizowane przez interakcje hydrofobowe z resztami β -tubuliny (Leu242, Leu248, Ala250, Lys254, Leu255, Lys352, Ile378, Val318). Cztery z tych aktywnych związków (**1d**, **1i**, **2i**, **3h**) były dodatkowo stabilizowane przez wiązania wodorowe z heterodimerem tubuliny (Rysunek 13). Jednak nie przewidziano żadnych wiązań wodorowych z Cys241, które są uważane za kluczowe dla wysokiej aktywności biologicznej, co może częściowo tłumaczyć wysoką mikromolarną cytotoksyczność testowanych związków [78,79].



Rysunek 13. Wizualizacja wyników dokowania molekularnego oraz przewidziane interakcje związków a) **1d**, b) **1i**, c) **2i**, d) **3h** z heterodimerem $\alpha\beta$ -tubuliny (PDB: 1SA0). Dla przejrzystości pokazano tylko aminokwasy wchodzące w interakcje z ligandami. Niebieskie linie oznaczają interakcje wiązań wodorowych; przerywane szare linie oznaczają interakcje hydrofobowe.

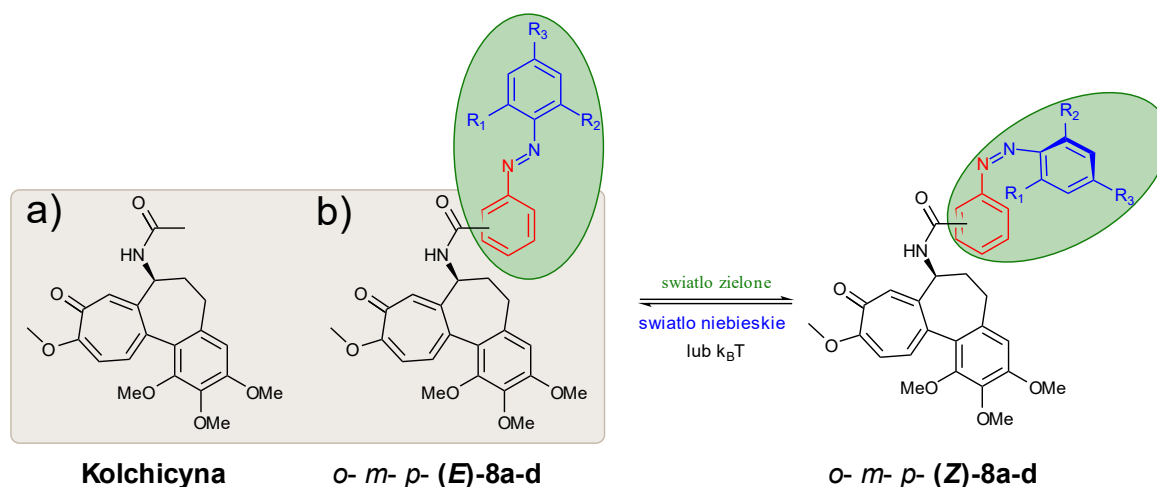
2. Synteza i badanie właściwości spektroskopowych i biologicznych fotochromowych pochodnych kolchicyny [P4]

2.1. Projektowanie i synteza fotochromowych pochodnych kolchicyny

Kontynuując badania w poszukiwaniu innowacyjnych rozwiązań terapeutycznych postanowiłem rozszerzyć tematykę zaprezentowaną w poprzednim rozdziale, w którym omówiłem wyniki dotyczące przeciwnowotworowego potencjału analogów stilbenów i dibenzo[*b,f*]oksepin o nowatorskie podejście wykorzystujące fotoprzełączalne związki oparte o strukturę kolchicyny. W prezentowanym badaniu moim celem było zaprojektowanie oraz synteza fotochromowych przełączników molekularnych zawierających układ kolchicyny w swojej strukturze. Drugim celem prezentowanych badań była ocena aktywności antyproliferacyjnej wobec wybranych nowotworowych linii komórkowych.

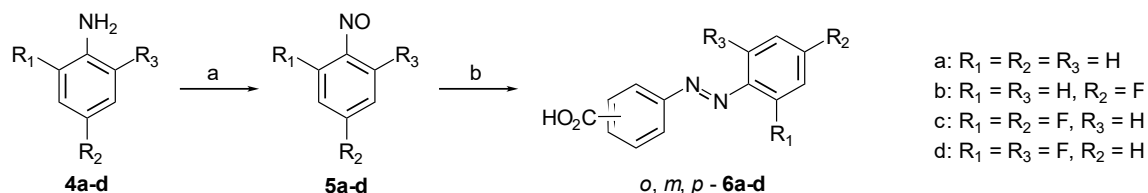
Zastosowana strategia syntetyczna opierała się na zastąpieniu grupy acetamidowej kolchicyny ugrupowaniem azobenzenowym, które będzie pełniło rolę przełącznika molekularnego (Rysunek 14). Na podstawie wcześniejszych danych literaturowych można przypuszczać, że podstawienia w tej pozycji są dobrze tolerowane i nie prowadzą do utraty bioaktywności

[60,61]. Dlatego spodziewałem się, że takie podejście pozwoli na utrzymanie działania antyproliferacyjnego i jednocześnie umożliwi precyzyjną czasoprzestrzenną kontrolę nad otrzymanymi związkami za pomocą napromieniania światłem o określonej długości fali.



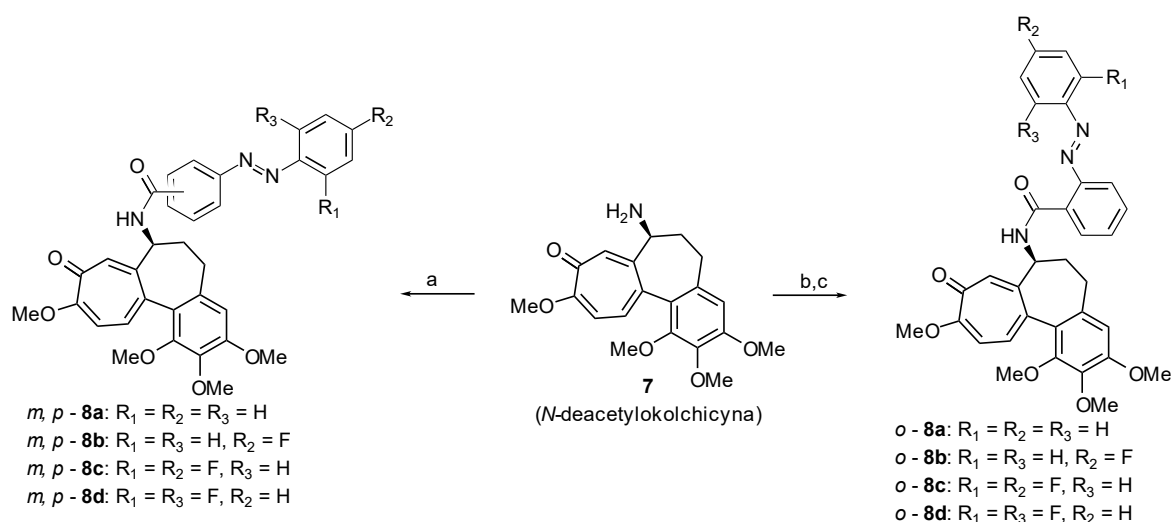
Rysunek 14. a) Struktura kolchicyny oraz b) fotochromowych przełączników molekularnych opartych na jej strukturze.

W pierwszym etapie syntetycznym otrzymano wybrane pochodne kwasu (fenyloazo)benzoesowego *o*-, *m*-, *p*-**6a-d** w tzw. reakcji Baeyer–Mills’a opisaney po raz pierwszy w 1874 roku. Jest to reakcja kondensacji amin aromatycznych oraz pochodnych *N*-nitrozobenzenu w środowisku słabo kwasowym, w której powstają odpowiednie pochodne azobenzenu zwykle z dużymi wydajnościami [80,81]. Wybrane pochodne aniliny **4a-d** utleniono do odpowiednich *N*-nitrozo pochodnych **5a-d** za pomocą łagodnego, nietoksycznego utleniacza jakim jest Oxone (sól potrójna - $2\text{KHSO}_5 \cdot \text{KHSO}_4 \cdot \text{K}_2\text{SO}_4$) w dwufazowym roztworze równych objętości wody oraz dichlorometanu. A następnie bez wyodrębniania z surowej mieszaniny reakcyjnej przeprowadzono reakcję kondensacji z odpowiednią pochodną kwasu *orto*-, *meta*- lub *para*-aminobenzoesowego (Rysunek 15).



Rysunek 15. Schemat ścieżki syntetycznej przeprowadzonej w celu otrzymania pochodnych *o*-, *m*-, *p*-**6a-d**. (a) Oxone, dichlorometan/woda (1:1 v:v), (b) kwas *orto*-, *meta*- lub *para*-aminobenzoesowy, AcOH, 24h.

Produkt reakcji – azobenzeny *o*-, *m*-, *p*-**6a-d** wydzielono z zastosowaniem chromatografii kolumnowej. Drugi etap syntetyczny zakładał kondensację *N*-deacetylokolchicyny **7** z otrzymanymi uprzednio azobenzenami *o*-, *m*-, *p*-**6a-d**. Reakcja ta podczas użycia izomerów *meta*- oraz *para*- azobenzenów (*m*-, *p*-**6a-d**) przebiegła z zadowalającymi wydajnościami produktów (*m*-, *p*-**8a-d**). W reakcji z użyciem izomerów *o*-**6a-d** uzyskano wiele produktów ubocznych trudnych do oddzielenia od spodziewanego produktu reakcji. W związku z tym zdecydowałem się poddać reakcji *N*-deacetylokolchicynę **7** z chlorkiem kwasowym kwasu 2-aminobenzoowego w obecności trietyloaminy, a następnie w kolejnym etapie przeprowadzić reakcję Baeyer–Mills’a z nitrozobenzenami **5a-d**. Ta ścieżka syntetyczna pozwoliła uzyskać zakładane związki docelowe *o*-**8a-d** (Rysunek 16).

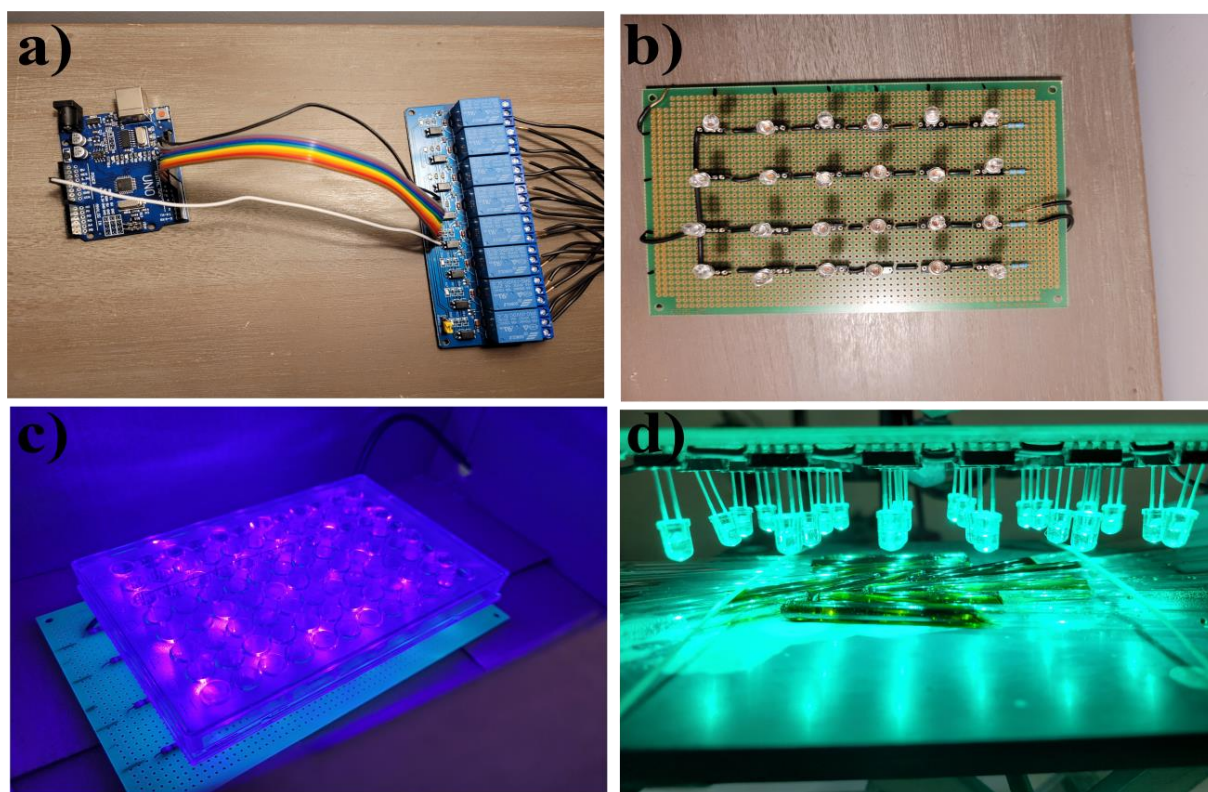


Rysunek 16. Schemat ścieżki syntetycznej przeprowadzonej w celu otrzymania przełączników molekularnych *o*-, *m*-, *p*-**8a-d**; (a) *m*-, *p*-**6a-d**, HATU, DIPEA, DMF, RT, 24h; (b) chlorek kwasowy kwasu 2-aminobenzoowego, NEt_3 , DCM, $0^\circ C \rightarrow RT$, 12h; (c) **5a-d**, AcOH/DCM (1:1 v:v), 24h.

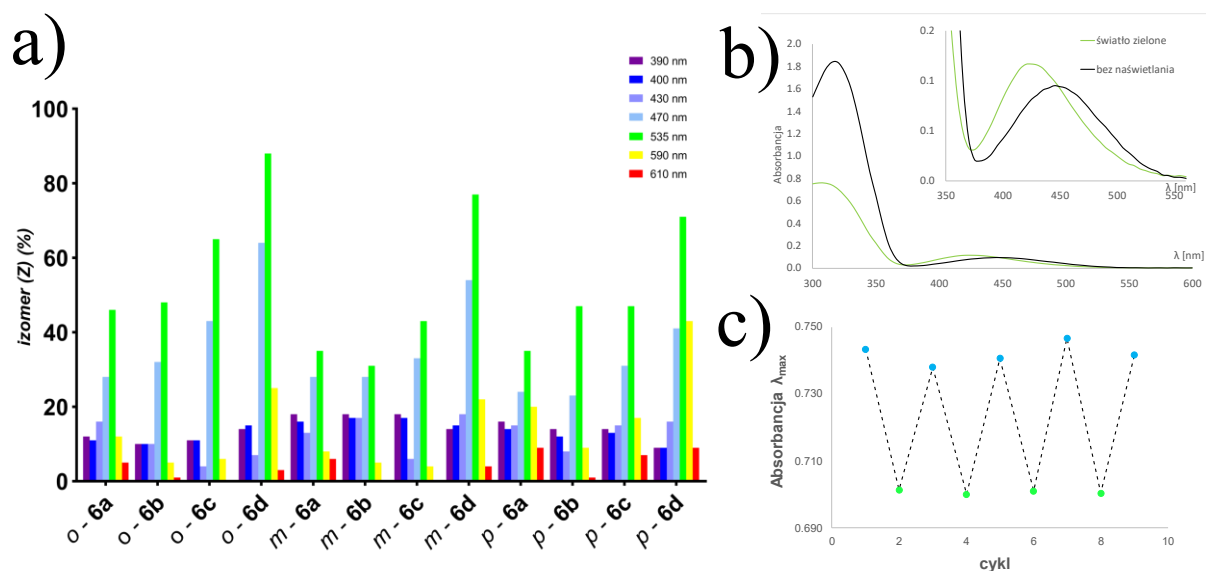
2.2. Charakterystyka spektroskopowa otrzymanych pochodnych

Właściwości spektroskopowe przełączników *o*-, *m*-, *p*-**8a-d** są zależne od typu azobenzenu (*o*-, *m*-, *p*-**6a-d**) wbudowanego w strukturę macierzystego farmakoforu – kolchicyny. Dlatego też dokonano oceny właściwości spektroskopowych uzyskanych azobenzenów przy użyciu spektroskopii magnetycznego rezonansu jądrowego (NMR) oraz spektroskopii UV-Vis. Do naświetlania próbek podczas eksperymentów użyto samodzielnie wykonanego zestawu diod LED (ang. *light-emitting diode*). Zestaw ten składał się z płytek, w które wlutowano cztery rzędy po 6 diod LED. Sterowania pracą odpowiednich płytek

umożliwiała platforma rozwojowa Arduino Uno (zawierająca mikrokontroler) połączona z modulem 8 przekaźników (Rysunek 17). Proces fotoizomeryzacji azobenzenów *o*-, *m*-, *p*-**6a-d** nie ulega istotnym zmianom po kondensacji z cząsteczką kolchicyny. Założono, że promieniowanie ultrafioletowe spowoduje fotokatalizowaną degradację pochodnych kolchicyny *o*-, *m*-, *p*-**8a-d**. Rzeczywiście naświetlenie pochodnej *p*-**8b** promieniowaniem ultrafioletowym o długości fali $\lambda=365\text{nm}$ prowadziło do powstania złożonej mieszaniny produktów. Na podstawie uzyskanego wyniku potwierdzono, że światło ultrafioletowe jest niekompatybilne z przełącznikami molekularnym opartymi o strukturę kolchicyny. Wobec tego określono rozkład procentowy izomerów *E* oraz *Z* azobenzenów *o*-, *m*-, *p*-**6a-d** dla stanu fotostacjonarnego podczas ciągłego naświetlania światłem z zakresu światła widzialnego (390-610nm) stosując analizę widm ^1H oraz ^{19}F NMR



Rysunek 17. (a) Arduino Uno połączone z modulem 8 przekaźników. (b) płytki z wlutowanymi diodami LED. (c) zestaw podczas naświetlania płytki do badań biologicznych. (d) zestaw podczas naświetlania probówek do spektroskopii NMR.



Rysunek 18. (a) Rozkład procentowy izomerów *E* / *Z* azobenzenów *o*-, *m*-, *p*-**6a-d** dla stanu fotostacjonarnego z zakresu światła widzialnego ($\lambda=390\text{--}610\text{nm}$). (b) Widmo UV-VIS 500 μM roztworu związku *o*-**6d** w DMSO po naświetleniu próbki światłem zielonym ($\lambda=535\text{nm}$) oraz bez naświetlania. (c) Proces fotoizomeryzacji obserwowany jako zmiana absorbancji 50 μM roztworu związku *o*-**6d** w DMSO podczas naprzemiennego naświetlania światłem zielonym ($\lambda=535\text{nm}$) i niebieskim ($\lambda=430\text{nm}$) przez 5 minut.

Uzyskane wyniki podsumowano na Rysunku 18. Gdy stosowano światło zielone ($\lambda=535\text{ nm}$) uzyskano fotokonwersję izomeru *E* do izomeru *Z* z największą wydajnością. Natomiast, gdy użyto światła niebieskiego ($\lambda=390\text{--}430\text{ nm}$) uzyskano odwrotną fotokonwersję – niewielki procent izomeru *Z*. Najniższą zawartość procentową izomeru *Z* otrzymano dla pochodnych niezawierających atomów fluoru w swojej strukturze tj. pochodnych *o*-, *m*-, *p*-**6a** (odpowiednio 46%, 35% oraz 35% dla $\lambda=535\text{nm}$). Pochodne *o*-, *m*-, *p*-**6b-c** zawierające w pozycji 4- oraz 2,4-atom fluoru wykazywały jedynie nieznacznie większy procent izomeru *Z* w stanie fotostacjonarnym w stosunku do pochodnych *o*-, *m*-, *p*-**6a**. Azobenzeny zawierające podstawnik fluorowy w pozycji 2,6- czyli *o*-, *m*-, *p*-**6d** odznaczały się największą zawartością izomeru *Z* dla stanu fotostacjonarnego (odpowiednio 88%, 77% oraz 71% dla $\lambda=535\text{nm}$). Po określeniu optymalnej długości fali (dla której stosunek izomerów *Z/E* jest największy) zarejestrowaliśmy widma absorpcyjne UV-VIS dla 500 μM roztworów azobenzenów *o*-, *m*-, *p*-**6a-d** w stanie podstawowym oraz w stanie fotostacjonarnym ($\lambda=535\text{nm}$) w celu wizualizacji oraz analizy pasma odpowiadającego przejściu $n \rightarrow \pi^*$ o niskiej intensywności. Jako rozpuszczalnik zastosowano dimetylosulfotlenek. Otrzymane dane spektroskopowe podsumowano w Tabeli 3.

Tabela 3. Dane spektroskopowe dla 500 μM roztworów związków *o*-, *m*-, *p*-**6a-d** w DMSO w temperaturze 25°C. Rozkład izomerów w stanie fotostacjonarnym został wyznaczony za pomocą spektroskopii ^{19}F lub ^1H NMR ($c \approx 10 \text{ mM}$ in DMSO- d_6). Czas półtrwania ($t_{1/2}$) został wyznaczony dla 100 μM roztworów związków *o*-, *m*-, *p*-**6a-d** w DMSO w 37°C.

Związek	izomer (<i>E</i>) ^a		izomer (<i>Z</i>) ^b		$t_{1/2}$ [h]	PPS(<i>E</i>) ^c (%)	PPS(<i>Z</i>) ^d (%)
	$\lambda_{\text{max}} (\pi-\pi^*)$ [nm]	$\lambda_{\text{max}} (n-\pi^*)$ [nm]	$\lambda_{\text{max}} (n-\pi^*)$ [nm]	$\Delta\lambda (n-\pi^*)$ [nm]			
<i>o</i> - 6a	324	448	440	8	6	95	46
<i>o</i> - 6b	326	445	437	8	5	99	48
<i>o</i> - 6c	330	444	433	11	23	96	66
<i>o</i> - 6d	315	450	420	30	>48	97	88
<i>m</i> - 6a	321	437	429	8	>48	94	35
<i>m</i> - 6b	322	431	423	8	>48	95	32
<i>m</i> - 6c	326	440	423	17	>48	96	46
<i>m</i> - 6d	315	448	417	31	>48	96	77
<i>p</i> - 6a	330	447	440	7	17	91	35
<i>p</i> - 6b	330	446	440	6	16	99	47
<i>p</i> - 6c	334	448	437	11	23	93	47
<i>p</i> - 6d	322	454	421	33	>48	91	71

a – przystosowany termicznie; b – PPS po naświetleniu światłem zielonym (535nm); c – procent izomeru (*E*) w stanie fotostacjonarnym dla $\lambda=430\text{nm}$; d – procent izomeru (*Z*) w stanie fotostacjonarnym dla $\lambda=535\text{nm}$.

Obserwowano intensywne pasmo odpowiadające przejściu $\pi \rightarrow \pi^*$ przy 315 – 334nm oraz pasmo o mniejszej intensywności odpowiadające przejściu $n \rightarrow \pi^*$ przy około 445 nm dla stanu podstawowego (izomer *E*). Po naświetlaniu próbki światłem zielonym ($\lambda=535\text{nm}$, powodującym fotoizomeryzację $E \rightarrow Z$) następuje spadek intensywności pasma $\pi \rightarrow \pi^*$ oraz wzrost intensywności pasma $n \rightarrow \pi^*$. Co jednak istotniejsze dla związków *o*-, *m*-, *p*-**6d** na skutek wprowadzenia podstawnika fluorowego w pozycji 2,6- powodującego stabilizację niewiążących par elektronowych wiązania azowego, zaobserwowano znaczną separację (o około 30nm) pasma odpowiadającemu przejściu $n \rightarrow \pi^*$ dla stanu podstawowego oraz stanu fotostacjonarnego ($\lambda=535\text{nm}$) dzięki czemu możliwa jest **selektywna fotoizomeryzacja obydwu izomerów z zastosowaniem światła widzialnego**. Zastosowanie naprzemiennego naświetlania światłem niebieskim ($\lambda=430\text{nm}$) oraz zielonym ($\lambda=535\text{nm}$) roztworów badanych związków pozwala na fotoodwracalną izomeryzację $E \leftrightarrow Z$ bez zauważalnej degradacji bądź dezaktywacji związków. Jako, że syntezowane pochodne kolchicyny miały zostać zastosowane w badaniach biologicznych sprawdzona została ich stabilność w warunkach zbliżonych do

warunków obecnych w hodowlach komórkowych (pożywka o wysokiej zawartości glukozy, 4mM stężenie L-glutaminy, 10% dodatek DMSO, naświetlanie badanego roztworu impulsami światła niebieskiego ($\lambda=430\text{nm}$) lub zielonego ($\lambda=535\text{nm}$) o czasie trwania 500 ms co 15 sekund przez 48 godzin). Nie zaobserwowano żadnych zmian w widmie UV-Vis wskazujących na degradację badanych związków. Obserwowano jedynie zmianę w widmie związaną z fotoizomeryzacją $E \leftrightarrow Z$. Wszystkie badane przełączniki molekularne wykazywały znacznie wolniejszą spontaniczną relaksację izomeru Z do izomeru E niż skala czasowa procesów biologicznych w żywych komórkowych (czas półtrwania w 37°C wynosił od 5 do ponad 48 godzin).

2.3. Ocena aktywności cytotoksycznej na wybranych liniach nowotworowych.

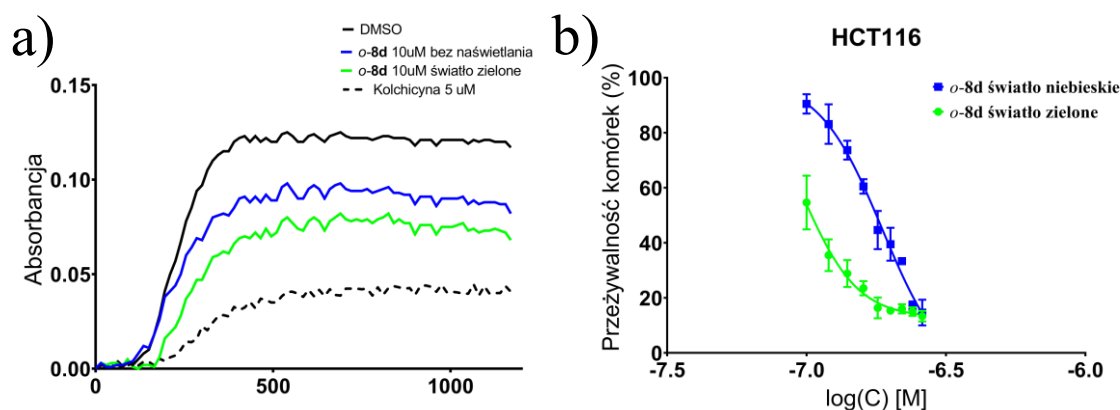
Kolchicina jest aktywnym inhibitorem polimeryzacji tubuliny, co prowadzi do zatrzymania cyklu komórkowego w fazie mitozy, a następnie śmierci komórki. Oczekiwano, że fotochromowe przełącznik molekularne zawierające w swojej strukturze fragment kolchicyny *o*-, *m*-, *p*-**8a-d** będą wykazywać podobną aktywność antyproliferacyjną. Dlatego postanowiono ocenić aktywność cytotoksyczną otrzymanych dwunastu pochodnych kolchicyny w warunkach naświetlania światłem niebieskim ($\lambda=430\text{nm}$ – większa zawartość izomeru E) oraz światłem zielonym ($\lambda=535\text{nm}$ – większa zawartość izomeru Z). Do naświetlania hodowli komórkowych wykorzystano samodzielnie zbudowany układ 24 diod elektroluminescencyjnych (LED) o niskiej mocy sterowanych za pomocą płytki drukowanej Arduino. Tak skonstruowany oraz zautomatyzowany system umożliwił precyzyjne, pulsacyjne oświetlanie płytek do hodowli komórkowych w długookresowych (>24 godzin) testach biologicznych. W pierwszym kroku przeprowadzono przesiewową ocenę aktywności biologicznej badanych związków na nowotworowej linii komórkowej MCF-7 (ludzki gruczolakorak piersi). Dla związków wykazujących zależną od światła cytotoksyczność rozszerzono badania o linię nowotworową HCT116 (ludzki gruczolakorak jelita grubego) oraz linię nienowotworową HEK293 (ludzkie komórki embrionalne nerki). Otrzymane wyniki zostały podsumowane w Tabeli 4.

Tabela 4. Stężenie hamujące żywotność komórek w 50% wyznaczone na podstawie testu MTT.

Związek	Linia komórkowa	IC ₅₀ (λ=430nm) (nM)	IC ₅₀ (λ=535nm) (nM)	stosunek IC ₅₀
<i>p</i> – 8a	MCF-7 (N=1)	48 ± 2	44 ± 2	1.1
<i>p</i> – 8b	MCF-7 (N=1)	44 ± 1	43 ± 1	1.0
<i>p</i> – 8c	MCF-7 (N=3)	50 ± 1	36 ± 1	1.4
<i>p</i> – 8d	MCF-7 (N=1)	55 ± 1	54 ± 1	1.0
<i>m</i> – 8a	MCF-7 (N=1)	31 ± 2	27 ± 1	1.1
<i>m</i> – 8b	MCF-7 (N=1)	42 ± 1	40 ± 1	1.1
<i>m</i> – 8c	MCF-7 (N=1)	49 ± 1	47 ± 1	1.0
<i>m</i> – 8d	MCF-7 (N=1)	45 ± 2	46 ± 1	1.0
<i>o</i> – 8a	MCF-7 (N=1)	nc	nc	nc
<i>o</i> – 8b	MCF-7 (N=1)	nc	nc	nc
<i>o</i> – 8c	MCF-7 (N=1)	183 ± 2	174 ± 1	1.1
<i>o</i> – 8d	MCF-7 (N=3)	184 ± 4	126 ± 2	1.5
<i>o</i> – 8d	HCT116 (N=2)	187 ± 9	97 ± 2	1.9
<i>o</i> – 8d	HEK293 (N = 3)	>250	>250	-
Kolchicyna	MCF-7	12 ^a	-	-
Kolchicyna	HCT116	11 ^b	-	-

a – bez naświetlania [82]; b – bez naświetlania [83]; nc – nie obliczono

Wszystkie badane związki wykazywały silną aktywność antyproliferacyjną (IC₅₀ w zakresie od 27 do 187 nM) co wskazuje, że wprowadzenie ugrupowania azobenzenowego do struktury kolchicyny nie wpływa w znacznym stopniu na wiązanie się z tubuliną ani na przenikanie przez błonę cytoplazmatyczną. Dla serii związków zawierających wiązanie azowe w pozycji *meta* **m-8a-d** zaobserwowano zbliżoną aktywność cytotoksyczną zarówno w warunkach naświetlania światłem zielonym jak i niebieskim. Dla związków **o-8a-b** otrzymano wyniki o dużym rozrzucie, które uniemożliwiały wyznaczenie wartości IC₅₀. Natomiast w przypadku izomerów *para*- oraz *orto*- zauważono dwa związki **p-8c** oraz **o-8d** różniące się w cytotoksyczności w zależności od warunków naświetlania. Podczas naświetlania światłem zielonym (λ=535nm – stan fotostacjonarny z przewagą izomeru *Z*) **p-8c** oraz **o-8d** wykazywały większą aktywność cytotoksyczną niż podczas naświetlania światłem niebieskim (λ=430nm – stan fotostacjonarny z przewagą izomeru *E*). Dla najbardziej obiecującego związku **o-8d** zauważono około dwukrotny wzrost aktywności antyproliferacyjnej wobec komórek HCT116 pod wpływem naświetlania światłem zielonym w porównaniu do naświetlania światłem niebieskim (Rysunek 19a).

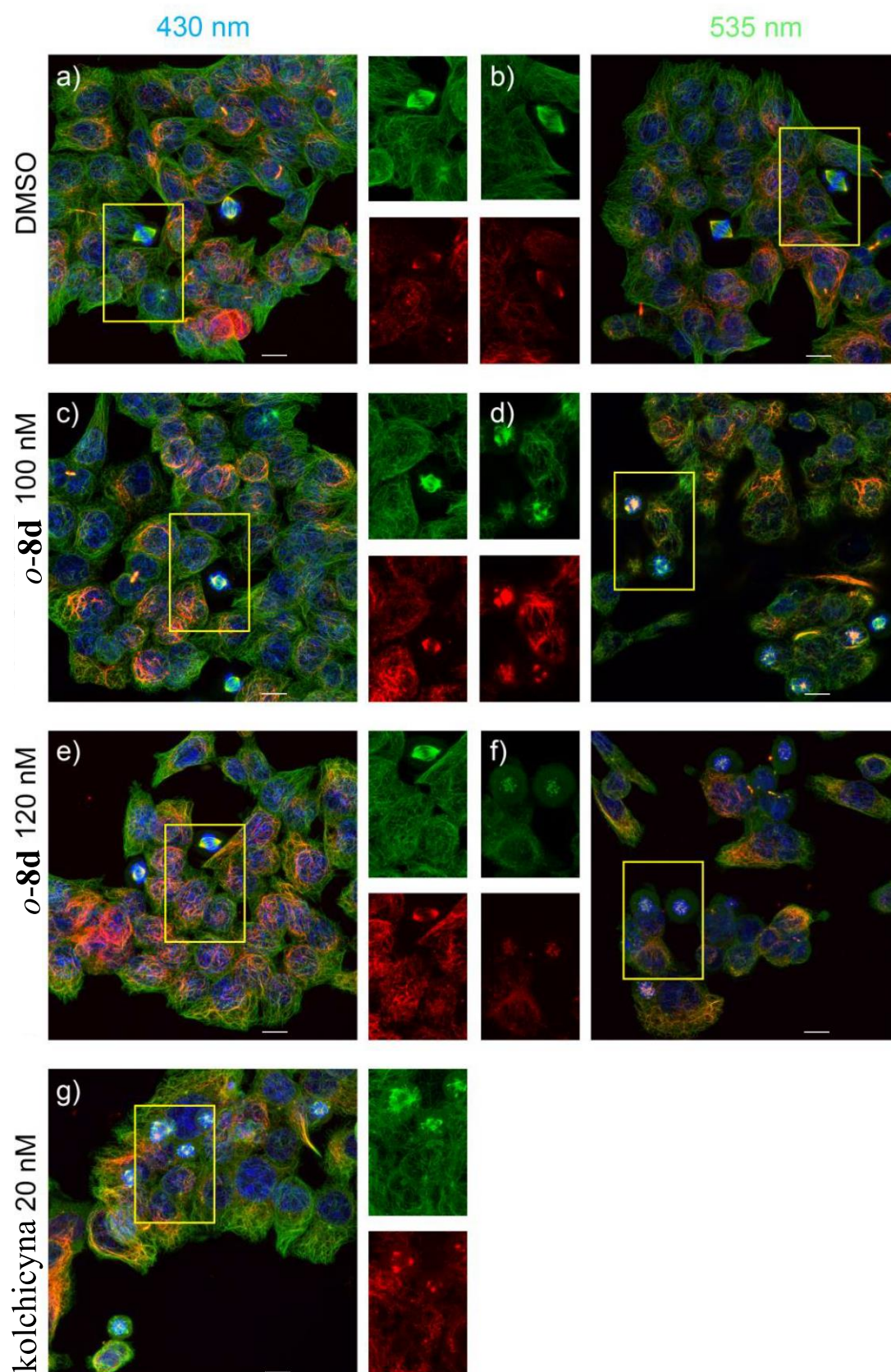


Rysunek 19. (a) Krzywe przeżywalności komórek linii HCT116 dla związku *o-8d* podczas naświetlania światłem zielonym ($\lambda=535\text{nm}$) lub niebieskim ($\lambda=430\text{nm}$). (b) Wpływ naświetlania światłem zielonym ($\lambda=535\text{nm}$) lub braku naświetlania na polimeryzację oczyszczonej tubuliny. Roztwór $5\text{ }\mu\text{M}$ kolchicyny oraz kosolwent (DMSO) zostały użyte jako odpowiednio pozytywna i negatywna kontrola.

2.4. Zaburzenie polimeryzacji tubuliny oraz wewnątrzkomórkowej organizacji mikrotubul w sposób zależny od światła

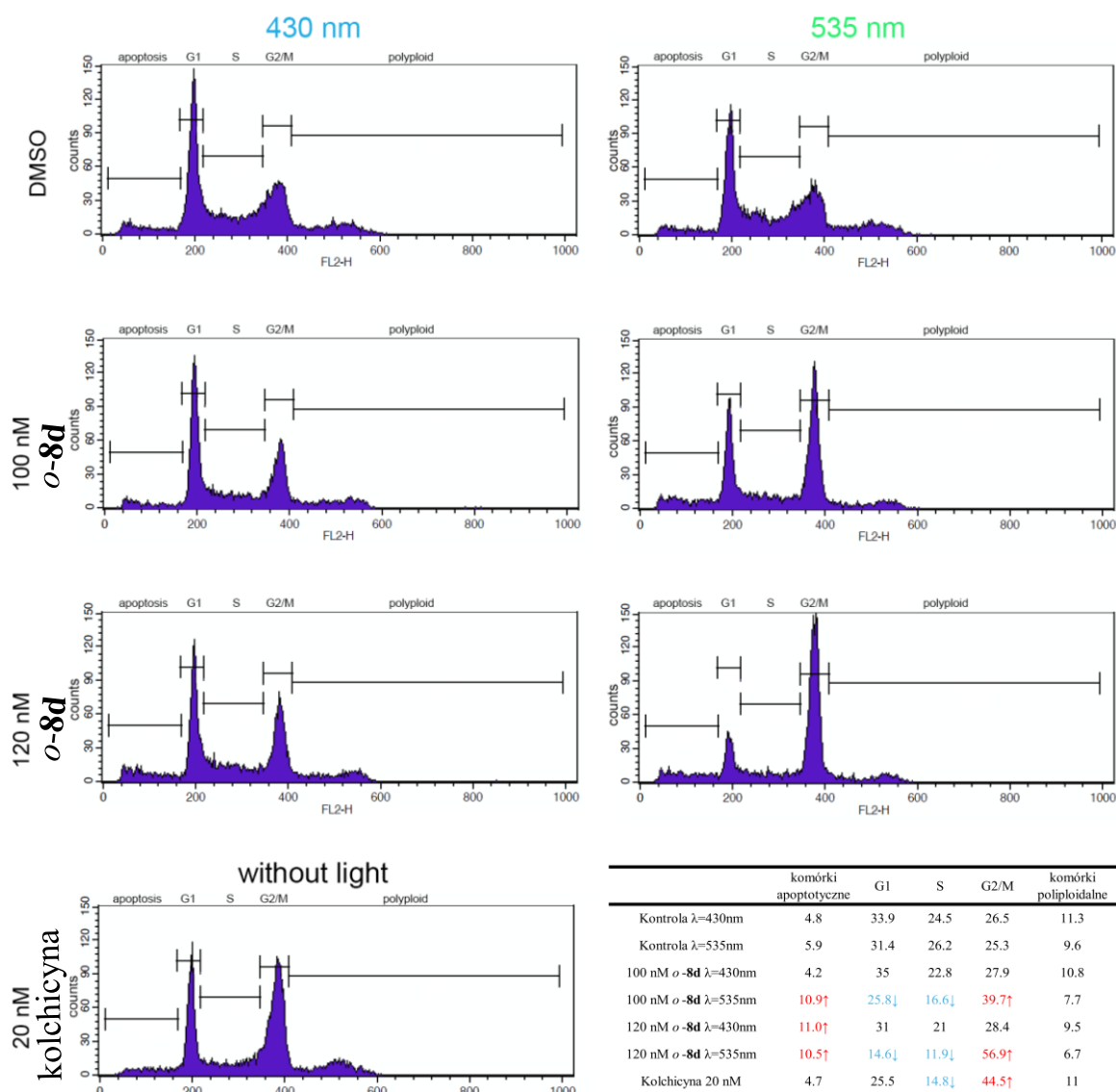
Aby dokładniej zbadać molekularny mechanizm zależnej od światła aktywności cytotoksycznej *o-8d*, przeanalizowano wpływ badanego związku na polimeryzację tubuliny w testach *in vitro* z użyciem oczyszczonej tubuliny. W stanie fotostacjonarnym z przewagą izomeru Z (po naświetleniu światłem zielonym) $10\text{ }\mu\text{M}$ roztwór *o-8d* spowodował około 40% inhibicję polimeryzacji tubuliny w porównaniu do kontroli (odnosząc się do DMSO jako 0%), podczas gdy $10\text{ }\mu\text{M}$ roztwór *o-8d* zawierający jedynie izomer E spowodował jedynie około 30% inhibicję polimeryzacji tubuliny. Dla porównania, $5\text{ }\mu\text{M}$ roztwór kolchicyny spowodował około 70% inhibicji polimeryzacji tubuliny (Rysunek 19b). Aby zweryfikować wpływ związku *o-8d* na cytoszkielet mikrotubularny *in vivo*, przeprowadzono 24-godzinną inkubację komórek HTC116 z dodatkiem DMSO (kontrola) lub *o-8d*, a następnie zwizualizowano go za pomocą immunofluorescencji oraz fluorescencyjnej mikroskopii konfokalnej. Wszystkie próbki komórek były naświetlane zielonym lub niebieskim światłem podczas inkubacji. Jako dodatkową pozytywną kontrolę zastosowano komórki traktowane 20 nM roztworem kolchicyny bez naświetlania. W obu próbach kontrolnych zaobserwowano, że komórki niedzielące się miały gęstą, dobrze rozwiniętą sieć mikrotubul interfazowych, natomiast komórki dzielące się tworzyły prawidłowe dwubiegunowe wrzeciono mitotyczne (Rysunek 20a,b). Podobny fenotyp komórek zaobserwowano dla komórek inkubowanych z 100 nM oraz

120 nM roztworem **o-8d**, które naświetlano niebieskim światłem (Rysunek 20c,e). Natomiast, w komórkach poddanych działaniu 100 nM roztworu **o-8d** i naświetlonych zielonym światłem, cytoszkielet mikrotubularny był tylko nieznacznie zaburzony w stosunku do próby kontrolnej. Jednak zaobserwowano więcej dzielących się komórek, u których struktura wrzeciona mitotycznego była często nieprawidłowa, z więcej niż dwoma biegunami lub nieprawidłowo rozmieszczonymi mikrotubulami. (Rysunek 20d). Fenotyp ten był jeszcze bardziej widoczny w komórkach inkubowanych z 120 nM roztworem **o-8d** i naświetlanych światłem zielonym (Rysunek 20f). Podobne zmiany zaobserwowano w komórkach inkubowanych z 20 nM roztworem kolchicyny (Rysunek 20g). W celu weryfikacji tych obserwacji przeprowadzono analizę odsetka faz cyklu komórkowego w komórkach linii HCT166 z użyciem cytometrii przepływowej FACS (ang. Fluorescence-Activated Cell Sorting; Rysunek 21). W próbach kontrolnych około 31-34% komórek było w fazie G1, 22-26% w fazie S, oraz 25-26% w fazie G2/M. Zaobserwowano również około 10-11% komórek poliploidalnych oraz 5-6% komórek będących w stadium apoptozy. Dla komórek inkubowanych z 100 nM roztworem **o-8d** oraz naświetlane światłem niebieskim zaobserwowano podobną dystrybucję faz cyklu komórkowego jak w próbach kontrolnych. Natomiast w hodowli komórek inkubowanych z 100 nM roztworem **o-8d** oraz naświetlanych światłem zielonym niemal dwukrotnie więcej komórek znajdowało się w stadium apoptozy (około 11%) lub w fazie G2/M ~40%. Podobny efekt zaobserwowano dla 20 nM roztworu kolchicyny (zwiększenie liczby komórek będących w fazie G2/M do około 45%; Rysunek 21).



Rysunek 20. Analiza obrazu cytoszkieletu komórek HTC116 po inkubacji z DMSO, *o*-8d lub kolchicyną. Komórki znakowano przeciwciałem monoklonalnym przeciwko

α -tubulinie (kolor zielony) oraz przeciwciałem przeciwko acetylowanej α -tubulinie (kolor czerwony). Jądra komórkowe wybarwiono DAPI (kolor niebieski). Wstawki pokazują powiększenie struktury wrzeciona mitotycznego. Powiększenia pokazują struktury wrzeciona mitotycznego. Skala na obrazach = 10 μ m. Komórki kontrolne naświetlano światłem (a) niebieskim lub (b) zielonym. Komórki wykazują gęsty mikrotubularny cytoszkielet i normalny kształt komórki. Widoczna jest gęsta sieć mikrotubul oraz normalny kształt wrzeciona mitotycznego. (c) Komórki inkubowane z 100nM roztworem związku **o-8d** naświetlane światłem niebieskim. Cytoszkielet mikrotubularny zbliżony do próby kontrolnej. (d) Komórki inkubowane z 100nM roztworem związku **o-8d** naświetlane światłem zielonym. Wrzeciona mitotyczne często wykazują aberrację, wielobiegunowość i nieprawidłowe ułożenie, a komórki są zaokrąglone. (e) Komórki inkubowane z 120nM roztworem związku **o-8d** naświetlane światłem niebieskim. (f) Komórki inkubowane z 120nM roztworem związku **o-8d** naświetlane światłem zielonym. Obecnych jest wiele komórek mitotycznych z nieuporządkowanym i/lub prawie całkowicie zdepolimeryzowanym wrzecionem mitotycznym. (g) Komórki inkubowane z 20 nM roztworem kolchicyny. Układ wrzecion mitotycznych jest podobny jak w komórkach inkubowanych z 100nM roztworem związku **o-8d** naświetlanym światłem zielonym.



Rysunek 21. a) Analiza faz cyklu komórkowego komórek HTC116 po inkubacji z DMSO, roztworem *o*-8d lub roztworem kolchicyny i po wystawieniu na działanie światła niebieskiego lub zielonego. Wykresy przedstawiają reprezentatywną analizę cytometrii przepływowej dla każdej próbki. W tabeli przedstawiono średnią z co najmniej dwóch eksperymentów dla każdej próbki. Wzrost w stosunku do kontroli oznaczono kolorem czerwonym i strzałką w górę, a spadek kolorem niebieskim i strzałką w dół. Co istotne zwiększenie stężenia *o*-8d do 120 nM spowodowało dalszy wzrost ilości komórek będących w fazie G2/M do około 60% dla komórek będących równocześnie naświetlanych światłem zielonym. Natomiast w populacji komórek inkubowanych z takim samym stężeniem *o*-8d ale naświetlane światłem niebieskim zaobserwowano zbliżony procent komórek będących w fazie G2/M (~29%) co w próbie kontrolnej (~25%), jednakże zaobserwowano zwiększoną liczbę komórek apoptotycznych (~11%).

WNIOSKI ORAZ PODSUMOWANIE

Podsumowując w pracach przeglądowych [P1] [P2] omówiono budowę oraz dynamiczną niestabilność mikrotubul oraz czynniki biologiczne regulujące stabilność oraz dynamikę mikrotubul takie jak modyfikacje potranslacyjne tubuliny oraz ekspresja różnych izotypów tubuliny. Następnie skupiono się na czynnikach chemicznych regulujących stabilność oraz dynamikę mikrotubul, w szczególności związkach będących pochodnymi (izomerami oraz izosterami) stilbenu. Omówiono w szczególności związki oddziałujące z domeną kolchicynową. W następnej części rozprawy doktorskiej zaprezentowano koncepcję fotofarmakologii oraz fotoprzełączalne inhibitory/stabilizatory dynamiki mikrotubul z szczególnym uwzględnieniem związków zawierających fragment azobenzenu w swojej strukturze.

W pracy eksperymentalnej [P3] zaprojektowano i zsyntetyzowano bibliotekę częściowo nieopisanych wcześniej w literaturze, dziewięciu pochodnych stilbenu, dwunastu pochodnych dibenzo[*b,f*]oksepin oraz nieopisanych wcześniej w literaturze dwunastu fotochromowych przełączników molekularnych opartych na strukturze azobenzenu połączonego z kolchicyną, które powodują zakłócenie dynamiki mikrotubul.

Dla pochodnych stilbenu oraz dibenzo[*b,f*]oksepin przeprowadzono badania dokowania molekularnego do heterodimeru $\alpha\beta$ -tubuliny, aktywności cytotoksycznej otrzymanych związków na liniach komórkowych zarówno nowotworowych jak i prawidłowych oraz przeprowadzono analizę struktury cytoszkieletu mikrotubularnego za pomocą metody immunofluorescencji. Na podstawie otrzymanych wyników wykazano, że pochodne stilbenu odznaczały się wyższą aktywnością cytotoksyczną niż pochodne dibenzo[*b,f*]oksepin. Indeks selektywności w stosunku do komórek nowotworowych (HCT116 i MCF7) w porównaniu do komórek nienowotworowych (HEK293 i HDF-A) został określony dla związków wykazujących największą aktywność cytotoksyczną – czterech pochodnych stilbenu (**1a**, **1c**, **1d**, **1i**) oraz trzech pochodnych dibenzo[*b,f*]oksepin (**2i**, **2j**, **3h**). Na podstawie dokowania molekularnego i wizualizacji cytoszkieletu mikrotubularnego udowodniono, że cytotoksyczność otrzymanych molekuł była związana z oddziaływaniem z tubuliną i w konsekwencji depolimeryzacją mikrotubul.

W następnej części badań [P4] zaprojektowano oraz syntezyowano dwanaście fotochromowych przełączników molekularnych opartych na strukturze kolchicyny

o-, *m*-, *p*-**8a-d**. Właściwości spektroskopowe tych związków są zależne od typu azobenzenu (*o*-, *m*-, *p*-**6a-d**) wbudowanego w strukturę macierzystego farmakoforu – kolchicyny. Dlatego też dokonano oceny właściwości spektroskopowych uzyskanych azobenzenów przy użyciu spektroskopii magnetycznego rezonansu jądrowego (NMR) oraz spektroskopii UV-Vis. Naświetlanie falą elektromagnetyczną o długości $\lambda=535$ nm powoduje fotokonwersję izomeru *E* do izomeru *Z*. Natomiast światło niebieskie ($\lambda=390 - 430$ nm) skutkuje odwrotną fotokonwersją – niewielki procent izomeru *Z*. Opracowane przełączniki molekularne mogą być fotoizomeryzowane za pomocą światła widzialnego, zamiast promieniowania UV, które jest stosowane w klasycznych przełącznikach molekularnych. Jest to kluczowe, ponieważ kolchicina ulega degradacji pod wpływem promieniowania UV. Co więcej, promieniowanie ultrafioletowe jest również szkodliwe dla organizmów żywych i może prowadzić do uszkodzenia DNA w zdrowych komórkach. Za pomocą testu MTT wyznaczono aktywność cytotoksyczną otrzymanych pochodnych kolchicyny w warunkach naświetlania światłem niebieskim ($\lambda=430$ nm – większa zawartość izomeru *E*) oraz światłem zielonym ($\lambda=535$ nm – większa zawartość izomeru *Z*). Dla wiodącego związku, *o*-**8d**, wykazano zależną od światła cytotoksyczność zarówno w linii komórkowej HCT116, jak i MCF-7 (zwiększenie aktywności cytotoksycznej podczas naświetlania światłem zielonym). Na podstawie przeprowadzonych badań wykazano około dwukrotny wzrost aktywności antyproliferacyjnej związku *o*-**8d** wobec komórek HCT116 pod wpływem naświetlania światłem zielonym. Inhibicja polimeryzacji oczyszczonej tubuliny oraz zaburzenie organizacji mikrotubul podczas inkubacji z związkiem *o*-**8d** potwierdzają, że przełącznik molekularne oparte na strukturze kolchicyny mogą być używane jako światłoczułe związki zakłócające dynamikę mikrotubul.

Podsumowując, udowodniono, że fotochromowe przełączniki molekularne oparte na strukturze kolchicyny stanowią podstawę do dalszych badań i rozwoju nowych klasy światłoczułych inhibitorów polimeryzacji tubuliny, które mogą znaleźć zastosowanie w przyszłych badaniach i aplikacjach klinicznych.

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KOPIA PUBLIKACJI BĘDĄCYCH PODSTAWĄ ROZPRAWY DOKTORSKIEJ

Review

Intrinsic and Extrinsic Factors Affecting Microtubule Dynamics in Normal and Cancer Cells

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Received: 19 June 2020; Accepted: 8 August 2020; Published: 14 August 2020



Abstract: Microtubules (MTs), highly dynamic structures composed of α - and β -tubulin heterodimers, are involved in cell movement and intracellular traffic and are essential for cell division. Within the cell, MTs are not uniform as they can be composed of different tubulin isoforms that are post-translationally modified and interact with different microtubule-associated proteins (MAPs). These diverse intrinsic factors influence the dynamics of MTs. Extrinsic factors such as microtubule-targeting agents (MTAs) can also affect MT dynamics. MTAs can be divided into two main categories: microtubule-stabilizing agents (MSAs) and microtubule-destabilizing agents (MDAs). Thus, the MT skeleton is an important target for anticancer therapy. This review discusses factors that determine the microtubule dynamics in normal and cancer cells and describes microtubule–MTA interactions, highlighting the importance of tubulin isoform diversity and post-translational modifications in MTA responses and the consequences of such a phenomenon, including drug resistance development.

Keywords: α -tubulin; β -tubulin; microtubule; post-translational modifications; cancer; microtubule-targeting agents (MTAs); tubulin-binding agents (TBAs); resistance

1. Introduction

Microtubules (MTs), which are dynamic cytoskeletal components, are hollow-tube filaments usually built up of 13 protofilaments composed of α - and β -tubulin heterodimers connected by noncovalent bonds (Figure 1). In eukaryotic cells, the initiation of tubulin polymerization, so-called nucleation, is associated with the presence of templates, including the γ -tubulin ring complex (γ -TuRC) or newly severed microtubule ends (so-called seeds) [1]. The elongation of polymerizing microtubules results in the formation of a microtubular network which is involved in many cellular processes, such as cell movement, cell shape determination, distribution of organelles, intracellular transport, and the formation of the mitotic spindle, the key structure in chromosome segregation during cell division [2,3]. Moreover, microtubules form skeletons of complex eukaryotic structures such as centrioles, basal bodies, and cilia [4]. Not surprisingly, defects in microtubule assembly or properties can lead to severe diseases, including cancer [5].

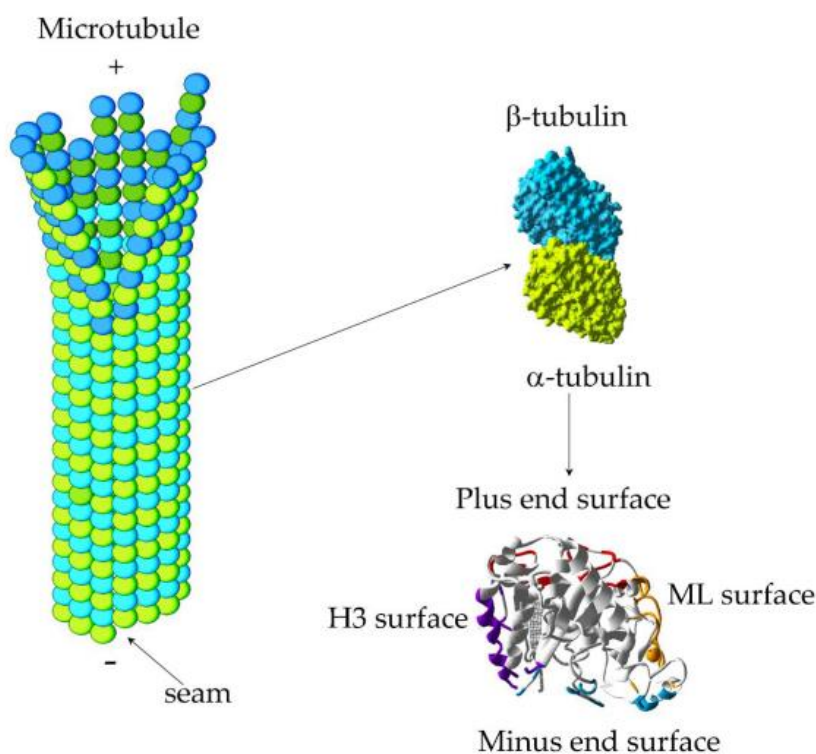


Figure 1. Scheme of the structure of microtubules, tubulin heterodimers, and functional surfaces of tubulin.

Performing such different functions requires both the stability of microtubules and the ability to quickly respond to cellular cues by shortening or directional growth. Within the cell, microtubule dynamics depends on three intrinsic factors: (i) the isotype of the incorporated α - and β -tubulins (as in most organisms, tubulins are encoded by multiple genes) [6–10]; (ii) the type and level of post-translational tubulin modifications [11,12]; and (iii) interactions with non-tubulin proteins, so-called microtubule-associated proteins (MAPs) [13,14]. Besides the intrinsic factors that generate heterogeneous MTs, extrinsic physical factors (e.g., temperature) and chemical factors, so-called microtubule-targeting agents (MTAs; also known as tubulin-binding agents (TBAs), microtubule-interfering drugs, anti-microtubule drugs, or microtubule poisons) can influence MT dynamics. Taking advantage of the possibility to externally modulate MT dynamics, several microtubule-targeting agents have been employed as chemotherapeutic agents [15–17].

The effect of an individual anti-tubulin/anti-microtubule compound on the polymer mass, stability, and dynamics of microtubules is very complex. Basically, MTAs can be divided into two main categories. Compounds of the first category (microtubule-stabilizing agents (MSAs)), after binding to the tubulin heterodimer, increase the lateral interactions between heterodimers, which at high compound concentration leads to increased polymerization and stabilization of MTs, resulting in increased polymer mass within the cell. Compounds from the second group (microtubule-destabilizing agents (MDAs)) decrease or inhibit mainly longitudinal interactions between heterodimers at high concentrations, resulting in MT depolymerization and decreased polymer mass. Notably, at lower concentrations used in clinical applications, both types of MTAs only finely tune MT dynamics, with no

effect on polymer mass or overall cytoskeleton features [16,18]. To date, six binding sites are recognized in the tubulin surface and numerous small molecules or macrocyclic compounds that alter intra- and intertubulin interactions by binding with these pockets [18].

In this review, we focus on the factors that determine microtubule dynamics in normal and cancer cells. We describe the microtubule–MTA interplay, highlighting the significance of the diversity of tubulin isoforms and their post-translational modifications in MTA response, and the consequences of this phenomenon, including the development of drug resistance. Due to the large number of chemical compounds used in cancer therapies, this paper covers only a narrow range of molecules.

2. Structure of Tubulin: Molecular Basis of MT Dynamics

The 3D structure of microtubule subunits was resolved in 1998 [19]. That study and subsequent studies showed that α - and β -tubulins share high structural similarity; both are composed of several large secondary structures, including 10 β -strands (S1–S10) and 12 α -helices (H1–H12) linked by loops [19]. Additionally, six small helices (H1', H2', H2'', H3', H9', and H11') are located on loops near the larger structures [20]. Helices H1–H12 and strands S1–10 constitute the protein body, which can be divided into three main domains: an N-terminal, also called a nucleotide-binding domain, a so-called intermediate domain, and a C-terminal part. The N-terminal domain (amino acids 1 to ~205) comprises six parallel β -strands (S1–S6) alternating with α -helices (H1–H7) linked by loops. The intermediate domain (aa ~206–381) is formed by three helices (H8–H10) alternating with four β -strands (S7–S10). The C-terminal domain (aa ~382 to ~451) is composed of two α -helices (H11–H12), followed by several unstructured amino acids that extend beyond the globular part as the so-called tubulin tail [19,20].

The nucleotide-binding and intermediate domains form the main part of the globular protein body with four distinguishable functional surfaces: plus end, minus end, H3 surface, and ML surface (Figure 1) [19,21].

The plus and minus ends of adjacent tubulins bind to each other, participating in longitudinal tubulin interactions necessary for the formation of both heterodimers and protofilaments. The minus end surface includes the amino acids of helix H10 and loops H3–S4, T7, and H10–S9 (Figure 1). The plus end is composed of residues from helices H1, H6, and H11; strand S3; and loops T3, T5, H6–H7, and H11–H12 (Figure 1). Therefore, the plus end surface also includes the nucleotide-binding pocket (NBP) where GTP/GDP is tethered (Figure 1). Importantly, the α -tubulin plus end is tightly associated with the minus end of the β -tubulin within the heterodimer and, thus, its NBP is permanently associated with GTP (so-called non-exchangeable or N-site). Its precise function is unknown, although mutations that alter the GTP-binding site on α can be highly deleterious [22]. By contrast, the β -tubulin plus end, including NBP, is exposed, allowing exchange of GTP to GDP (so-called exchangeable or E-site). The GTP-bound dimer acquires a so-called curved conformation, which allows it to be accommodated at the microtubule tip. In fact, cryo-EM studies show that the microtubule tip has a slightly “open” morphology (see Figure 1) (reviewed in [18,23]). After being incorporated into a microtubule wall, a heterodimer acquires a so-called straight conformation, which requires “compression” of the α -tubulin intermediate domain by moving S8 and S9 closer to H8 [24].

The hydrolysis of α -tubulin–GTP causes compaction of the heterodimer and sub-nanometer shortening of the protofilament, changing longitudinal but not lateral forces within the microtubule [25]. Although GTP-to-GDP conversion is an important factor that regulates microtubule dynamics by triggering conformational changes in tubulin dimer, several studies suggest that other tubulin regions, including intermediate and C-terminal domains, could also be involved in this process [26–28].

The H3 and ML surfaces are involved in lateral interactions between heterodimers of neighboring protofilaments, leading to the arrangement of the protofilaments into a cylindrical structure [19]. The H3 surface consists of helix H3 and strand S3 as well as loops H1–B1, H2–B2, and H4–S4, while the ML surface contains helices H6, H9, and H10 and loops H6–H7, S7–H9 (M-loop), and H9–S8 (Figure 1) [19].

The secondary structures of the C-terminal domain, helices H11–H12, are organized into the so-called outside of the tubulin, which in the MT, is exposed to the cytoplasm. The two helices form a

surface on which microtubule-associated and motor proteins can bind [19,20], while the unstructured C-terminal amino acids (10 in α -tubulin and 18 in β -tubulin) form the tubulin tail. This region is particularly variable, showing the most amino acid sequence differences between tubulin isotypes (Table 1). Such sequence divergence may provide the structural basis for the construction of specific types of microtubules and determination of their particular properties and functions [19,20].

3. Intrinsic Factors Affecting Microtubule Dynamics

3.1. Tubulin Isotypes and Microtubule Dynamics

The human genome encodes seven α - and nine β -tubulin isotypes [29]. As a consequence, MTs composed of mixed combinations of α - and β -tubulin isotypes show tissue and developmental stage specificity and different properties in different cell types [30,31].

Human α -tubulins belong to four classes (out of nine characterized in mammals; see [32,33]): α I (isotypes α Ia and α Ib), α III, α IV (isotypes α IVa and α IVb), and α VIII (Table 1). Interestingly, two classes show tissue-specific distribution, with α III restricted mainly to testis and α VIII to heart and skeletal muscle, while the other two are widely distributed [34–39]. Although the tissue or cell type-specific functions of α -tubulin isotypes were only recently addressed for several isotypes (α Ia in neuron migration [40], α IVa in platelets [41], and α VIII in brain and testis [42]), the role of α -tubulin isotypes in MT dynamics remains unknown.

Human β -tubulins belong to seven classes (out of nine in mammals): I–VI and VIII (Table 1). From published data and the publicly available National Center for Biotechnology Information (NCBI) database, it seems that the most ubiquitously expressed isotypes are β I, β IVb, β V, and β VIII, while other β -tubulins have more specific tissue distribution (Table 1) [43]. Interestingly, although they are widely distributed, β I, β V, and β VIII were shown to have nonredundant roles in specific cellular or developmental processes. The silencing of β I expression in differentiating human neuroblastoma cells was lethal, while depletion of β I in undifferentiated cells had no apparent effect on cell survival [44]. On the other hand, β VIII is essential for oocyte maturation and early embryo development [45].

β II, β III, and β IVa are mainly expressed in the brain (Table 1) [43,46,47]. Accordingly, experimental data indicate their specific role in processes related to the nervous system. The silencing of β II inhibits neurite outgrowth in differentiating neuroblastoma cells, while β III was suggested to protect cells against oxidative stress [44]. However, more recent data with the use of *Tubb3* knockout mice indicate that the β III isotype functions in the process of axon growth and nerve regeneration [48]. An interesting observation was made for β IVa: mutations in this tubulin can cause dysfunction of neurons, oligodendrocytes, or both [49].

Not much is known about the functions of other β -tubulin isotypes, β IVb and β VI. The latter shows slightly higher expression in bone marrow (Table 1) and is essential for the formation of platelet cytoskeleton (reviewed in [50]); in many publications, the *TUBB1* gene product, following protein names in the database, is called β 1 or β -1 instead of β VI).

How do particular α - and β -tubulin isotypes relate to MT dynamics? The answer to this question came from studies focusing mainly on the comparison of the properties of three neuronal isotypes, β II, β III, and β IV. The molecular surfaces of tubulins are very similar, considering the level of amino acid similarity, which for α -tubulins reaches approximately 90–95%, and is slightly lower for β -tubulins at ~85–95% (with β VI being the most divergent, with approximately 75% similarity to other isotypes). Most amino acid substitutions are accumulated within the C-terminal tubulin tail (Table 1), suggesting that this part could be responsible for the potential variation of properties of tubulin isotypes and, subsequently, the differences in MT dynamics [29]. However, several β isotypes also have unique substitutions in other regions important for the formation of functional surfaces and pockets.

Table 1. Features of human tubulin isotypes.

Tubulin Isotype	Gene Name	Expression	Cell Type-Specific Functions	Sequence of C-Terminus *	Reference
α Ia	TUBA1A	ubiquitous	neuron migration	VEGE ^{blue} EEEE ^{blue} GEEY ^{yellow}	[40,51]
α Ib	TUBA1B	ubiquitous		VEGE ^{blue} EEEE ^{blue} GEEY ^{yellow}	[52]
α Ic	TUBA1C	ubiquitous		ADGE ^{blue} DFE ^{blue} GEEY ^{yellow}	[53]
α IIc	TUBA3C	testis		VEAE ^{blue} AE ^{blue} GEEY ^{yellow}	[54]
α IIe	TUBA3E	testis		VEAE ^{blue} AE ^{blue} GAY ^{yellow}	[55]
α IVa	TUBA4A	ubiquitous	platelet biogenesis	YE ^{blue} DE ^{blue} EGEE ^{blue}	[41,56]
α VIII	TUBA8	high: heart and skeletal muscle moderate: brain, testis, and thyroid very low: all other tissues	spermatogenesis	FE ^{blue} EE ^{blue} NE ^{blue} GEEF ^{blue}	[35,42,57]
β I	TUBB	ubiquitous	survival of differentiated neuroblastoma	EEEE ^{blue} DFGEE ^{blue} AEEEA ^{blue}	[43,44,58]
β IIa	TUBB2A	high: brain very low: all other tissues	neurite outgrowth	DE ^{blue} QGE ^{blue} FE ^{blue} EE ^{blue} GE ^{blue} DEA ^{blue}	[43,44,59]
β IIb	TUBB2B	high: brain very low: all other tissues	neurite outgrowth	DE ^{blue} QGE ^{blue} FE ^{blue} EE ^{blue} GE ^{blue} DEA ^{blue}	[43,44,60]
β III	TUBB3	moderate: brain low: testis	oxidative stress axon and nerve regeneration	EE ^{blue} GE ^{blue} MY ^{blue} ED ^{blue} EE ^{blue} EE ^{blue} SE ^{blue} AQGPK ^{red}	[43,44,48,61]
β IVa	TUBB4A	high: brain moderate/low: other tissues ubiquitously expressed	oligodendrocyte function	EE ^{blue} GE ^{blue} FE ^{blue} EE ^{blue} AEE ^{blue} VA ^{blue}	[43,49,62]
β IVb	TUBB4B/ TUBB2C	high: testis, bone marrow, and heart moderate/low: other tissues		EE ^{blue} GE ^{blue} FE ^{blue} EE ^{blue} AEE ^{blue} VA ^{blue}	[43,63]
β V	TUBB6	ubiquitous at low levels	related to skeletal muscle regeneration	NDGE ^{blue} EE ^{blue} AFE ^{blue} DE ^{blue} EE ^{blue} IDG ^{blue}	[43,64,65]
β VI	TUBB1	very low level in all tissues, highest in bone marrow and spleen	platelet cytoskeleton	VLEE ^{blue} DE ^{blue} EV ^{blue} TTEE ^{blue} AEME ^{blue} PE ^{blue} DKGH ^{red}	[43,66]
β VIII	TUBB8	very low in all tissues, highest in testis	oocyte maturation, early development	EEEE ^{blue} DEE ^{blue} YA ^{blue} EEEE ^{blue} VA ^{blue}	[67]

Blue: negatively charged amino acids, red: positively charged amino acids, yellow: C-terminal tyrosine residue, purple: phosphorylatable residue, * sequences were taken from the NCBI database.

Among the investigated β -tubulin isotypes (β II, β III, and β IV), β III is the most divergent, with substitutions in globular protein body including: (i) helix H3 (serine in positions 124 and 126 is substituted by cysteine and asparagine, respectively), (ii) loops H1–S2 and H2–S3 (structures involved in the formation of H3 surface), (iii) structures located proximal to the ML surface (H6–H7 loop threonine 217 and S7–H9 loop serine 275 substituted to alanine) [68], and (iv) T7 loop (minus end surface) near colchicine-binding site (cysteine 239 substituted by serine). Moreover, the C-terminal tail of β III is very divergent. It contains positively charged lysine (Table 1) and a phosphorylatable serine [69,70]. These variations are potentially important for the overall tubulin structure; in fact, an atomic model of β III-containing MTs shows slight but significant displacement of the H1–S2 loop and part of the ML surface-forming structures with respect to β II-containing MTs [68]. However, how β III substitution relates to its specific properties is mainly unknown.

For more than two decades, it has been known that MTs assembled from β III tubulin are more dynamic than MTs containing β II or β IV [9]. More recent data indicate that the main difference is caused by the significantly increased catastrophe rate (depolymerization) of β III-containing MTs [27,71,72],

while the growth rate seems to be similar [27,71] or only slightly lower [72]. The dynamic features of MTs assembled with specific isotypes were retained when the C-terminal tails of β II and β III were interchanged, indicating that the dynamic properties of these two tubulins are “encoded” within the main globular protein body [71]. Remarkably, not only dynamics, but also resistance to depolymerizing factors and structural features vary between β II- and β III-containing MTs, with the former showing lower resistance to depolymerizing agents and more protofilaments in the MT wall (14) than the latter (β III contains 13 protofilaments and shows more resistance to depolymerizing factors) [68]. Interestingly, when two populations of tubulin heterodimers are mixed with different stoichiometry, assembled MTs show intermediate dynamics [68,71,72].

On the other hand, at low tubulin concentration, the MTs nucleate much more slowly if the tubulin heterodimers contain β III-tubulin compared to β II or β IV. Interestingly, this difference can be abolished by the proteolytic removal of C-terminal tail [8]. This result indicates that although it is not crucial for dynamic tubulin properties, the tubulin C-terminus can influence other features that could be important for MT cytoskeleton formation within the cell.

To summarize, growing evidence indicates that the intrinsic properties of tubulin isotypes, the expression of specific isotypes, and their ratio within the cell are significant factors influencing MT dynamics.

3.2. Post-Translational Modifications of Microtubules

Tubulin modification sites, modifying enzymes, and functions of post-translational modifications (PTMs), including the impact on MT dynamics, have recently been broadly reviewed [12,33,73,74]. Thus, here, we will only briefly summarize how PTMs affect MT dynamics.

Both α - and β -tubulin undergo a number of post-translational modifications that change the properties of the free tubulin heterodimers and microtubules. Tubulin PTMs can modulate MT dynamics directly or indirectly by influencing the interactions between MTs and microtubule-interacting proteins (which can stabilize, destabilize, or cut microtubules).

While for the vast majority of modifying enzymes, a tubulin heterodimer already incorporated into the microtubule lattice is a preferred substrate, in some cases, free tubulin heterodimers are effectively modified. The latter can affect the binding of tubulin heterodimers to the microtubule plus end and, thus, affect microtubule growth and stability. For instance, acetylation of lysine 252 of β -tubulin by *San* acetyltransferase slows down the rate of tubulin incorporation into the microtubule and consequently reduces the rate of MT assembly [75]. Phosphorylation of serine 172 of β -tubulin by minikinase/DYRK1a (neurons) or cyclin-dependent kinase Cdk1 (mammalian mitotic cells) inhibits the incorporation of tubulin heterodimers [76,77], while heterodimers containing α lc-tubulin phosphorylated at serine 165 assemble more effectively than unmodified ones [78,79]. Serine and threonine residues of α - and β -tubulin can also be modified by *O*-GlcNAcylation [80,81] and, in vitro, such modified tubulins are not incorporated into microtubules [81].

The presence of differentially modified MTs within the cell is crucial in the assembly, disassembly, and rearrangement of the microtubular cytoskeleton [82]. Newly polymerized dynamic microtubules are highly tyrosinated (besides α IVa, tyrosine is encoded in the C-terminus of α -tubulins, see Table 1). With time, the tyrosine is removed by the vashohin family, VASH1 and VASH2 tubulin detyrosinases, generating so-called detyrosinated tubulin. Detyrosinated tubulin is found in spindle and is essential for correct chromosome congression [83]. Glutamic acid residue, which is the most frequent penultimate residue in α -tubulin, can also be removed by cytosolic carboxypeptidases, irreversibly forming so-called Δ 2-tubulin [84–87]. After depolymerization, free detyrosinated α -tubulin can be re-tyrosinated by tubulin tyrosine ligase (TTL) [88,89].

The α -tubulins I, II, III, IV, and VII have a lysine at position 40. Acetylation of lysine 40 residue by α -tubulin N-acetyltransferase 1 (ATAT1) has been known to mark stable microtubules [90–93]. The modification is reversible, and tubulin deacetylation is carried out by histone deacetylase 6 (HDAC6) [94] and Sirt2 deacetylase [95]. Recent analysis using high-resolution cryo-electron microscopy

showed that the acetylation of lysine 40 restricts the range of motion of the loop containing K40, likely weakening the lateral contacts between protofilaments [96] and thus increasing MT flexibility [97,98].

Polyamination of α - and β -tubulin by a transglutaminase causes the formation of hyperstable, cold-resistant microtubules. This modification is important for neuronal development and axon maturation [99]. The positions of the main polyamination sites near the GTP pocket (glutamine 15 in β -tubulin) and α -tubulin minus end suggest that tubulin polyamination could affect GTP binding or hydrolysis and microtubule lattice stabilization [99].

Glycylation and glutamylation of α - and β -tubulin can occur as mono- or polymodification, and glycylyl or glutamyl residues are ligated to the glutamic acid residues within the C-terminal tail [73,92]. These tubulin modifications are catalyzed by enzymes related to TTL, called tubulin tyrosine ligase-like (TTL) [73]. The reverse reaction (deglutamylation) is carried out by cytoplasmic carboxypeptidases (CCPs). To date, the identity of tubulin deglycylase remains unknown [92].

3.3. Microtubule-Associated Proteins and Microtubule Dynamics

Microtubule-associated proteins (MAPs) are another intrinsic factor affecting microtubule dynamics (reviewed in [100–102]). Generally, MAPs function as MT stabilizers or destabilizers; however, stabilization/destabilization of MTs can be achieved by affecting one of several processes, including MT nucleation [1] and stabilization/destabilization of the MT ends [2] or of the MT lattice [102,103]. For this reason, MAPs are divided into several functional categories: (i) microtubule nucleators, (ii) MT end-binding proteins, (iii) lattice-binding proteins also known as structural MAPs, (iv) enzymes severing or depolymerizing microtubules, and (v) motor MAPs (kinesin, dynein) that generate forces and use microtubules as tracks for intracellular transport [102]. Intriguingly, some MAPs can participate in several MT dynamics-related processes. For example, XMAP215 can be classified as both an MT nucleator and a plus end-binding protein [1,2] while kinesin-13 family proteins are motor proteins that bind to microtubule plus ends and have MT-depolymerizing properties [104,105]. Because MAPs form a large class of proteins and a number of high-quality reviews on this topic are already available, we will only provide a short overview of these proteins, highlighting the relationship between MAPs and MT dynamics.

Microtubule nucleators enhance the initiation of MT formation from both γ -TuRCs and microtubule seeds and stabilize growing MTs. This category of MAPs includes XMAP215/CKAP5, TPX2 (targeting factor for Xklp2), DCX (doublecortin), CAMSAP (calmodulin-regulated spectrin-associated protein)/Patronin, CLASP (cytoplasmic linker associated protein), and p150Glued proteins [1,23]. They are believed to act mainly as enhancers of longitudinal and lateral contacts between tubulin dimers [1]. Interestingly, TPX2, besides its nucleating activity, also suppresses MT depolymerization and shrinkage, and thus increases MT stability [106].

Proteins that bind to MT ends are specific to either the plus (so-called +TIPs [107]) or minus end (so-called −TIPs [108]). Plus TIPs belong to approximately 20 different families of proteins [107]. EB (end-binding) proteins form a core of +TIP network and the majority of studies suggest that they stabilize or protect the MT plus end [107]. EB proteins interact with both the MT plus end and other +TIP proteins that can be either stabilizers (as CLIP-170 (cytoplasmic linker protein 170)/CLIP1 and CLASP proteins), destabilizers (as kinesin-13 family proteins), or polymerases (as XMAP215 protein). Within the cell, the interplay between these proteins results in MT growth or shrinkage (reviewed in [2,23,107]).

Stathmin-1, also known as Op18 (oncoprotein 18), binds to the MT plus end but is not included in the +TIP protein class. It was first discovered as an oncoprotein highly expressed in some types of leukemia, breast, and ovarian cancers [109]. Stathmin-1 causes a decrease of the MT polymer mass by two mechanisms: (i) by binding two tubulin dimers in a curved conformation and inhibition of their incorporation into microtubule and (ii) by interfering with the lateral bonding between tubulin subunits, leading to destabilization of the microtubule tip and MT shrinkage [110,111].

A recently described class of γ -TIP, includes CAMSAP proteins and the KANSL complex [108]. In mammals, CAMSAP proteins protect the MT minus end against the depolymerizing activity of kinesin-13. Additionally, CAMSAP2 and 3 proteins decrease the rate of tubulin incorporation at the minus end, decreasing its dynamics [112]. The functions of KANSL complex are still unknown [108].

The lattice-binding MAPs include classical MAPs, MAP1, MAP2, MAP4, MAP6, MAP7, and Tau, which promote polymerization, stabilization, and bundling of microtubules (reviewed in [102]). They also regulate the association of MTs with other cytoskeletal fibers, organelles, and membranes, and influence the ratio of transport along MTs and MT severing by physically blocking the access of motors and severing enzymes. Additionally, structural MAPs can regulate the number of MT protofilaments [102]. Interestingly, it seems that MAP6 has unique properties and functions as it is a microtubule luminal protein and protects MTs against drug and cold-dependent destabilization [113]. With the exception of MAP4, which is a ubiquitous protein, and the expression of MAP7 in epithelial cells, the expression of structural MAPs is mainly restricted to the brain [114].

Microtubule organization is also regulated through the microtubule-severing proteins katanin, spastin, and fidgetin, whose activity can lead to MT shortening or even depolymerization, but also to the formation of numerous microtubule seeds that serve as MT nucleation templates and free tubulin dimers that can be incorporated into new microtubules. Therefore, severing activity can have both a negative and positive effect on MT dynamics and microtubule polymer mass (reviewed in [103]).

It should be noted that, within a cell, the organization and dynamics of MTs is a result of the interplay between tubulin isotypes, their posttranslational modifications, and microtubule-associated proteins. For example, tyrosination increases the affinity of MT to the stabilizing protein, CLIP-170/CLIP-1 but, also to depolymerizing proteins from the kinesin-13 family [115–117]. Similarly, tubulin polyglutamylation affects the interactions of MT with several MAPs, including Tau, MAP1, and MAP2, but also with MT-severing proteins [118–120].

4. Extrinsic Factors Affecting Microtubule Dynamics

4.1. Microtubule-Targeting Agents

The surface of the globular part of tubulins contains several pockets that can be intercalation sites for MTAs. These compounds, while embedded in the tubulin structure, can alter the microtubule dynamics. This feature of MTAs is used in cancer therapy, as it was shown that treatment of cancer cells with MTAs led to the mitotic arrest and consequent cell death [18,121]. Many MTA compounds are produced by plants, fungi, and invertebrates as a natural defense against antagonists, competitors, or parasites (for a review, see [122]).

Currently, six MTA-binding sites, named after the main compounds with affinity to them, have been described (Figure 2). Four pockets are located on β -tubulin: taxane, laulimalide/peloruside, vinca, and maytansine sites. The colchicine site is located near the intradimer interface between the α - and β -tubulin subunits, while the pironetin site is a binding pocket located on the α -tubulin surface (for a review, see [18]). Taxane and laulimalide/peloruside sites bind compounds that stabilize microtubules (MSAs), while the other four pockets accommodate factors that destabilize MTs (MDAs).

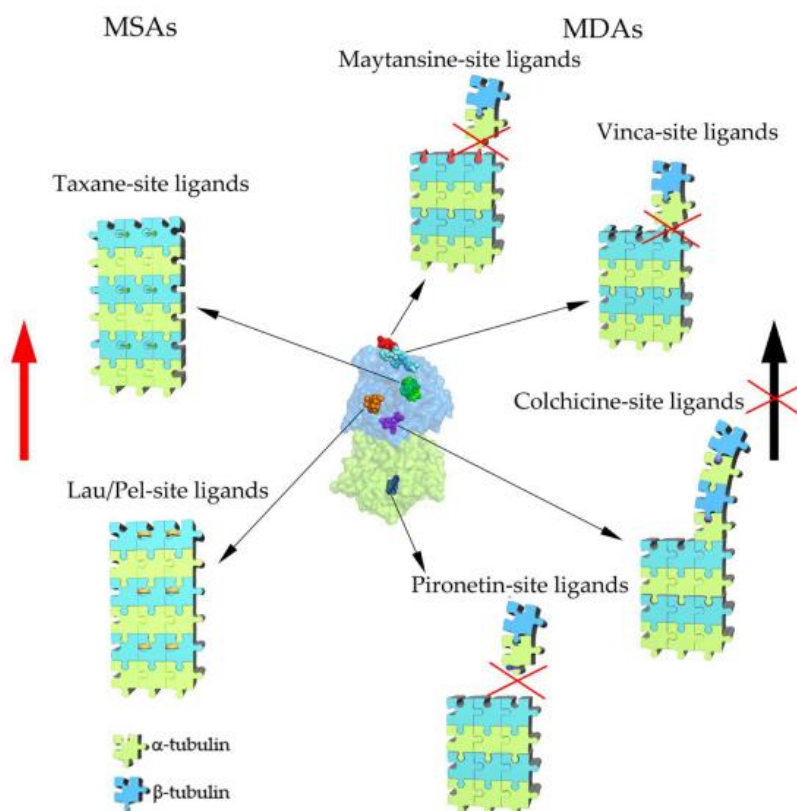


Figure 2. Location of the microtubule-targeting agent (MTA)-binding site on tubulin and the MTA mechanism of action. Microtubule-stabilizing agents (MSAs) (taxane and laulimalide/peloruside-site ligands) promote microtubule (MT) growth (red arrow) by stabilizing lateral contacts between neighboring heterodimers (see text). MDAs inhibit MT growth and destabilize MTs (crossed out black arrow) by inhibiting addition of new heterodimers at the MT plus end (maytansine- and pironetin-site ligands) or by inhibiting transition of heterodimer structure to straight conformation (colchicine- and vinca-site ligands) (see text).

4.2. Tubulin Pockets

4.2.1. Taxane Site

Paclitaxel, a tetracyclic diterpenoid originally isolated from *Taxus brevifolia* in the 1960s [123], was approved for the treatment of ovarian cancer in 1992 by the US Food and Drug Administration (FDA) as Taxol®. Now, paclitaxel is produced by a semi-synthetic route by modifying 10-deacetylbaccatins III derived from the European yew *Taxus baccata* [124].

Paclitaxel and its derivatives are used in diverse cancer therapies and are characterized by high neurotoxicity, myelosuppression, poor water solubility, and the occurrence of multidrug resistance (MDR) in treated tumors (see below). This led to the search for and discovery of other compounds that enhance microtubule stabilization, including epothilones A, B, and D; discodermolide (DDM) and the

DDM–paclitaxel hybrid KS-1-199-32; dictyostatin; taccalonolide A and J; and zampanolide (Figure 3) (for reviews, see [18,125,126]).

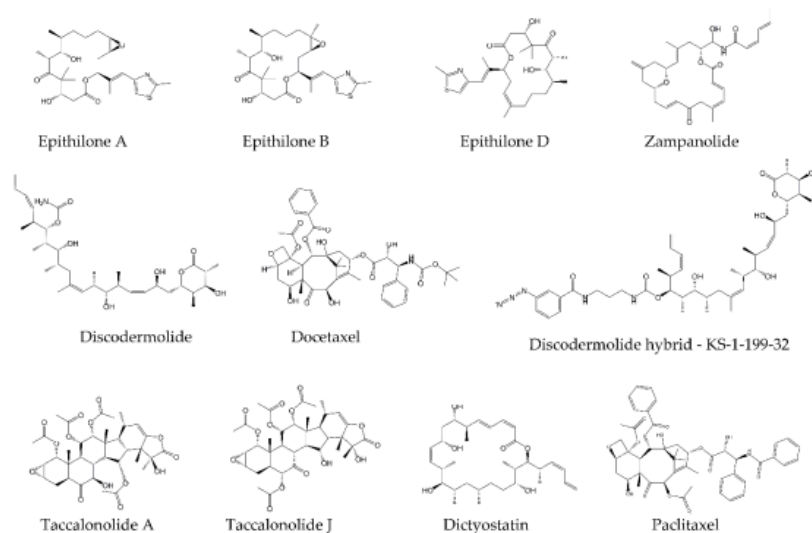


Figure 3. Structure of docetaxel; epithilones A, B, and D; discodermolide (DDM), KS-1-199-32; dictyostatin; taccalonolide A and J; zampanolide; and paclitaxel.

Epithilones A and B are macrolide drugs (natural products that consist of a large macrocyclic lactone ring) produced by the myxobacterium *Sorangium cellulosum* [127]. Unlike paclitaxel, they are highly soluble in water and are not a substrate for P-glycoprotein, which actively transports drugs out of the cell. A semi-synthetic derivative of epithilone B, ixabepilone, was approved for cancer treatment [128,129].

Zampanolide, a sponge-derived macrolide, and taccalonolide A and J, polycyclic steroids isolated from plants of the genus *Tacca*, were more recently discovered and are still under investigation, but are promising antitumor drug candidates [130]. They both have the unique ability to bind covalently to taxane-site residues asparagine 228/histidine 229 and aspartic acid 226 [130,131].

Taxane-site targets are used in the treatment of various cancers, including ovarian (paclitaxel), breast (paclitaxel, docetaxel, larotaxel, ixabepilone), lung (docetaxel, epithilone B, larotaxel), bladder (larotaxel), hormone-resistant prostate cancer (cabazitaxel), and others [128,129,132–137].

The taxane (Figure 2) site is located near the ML surface, on the “inside” side of the tubulin (which in MT, faces the lumen), and is formed by hydrophobic residues of H7, S7, and loops H6–H7, S7–H9 (M loop), and S9–S10 (Figure 2) [19,20,138]. All compounds (Figure 3) that bind to the taxane site form hydrophobic and polar contacts with pocket amino acids and strengthen the lateral contacts between heterodimers of adjacent protofilaments, leading to MT stabilization (Figure 2). The mechanism of microtubule stabilization is compound-specific [25,131,138,139]. Several compounds that contain side chains, such as epithilone A and zampanolide, engage with the M loop, stabilizing it into a short helix [138]. Significantly, a similar helical conformation of the M loop was observed in native polymerized MTs [25,138], indicating its importance in the formation of stable MT. Other compounds (e.g., taccalonolide A and J) cause displacement of the M-loop into a more open conformation, facilitating lateral interactions between adjacent protofilaments [131]. By contrast, paclitaxel was suggested to stabilize MTs via an allosteric mechanism by preventing dimer compaction after GTP

hydrolysis [25,138]. A similar indirect effect was also proposed as an additional mechanism of MT stabilization for taccalonolide A and zampanolide [130,131].

4.2.2. Laulimalide/Peloruside Site

Laulimalide and peloruside A (Figure 4), macrolides originally isolated from marine sponges (*Cacospongia mycofijiensis* and *Mycale hentscheli*, respectively) [140–142], are promising non-taxane-site MSAs. Although high-resolution X-ray crystallography and cryo-EM have resolved the tubulin–compound interactions in detail [138,143], biological investigation of these MTAs is still ongoing [144–146].

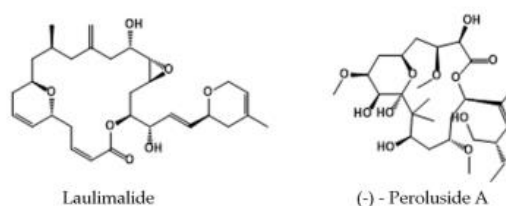


Figure 4. Structure of laulimalide and peloruside A.

The laulimalide/peloruside site is positioned at the opposite side of the ML surface with respect to the taxane site, i.e., the “outside” surface of the MT wall (Figure 2), and is formed by hydrophobic and polar residues of H9 (including a short loop that divides H9 into H9 and H9’), H10, and loop H10–S9 of β -tubulin (Figure 2) [138,143].

After binding laulimalide or peloruside to the β -tubulin pocket, MTs are stabilized by two main mechanisms which strengthen the lateral contacts between neighboring protofilaments. First, the β -tubulin M loop shifts to an “open” conformation (without forming the regular secondary structure). Second, the position of both agents within the pocket allows their interaction with the H3 surface of the adjacent heterodimer, which leads to bridging of neighboring protofilaments (Figure 2) [143]. In the case of peloruside A, an especially strong effect is observed in the seam, where the lateral contacts are weaker. It was also proposed that both compounds fix structures located near the M loop and, thus, have an additional allosteric effect on MT stabilization [143].

4.2.3. Vinca Site

The naturally occurring vinca alkaloids (vincristine and vinblastine) were discovered in periwinkle (*Catharanthus roseus* G. Don.) in the late 1950s (Figure 5). These are first-generation vinca alkaloids that have achieved significant clinical progress [147,148]. The therapeutic success of vinca alkaloids in the treatment of hematological cancers (mainly childhood leukemia) [149] led to the development of diverse semi-synthetic analogs (Figure 5) [150], including vindesine, vinorelbine, and vinflunine, the latter used for the treatment of solid tumors, particularly metastatic breast cancer [151]. However, similar to taxanes, vinca alkaloids have severe side effects (peripheral neuropathies and reversible myelosuppression) [152,153].

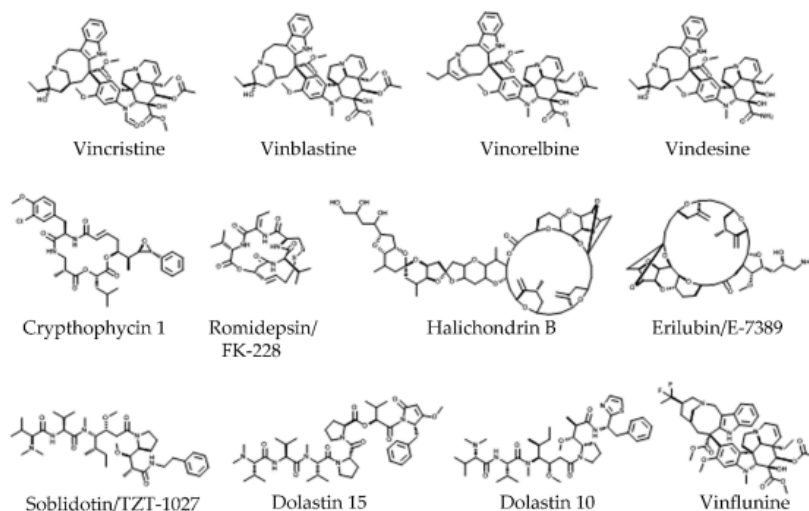


Figure 5. Structure of vincristine, vinblastine, vindesine, vinorelbine, vinflunine, dolastatin-10, dolastatin-15, TZT-1027, cryptophycin 1, FK228, halichondrin B, and E-7389.

Besides vinca alkaloids, several other groups of compounds were also shown to target the vinca site, including peptides, depsipeptides, and macrolides, and some have been used in clinical trials (reviewed in [18,125,154,155]). Currently, vincristine and vinblastine are used for the treatment of breast cancer, lymphomas, and sarcomas [150], vinorelbine for breast and lung cancer, sarcomas, and glioma [150,156], vindesine for lung cancer [150], vinflunine for urothelial cancer [157,158], vintafolide (vinflunine and folate) for lung, ovarian, and endometrial cancer [159], eribulin for liposarcomas, bladder cancer, and metastatic breast cancer [160,161], and dolastatin 10 for solid tumors [162].

The vinca site is located at the plus end surface of β -tubulin and is formed by residues of H6 and loops T5 and H6–H7; however, several agents also bind to H7 and β -tubulin-bound nucleotide sites [163–165]. The vinca-site ligands also form connections with α -tubulin of the subsequent dimer, interacting with its minus end surface structures, including H10, S9, and T7 loop [163].

The binding of ligands to the vinca site alter the surface of the β -tubulin plus end, forming a so-called wedge (Figure 2) [163], thus interfering with the incorporation of new heterodimers at the MT plus end. As a result, the plus end heterodimers remain in curved conformation, which inhibits formation of the MT wall and leads to destabilization [163]. It was also shown that vinca-site ligands can cause the formation of ring-like tubulin oligomers, decreasing the level of free tubulin available for polymerization (Figure 2) [163,166]. Additionally, several vinca-site compounds were shown to have an allosteric effect on the inhibition of lateral contacts between dimers by stabilizing the M loop in the interaction-incompetent conformation [165].

4.2.4. Maytansine Site

Maytansine and its derivatives were first isolated from an African shrub, *Maytenus ovatus* [167]. They belong to the natural product group of maytansinoids, macrolides of the ansamycin type (Figure 6). Later, other groups of compounds, including macrocyclic polyketides (disorazole Z, Figure 6), macrocyclic lactones (rhizoxin, Figure 6), lactones (plocabulin, PM060184, Figure 6), and macrocyclic lactone polyethers (spongistatin 1, Figure 6) were isolated.

It is worth noting that PM060184 is currently under clinical evaluation [168], while ado-trastuzumab emtansine was recently approved for adjuvant treatment of patients with HER2-positive early breast cancer [169] and was shown to prolong patient survival with a manageable safety profile [170].

The maytansine site is located in close vicinity to NBP and the vinca site, but is formed by other structures, including H3', H11, H11', and loops H11–H11', T3, and T5 (Figure 2) [171].

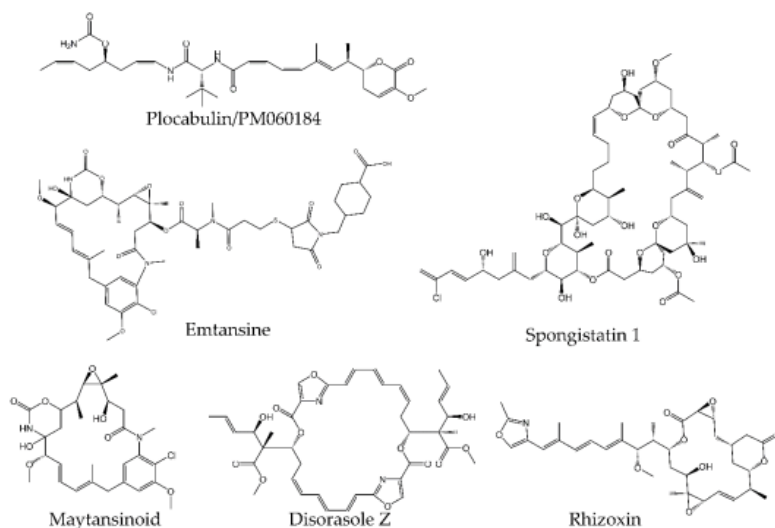


Figure 6. Structure of maytansinoid, disorazole Z, emtansine, rhizoxin, PM060184, and spongistatin 1.

The experimental evidence indicates that the inhibitory effect of maytansine-site ligands is a direct consequence of the occupation of the β -tubulin pocket. In growing microtubules, the maytansine-binding pocket of the MT plus end β -tubulin accommodates the minus end structures of the α -tubulin of a newly added heterodimer, including S8, H8, and loop H10–S9 [171]. Incorporation of maytansine-site ligands prevent this interaction, impeding MT elongation (Figure 2).

4.2.5. Colchicine Site

Colchicine (Figure 7) was isolated from autumn crocus *Colchicum autumnale*. This alkaloid contains three rings, of which rings A and C bind to β -tubulin, while aromatic ring B binds to α -tubulin [172]. Colchicine–tubulin binding is a slow, strongly temperature-dependent, and practically irreversible process [173]. Although colchicine has been used clinically in the treatment of nonneoplastic diseases (gout, familial Mediterranean fever), neither colchicine nor other related compounds were successful as chemotherapeutic agents owing to their severe toxicity to normal tissues at doses required for antitumor effects [174,175].

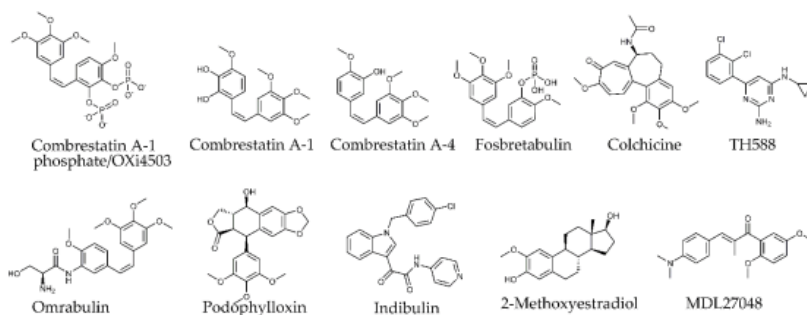


Figure 7. Structure of colchicine, combretastatin A-1, combretastatin A-4, OXi 4503, fosbretabulin, ombrabulin, 2-methoxyestradiol, chalcone: trans-1-(2,5-dimethoxy)-3-[4 (dimethylamino) phenyl]-2-methyl-2-propen-1-one (MDL 27048), podophyllotoxin, indibulin, and TH588.

Over the last few decades, compounds of low toxicity which target the colchicine site have been reported (Figure 7), including derivatives of stilbenoid–combretastatins (combretastatin A-1 phosphate/OXi4503, combretastatin A-1, combretastatin A-4, fosbretabulin, ombrabulin), chalcones (MDL 27048), compounds with furonaphthodioxole skeleton (podophyllotoxin), derivatives of indole (indibulin), and natural metabolite of estradiol (2-methoxyestradiol). While no colchicine-site MTAs are currently approved for cancer treatment, several are in phase I/III clinical trials [174] (for a review, see [175]).

Recently, a compound initially designed as MTH1 (Mut T homolog 1) inhibitor, TH588, was shown to dock into the colchicine-binding pocket [176]. By reducing microtubule plus end dynamics, this cyclopropyl analog affects tubulin polymerization, resulting in disruption of mitotic spindles, prolongation of mitosis and, eventually, apoptosis [176–178]. Preclinical studies show promising results for the use of TH588 as an anticancer drug [179,180].

The colchicine-binding site is located near the plus end surface of β -tubulin in the center of the tubulin heterodimer at the interface between α - and β -tubulin. It is a big pocket formed by the hydrophobic and polar residues of H7, H8, S7, S8, and loop H7–H8 (T7 loop) that can be divided into three zones: central zone 2 and two accessory zones, zone 1 facing α -tubulin and zone 3 buried deeper within the β -tubulin pocket [181,182].

Binding of colchicine-site ligands to heterodimer causes its stabilization in the curved conformation (Figure 2) [181]. As mentioned, during MT polymerization, tubulin dimers at the MT tip undergo a transition from curved to straight conformation, which requires a shift of several β -tubulin structures (S8–S9 and H8) closer to each other. As a result, the colchicine pocket is contracted [24,181]. While the colchicine pocket is occupied by a ligand, such conformational changes cannot occur, making colchicine ligand-bound heterodimer incompetent for polymerization [24,181].

4.2.6. Pironetin Site

Pironetin (Figure 8), a polyketide, is a natural product that was first extracted from fermentation broths of *Streptomyces* sp. [183,184]. It is worth noting that pironetin is, to date, the only known compound that exclusively targets the α -tubulin subunit and covalently binds to Cys316 of α -tubulin [185,186]. The molecule and its derivatives are currently under investigation and display promising anticancer properties (reviewed in [187]).

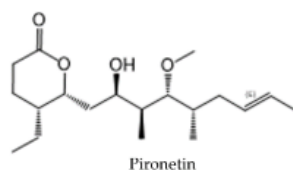


Figure 8. Structure of pironetin.

The pironetin-binding site is the only known pocket on α -tubulin targeted by MTAs. Its main part is formed by residues of S8, S10, and H7, but residues of S4, S5, and S6 also participate in the ligand accommodation [185]. The binding of pironetin leads to conformational changes of α -tubulin within the minus end, including disordering of loop H7–H8 (T7 loop) and part of H8 [185]. Since these structures are required for the formation of longitudinal interactions within protofilaments, it was proposed that pironetin prevents MT polymerization by the formation of assembly-incompetent pironetin-bound tubulin dimers (Figure 2) [185].

5. Factors Affecting Microtubule Dynamics in Cancer Cells

Carcinogenesis is a multistep process involving, among other actions, a remodeling of the cytoskeleton. The transformation from highly polarized epithelial cells to multipolar spindle-like metastatic cells that are able to detach from the extracellular matrix and migrate requires extensive reorganization of the cytoskeleton, including microtubules, during a process called epithelial–mesenchymal transition (EMT) (for a review, see [188]). The abnormalities of mitotic spindle and consequent aberrant cell cycle progression and division lead to genomic instability (for a review, see [189]). Thus, it is not surprising that numerous alterations in tubulins, including mutations and variations in isotype expression level, post-translational modifications, and MAP composition, were identified in cancer cells.

5.1. Tubulin Isoforms in Cancer and Anticancer Drug Resistance

Altered expression of tubulin isotypes is considered to be a hallmark in a range of cancers. Analysis of clinical specimens has shown that in many cancers, a high expression of several β -tubulin isotypes correlates with aggressive clinical behavior, chemotherapy drug resistance, and poor patient outcome [121]. Strikingly, little is known about the level of tubulin isoforms in primary nontreated cancers, while numerous studies indicate their variations after chemotherapy, especially after taxane-based treatment [121]. In fact, some data show that in cancer cell lines, paclitaxel can itself induce the expression of specific tubulin isoforms [190].

An increase in β I expression was observed in several cancers, including breast, colon, and kidney cancer, while its level was decreased in prostate cancer (Table 2) [43,191–193]. In the case of ovarian cancer, the data are inconsistent as both increased and invariant levels of β I expression were reported [43,191,194,195]. High β I expression is associated with the acquisition of chemoresistance to MTAs and poor prognosis in ovarian serous carcinoma and lung adenocarcinoma, but not in lung squamous cell carcinoma [191,193,196]. Interestingly, recent data show that experimentally lowering β I level by siRNA or mir-195 microRNA sensitizes adenocarcinoma cell lines to paclitaxel and eribuline [193], indicating a direct correlation between β I level and MTA resistance, at least in non-small-cell lung adenocarcinomas.

β IIa and b differ by only two amino acids and, thus, the expression of these two isotypes can be distinguished at the RNA level but not at the protein level. Using PCR, it was shown that β IIa is increased in NSCLC, prostate, and ovarian cancers, and decreased in kidney, colon, and breast cancers [43], while β IIb is increased in ovarian cancer and decreased in kidney, colon, and breast cancers [43]. Increased β IIb isoform was also recently associated with the metastatic stage of melanoma, indicating its role in EMT transition [197]. β II was also examined at the protein level in several cancer

types, including head and neck carcinomas (LASCCHN), ovarian carcinoma, colorectal cancer, and breast cancer cell lines [194,196,198].

Table 2. Tubulin isotypes as survival and MTA-resistance prognostic markers in cancer.

High Level of Tubulin Expression	Cancer	Prognosis	Resistance to	Reference
α Ia	renal	poor	n/a	[38]
α Ib	hepatocellular carcinoma	poor	paclitaxel	[199]
	renal, breast		n/a	[38]
α Ic	liver, renal, pancreatic, colon, breast, lung	poor	n/a	[38]
α IVa	liver	poor	n/a	[38]
β I	ovarian	not determined	paclitaxel	[191]
	breast	not determined	docetaxel	[200]
	NSCLC adenocarcinomas	poor	paclitaxel and eribulin	[193]
β II	breast	not determined	docetaxel	[196,201]
	lung adenocarcinoma cell line	not determined	paclitaxel	[191]
β IIa	urothelial	poor	n/a	[38]
	renal	good	n/a	[38]
β IIb	endometrial	poor	n/a	[38]
β III	prostate	poor	docetaxel	[202,203]
	colon	poor	paclitaxel	[204]
	bladder, cisplatin resistant	poor after paclitaxel chemotherapy	n/a	[205]
	gastric	poor	n/a	[206]
	gastric metastatic	poor after taxane chemotherapy	n/a	[207]
	uterine serous carcinoma	poor	paclitaxel, sensitivity to epothilone	[208]
	lung carcinoma cell line	n/a	epothilone	[209]
	NSCLC	poor	vinorelbine	[210]
	NSCLC stage III/IV	poor	vinorelbine	[211]
	NSCLC stage I/II	good after cisplatin/vinorelbine adjuvant chemotherapy	n/a	[212]
	ovarian	poor	n/a	[213]
	ovarian clear cell carcinoma	good after taxane based chemotherapy		[214]
	breast	poor	n/a	[215]
	breast	not determined	sensitivity to taxanes	[216] *
	metastatic breast	invariant	sensitivity to docetaxel treatment	[217]
	melanoma	difference not statistically significant	paclitaxel	[218]
β IVa	endometrial	poor	n/a	[38]
β IVb	liver	poor	n/a	[38]
	thyroid, endometrial	good	n/a	[38]
β V	renal, urothelial	poor	n/a	[38]
	NSCLC	good	good response to paclitaxel and vinorelbine	[219]
	breast	not determined	sensitivity to taxanes	[216] *

* only mRNA level was determined.

Similar to β I, in lung adenocarcinoma, breast cancer, and breast cancer cell lines, an increased level of β II was associated with resistance to MTA [191,196,201], while in LASCCHN, it was associated with poor survival after chemotherapy [198]. By contrast, in taxane-treated ovarian carcinomas, poor outcome is associated with a low level of β II [194]. Interestingly, silencing of β II by siRNA in NSCLC adenocarcinoma and large-cell carcinoma cell lines increases cell sensitivity to vinca alkaloids but not to paclitaxel treatment [220].

In a number of cancers (mainly of epithelial origin) β II was observed within the nucleus of both cancer cells and nontransformed cells in tissues adjacent to the cancer [221]. Nuclear localization of β II was recently associated with poor outcomes in colorectal cancer patients [222].

It was surprising when the neural tubulin isoform β III, which increases MT dynamics (see above), was discovered to be expressed in tumors with different origins. An analysis of the significant number of different types of tumors revealed that the contribution of β III to the total tubulin pool depended on the cancer type [223]. For example, in nearly 70–80% of the examined cases of small-cell lung cancer, mesothelioma, NSCLC, adenocarcinoma and large-cell cancer, neuroendocrine pancreatic cancer, malignant melanoma, and gallbladder carcinoma, β III was expressed at high levels [223]. By contrast, 70–95% of cases of breast cancer, colon adenoma, stomach cancer, basalioma, Warthin's tumor, and hepatocellular carcinoma were β III-negative [223].

In a wide range of tumors and cancer cell lines, including small-cell lung carcinoma, NSCLC, ovarian, prostate, bladder, uterine, upper gastrointestinal, colon, pancreatic, LASCCHN, and gastric cancer, β III upregulation is associated with the development of resistance to taxane-based chemotherapy and poor clinical outcome [121,200,202–208,210–213,215,223–233]. Moreover, an increased β III level was also shown to be associated with EMT and cell motility of colon cancer cell lines [234].

The role of the β III isoform in tumorigenesis was confirmed in a pancreatic cell line model. Silencing of β III expression by shRNA or mir-200c microRNA reduced cancer cell growth and tumorigenic potential both in vitro and in vivo in orthotopic and xenographic pancreatic cancer mouse models [190,230].

A high level of β III is generally believed to be a bad prognostic marker for MTA resistance and survival. However, it was recently shown that a paclitaxel-resistant NSCLC adenocarcinoma cell line with increased β III expression was sensitive to vinblastine and its analogs to the same extent as “parental” cells with low taxane resistance and lower β III expression [235]. This indicates that β III-induced taxane resistance may not influence resistance to other MTAs. In contrast to these observations, overexpression of β III-tubulin in ovarian clear cell adenocarcinoma is a predictor of a good response to taxane-based chemotherapy, and cases with higher β III-tubulin expression are associated with a significantly more favorable prognosis than those with lower β III-tubulin expression [214]. A similar observation was made for early stages (I/II) of breast cancer [212].

Altered expression of the β IV isotype was also reported in numerous cancers, including ovarian, lung, prostate, breast, and kidney cancers and breast and lung cancer cell lines [43,191,194,196,220]; the data, however, are frequently inconsistent. For example, some data indicate decreased β IV in lung and breast cancer [43], while studies on cancer cell lines indicate increased β IV expression, especially in taxane-resistant cell lines [191,196].

Similar to β II, β IVb overexpression is associated with resistance to vinca alkaloids rather than taxanes. In fact, siRNA knockdown of IVb β -tubulin expression in NSCLC and pancreatic ductal carcinoma cell lines increases the response to vinca alkaloids [220,236]. Interestingly, downregulation of β IVb was recently observed in an EMT-induced colon cancer cell line and transformation of epithelium-like to spindle-like cell morphology in these cells was reversed by β IVb overexpression [237].

The level of β V expression was tested on RNA and protein levels. The level of β V RNA was shown to be reduced in most tumors (colon, ovary, prostate, breast, lung) except for kidney [43], while β V protein level was shown to be elevated in lung, breast, and ovarian cancers and decreased in prostate cancer [238,239]. In NSCLC, a low level of β V-tubulin was associated with poor prognosis after paclitaxel-based chemotherapy [219].

Based on the available data, it appears that different β -tubulin isotypes play specific roles in the cancer cell response to extrinsic factors influencing MT dynamics. For example, in breast cancer patients with both β I and β III upregulation, response to taxane-based therapy was poor; in the group with a low level of both, the majority of patients responded well to the therapy, while in groups where one β -tubulin isotype high and another low, the response was intermediate [200]. Similar, in NSCLC patients with a low level of β V and high level of β III expression, the outcome was much worse than in

patients with high βV and low βIII , while patients with either a high or low level of both isotypes had an intermediate outcome [219].

Thus, the outcome of levels and ratios of particular β tubulin isotypes in terms of the progression of carcinogenesis appears to be specific to tumor type.

MTA resistance in cancer could also be related to mutations of β -tubulin. However, analyses of the clinical samples revealed that mutations in β -tubulin are either not present or very rare. Thus, it seems unlikely that mutations in β -tubulin could play an important role in drug resistance (reviewed in [240]).

Studies conducted on cell lines showed that mutations of predominating β -tubulin isotypes within the taxane-, colchicine-, and vinca-binding sites can be associated with altered MT dynamics and/or resistance to MTA (reviewed in [240–242]). Most β -tubulin mutations located within close proximity to the taxane-binding site did not change the affinity of tubulin to taxanes or epothilones, but probably destabilized MTs in the absence of any drugs [242]. Only mutation at F270V, T274I, and R282N residues were reported to have a direct effect on drug-binding affinity [242]. A similar effect was observed when point mutations were located in the M-loop (T274I, R282Q) or in helix H9, which is essential for interdimer interactions (Q292E) [242].

A recent study on a large number of samples of breast cancer tumors identified several mutations in βI , βIIa , and βIVb tubulins in which a gene-encoded residue was replaced by the amino acid present in the corresponding position in βIII [243]. It was proposed that such mutations could influence the clinical outcome in a similar manner as overexpression of βIII -tubulin [243].

5.2. Microtubule PTMs and Cancer

Changes in the level of tubulin modifications were linked to tumorigenesis (Table 3) [29,244]. Downregulation of TTL and increased α -tubulin detyrosination were reported during the epithelial–mesenchymal transition (EMT) that occurs during tumor invasion [245] in prostate cancer cells [246], in aggressive subtypes of breast cancer cells [247], and in primary neuroblastomas with poor prognosis [248]. The recent discovery of the vasohibin (VASH)/small vasohibin-binding protein (SVBP) complex, reported as a detyrosinating enzyme, tubulin carboxypeptidase (TCP) [249,250], provides new links between this tubulin modification and already known associations between vasohibin dysfunction and cancer [251,252].

A high $\Delta 2$ α -tubulin level in non-small-cell lung cancer (NSCLC) cells was associated with shorter overall patient survival and resistance to vinorelbine [211]. On the contrary, $\Delta 2$ α -tubulin was undetectable in prostate cancer cell lines (LNCaP and PC3), but was present in control cells [246].

Tubulin acetylation is associated with several types of cancer. An increased level of acetylation was reported in head and neck squamous cell carcinoma, for which it can be used as a prognostic marker [253]. An elevated level of tubulin acetylation in breast cancer cell line (MCF-7) is associated with the development of colchicine-resistance [254]. Additionally, a higher level of acetylated tubulin in primary breast tumors is linked to the basal-like subtype of breast cancer, in which it promotes adhesion and invasion of breast cancer cells, increasing the risk of disease progression and death [255]. Overexpression of ATAT1 in cultured nonmetastatic lines of breast cancer cells promoted the formation of microtubule-based membrane protrusions, structures characteristic of metastasis [255]. The level of microtubule acetylation was also shown to affect epithelial–mesenchymal transition and cell polarity [256].

Recent studies provide evidence that phosphorylation of serine 21 of HDAC6 by G protein-coupled receptor kinase 5 (GRK5) promotes deacetylase activity in ovarian (HeLa) and breast (MDA MB 231) cancer cell lines. An increased level of acetylated α -tubulin sensitizes these cells to the anti-apoptotic activity of paclitaxel [257]. The high expression of HDAC6 was also linked to poor prognosis of oral squamous cell carcinoma (OSCC) [258], oncogenic transformation [259], and EMT [260].

Table 3. Alterations in tubulin post-translational modifications (PTMs) in cancer.

PMT	Changes	Cancer	Outcome	Reference
α -, β III-, β IV-tubulin tyrosination α -tubulin detyrosination	elevated level	breast cancer cell lines	paclitaxel resistance	[261]
	TTL down regulation	breast cancer lines,	increasing metastasis and tumor aggressiveness	[245]
		non-epithelial tumor of different origin	tumor growth correlates with loss of TTL activity	[262]
		primary neuroblastomas	impaired neuronal differentiation and poor prognosis	[248]
$\Delta 2$ α -tubulin	elevated level	prostate cancer cell lines	n/a	[246]
		non-small-cell lung cancer	poor outcome, vinorelbine resistance	[211]
		breast cancers	high aggressiveness and poor prognosis	[247]
$\Delta 2$ β IVb-tubulin		hepatic carcinoma (rat)	increased in cancer with respect to healthy liver	[263]
α -tubulin acetylation	HDAC6 knockdown	ovarian, breast epidermoid carcinoma cell lines	mitotic arrest, and cell death	[259]
	HDAC6 inhibition	nsclc cell lines	marker of better prognosis	[264]
	HDAC6 overexpression	breast cancer	good prognosis	[265]
		oral squamous cell carcinoma	correlates with tumor stage	[258]
	MEC-17 overexpression	lung cancer animal model	cancer cells migration and facilitated invasiveness	[256]
	MEC-17 downregulation		higher tumor grade	[256]
	elevated level of tubulin acetylation	head and neck cancer	correlates with tumor grade	[253]
		primary breast tumors	correlates with metastatic phenotype	[255]
Glutamylation	elevated levels	breast cancer cell lines	colchicine-resistance	[254]
		ovarian and breast cancer cell lines	paclitaxel sensitivity	[257]
		prostate cancer cells	n/a	[246]
Glycylation	TTLL3 downregulation	breast cancer cell lines	colchicine-resistance	[254]
		colorectal cancer	risk factor for carcinoma development	[266]
Phosphorylation of α -tubulin (Ser 165)	dephosphorylated (S165D) α -tubulin	breast cancer cell lines	hyperproliferation and increased metastatic potential	[267]

Phosphorylation of α -tubulin at Ser 165 residue by protein kinase C, in turn, stimulates microtubule dynamics in human breast cancer cells [78,79,267]. It seems that phosphorylation of α -tubulin at Ser 165 can act as a switch that controls the expression of EMT markers in nontransformed human breast cells and the rate of proliferation of breast tumors [79,267].

A few studies suggested that changes in the levels of tubulin glutamylation [246] and glycylation [266] are observed during tumorigenesis. Some unusual post-translational modifications have been detected in lung and hepatic cancers. The removal of the final two residues of the β IVb-tubulin C-terminal tail was identified in more advanced stages of liver cancer and metastasis to lung in a rat model of hepatic carcinoma [263].

5.3. Microtubule-Associated Proteins and Cancer

The data concerning MAPs in cancer are limited. Up to now, only a few MAPs, including Stathmin-1, EB1, CLIP-170/CLIP1, and some structural MAPs, have been associated with cancerogenesis, outcome prognosis, and chemotherapy sensitivity [268–271].

As already mentioned, Stathmin-1 was originally identified as an oncoprotein. Elevated levels of Stathmin-1 is a poor prognostic factor in many cancers, including leukemia, prostate, breast, lung, ovarian, cervical, endometrial, oral nasopharyngeal gastric, and colorectal cancers [272–280].

Some studies suggested that the silencing of the Stathmin-1-encoding gene can inhibit cancer cell migration and metastatic potential [281]. The data concerning correlation of the level of Stathmin-1 expression and resistance of cancer to chemotherapy are contradictory. Several studies show that Stathmin-1 overexpression increases the sensitivity of breast and lung cancer cells to taxanes and/or vinca alkaloids [282,283]. However, in epithelial carcinomas, nasopharyngeal carcinomas, breast cancer, and esophageal squamous cell cancer, the increased taxane sensitivity was correlated with Stathmin-1 silencing [284–288]. Interestingly, not only the protein level but also its phosphorylation state was correlated with cancerogenesis and drug resistance [289].

Increased levels of EB1 and CLIP-170/CLIP1, two +TIP proteins, enhances paclitaxel sensitivity in breast cancer cell lines and the response to taxane-containing therapy in patients [290,291]. On the other hand, a decrease of CLIP-170/CLIP1 expression correlates with patients survival in the case of glioma [292].

Structural MAPs have also been related with carcinogenesis. The expression of neuronal MAPs, Tau, MAP2, and MAP4 was detected in non-neuronal cancer tissues. For example, Tau overexpression observed in breast and ovarian cancer cells was correlated with a poor outcome [293–295], while downregulation of Tau in breast and ovarian cancer cell lines increased sensitivity to paclitaxel [293,295]. Because Tau and taxanes bind to the same tubulin surface, it was proposed that Tau may compete with paclitaxel for binding to β -tubulin, causing taxane ineffectiveness [293,295]. On the other hand, in mice, docetaxel-sensitive pancreatic neoplasms show a higher level of Tau and MAP2 with respect to those that are docetaxel-resistant [296,297].

MAP2 was proposed as a diagnostic marker in pulmonary neuroendocrine carcinomas, some non-small-cell lung carcinomas [298], Merkel cell carcinomas [299], and oral squamous cell carcinoma [300], but not in metastatic melanomas (while abundant in primary melanomas) [301]. Overexpression of MAP2 in melanoma cell lines leads to microtubule stabilization, associated with G2–M phase cell cycle arrest, growth inhibition, and cancer cell apoptosis, both in vitro and in a nude mouse model [301,302]. Decrease of MAP2c accompanied with a decrease in β III-tubulin expression was also observed in vinca-resistant neuroblastoma cell lines [303].

Recently, also, MAP1B was shown to be expressed and a marker of a poor outcome in urothelial cancer [270]. Silencing of MAP1B in urothelial cancer cell lines decreased the cell migration and invasiveness [270].

An elevated level of MAP4 and resistance to vinca alkaloids have been observed in childhood acute lymphoblastic leukemia (ALL) cells [304], while leukemia cell lines resistant to the epothilone and hypersensitive to microtubule-destabilizing agents increased the levels of both MAP4 and β III tubulin [305]. In esophageal squamous cell carcinoma, an increased level of MAP4 was shown to be a poor outcome marker, and its intratumor silencing inhibited cell growth in nude mice [306]. Very similar observations were also made in lung adenocarcinoma [307].

An increased level of MAP7 is a marker of poor prognosis in leukemia and cervical cancers [269,308,309]. Moreover, it was shown that MAP7 promotes migration and invasiveness of cervical cancer cell lines by inducing EMT transition [309].

6. Conclusions and Perspectives

Microtubule dynamics play a key role in the proper execution of cell division. Thus, it is not surprising that a large number of MTAs have found application as clinical drugs against different types of cancers. Unfortunately, MTAs are also toxic to healthy tissues. Therefore, reducing the toxicity of anticancer MTAs and understanding the causes of cancer cell resistance are extremely important. The main direction of research worldwide includes: (i) a comprehensive understanding of the tubulin code in cancer cells and the selective manipulation of tubulin isotype expression [121], (ii) an improvement of the potency of drugs and increased tumor specificity [18], (iii) combination therapy, with nanoparticles and anticancer drugs working synergistically to delay the onset of drug

resistance [310], and (iv) the use of antibody–drug conjugates (ADCs) as a potent class of anticancer therapeutics that confer selective and sustained cytotoxic drug delivery to tumor cells.

Author Contributions: All authors searched and organized data concerning selected chapter. H.F. and E.J. prepared initial version of the manuscript, F.B. and E.J. also prepared figures. All authors worked on the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

Acknowledgments: We thank Dorota Włoga for a critical reading of the manuscript. This work implemented as a part of Operational Project Knowledge Education Development 2014–2020 co-financed by European Social Fund, and was also supported from National Science Centre, Poland, Opus15, 2018/29/B/NZ3/02443 to E.J. and statutory funds from the Nencki Institute of Experimental Biology of the Polish Academy of Sciences.

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

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Review

Development of novel derivatives of stilbene and macrocyclic compounds as potent of *anti*-microtubule factorsFilip Borys^{a,b}, Piotr Tobiasz^a, Marcin Poterała^a, Hanna Krawczyk^{a,*}^a Department of Organic Chemistry, Faculty of Chemistry, Warsaw University of Technology, Noakowskiego 3, 00-664, Warsaw, Poland^b The Nencki Institute of Experimental Biology Polish Academy of Sciences, Poland

ARTICLE INFO

Keywords:

New tubulin inhibitors
Small molecules
Photoswitchable inhibitors
Macromolecules inhibitors

ABSTRACT

Microtubules (composed of α - and β -tubulin heterodimers) ubiquitous cellular polymers are important components of the cytoskeleton and play diverse roles within the cell, such as maintenance of cell structure, protein trafficking or chromosomal segregation during cell division. The polymers of tubulin play a pivotal role in mitosis and are regarded as an excellent target for chemotherapeutic agents to treat cancer. This review presents a brief overview of the synthesis and mechanism of action of new compounds targeting the dynamic of microtubule – tubulin polymerization/depolymerization. It is divided into the following parts: section I concerns targeting microtubules- tubulin-binding drugs derivatives of stilbene. In section II there are presented photoswitchable inhibitors of microtubule dynamics. Section III concerns using macrocyclic compounds as tubulin inhibitors. In this review, the authors focused primarily on reports produced in the last five years and the latest strategies in this field.

1. Introduction

Microtubules ubiquitous cellular polymers are important components of the cytoskeleton and play diverse roles within a cell such as maintenance of cell structure, protein trafficking or chromosomal segregation during mitosis [1,2]. These are cylindrical polymers composed of α - and β -tubulin heterodimers (subunits). The dimensions of a heterodimer are: 4 nm x 5 nm x 8 nm and its molecular weight is about 100 kDa. These subunits associate longitudinally to form protofilaments and the protofilaments associate laterally to generate a microtubule. As a result of the association, most often of 13 protofilaments, a microtubule forms hollow tubes (hollow rods) whose length reaches several micrometres [3]. The result of this type of polymerization is that the two ends differ in their kinetics of attachment. At the so-called "plus" end, polymerization and depolymerization are faster than the "minus" end [4]. The ends of microtubules undergo rapid lengthening and shortening by the addition and removal of tubulin heterodimers, a phenomenon termed 'dynamic instability' (Scheme 1). Dynamic instability results from the hydrolysis of GTP bound to β -tubulin during or shortly after polymerization, which reduces its binding affinity for adjacent molecules. Growth of microtubules continues as long as there is a high concentration of tubulin bound to GTP.

New GTP-bound tubulin molecules are then added more rapidly than GTP is hydrolyzed, so a GTP cap is retained at the growing end. However, if GTP is hydrolyzed more rapidly than new subunits are added, the presence of GDP-bound tubulin at the end of the microtubule leads to disassembly and shrinkage. Microtubules play a pivotal role in cell division and are regarded as an excellent target for chemotherapeutic agents to treat cancer.

The compounds that target microtubules and disrupt the normal function of the mitotic spindle have proven to be one of the best class of cancer chemotherapeutic drugs available in clinics to date. This group includes tubulin-binding agents, containing the *Vinca* alkaloids (e.g., vinblastine (1) and vincristine (2), used to treat breast cancer, lymphomas, sarcomas or vinorelbine (3) administered in breast, lung cancer, sarcomas) interfering with microtubule structure as destabilizers, and the taxanes (e.g., paclitaxel (4) and docetaxel (5) treating ovarian, breast cancer, lung cancer and many others) which promote microtubule stabilization preventing their disassembly (Fig. 1). Because these compounds have been widely discussed in the literature [5–9] we focused primarily on the last five years of research and the latest strategies in this field [10–14]. We concentrated on novel derivatives as potent of microtubule-targeting agents (MTAs), on azo compounds and investigation of photopharmacology and macrocyclic compounds and their

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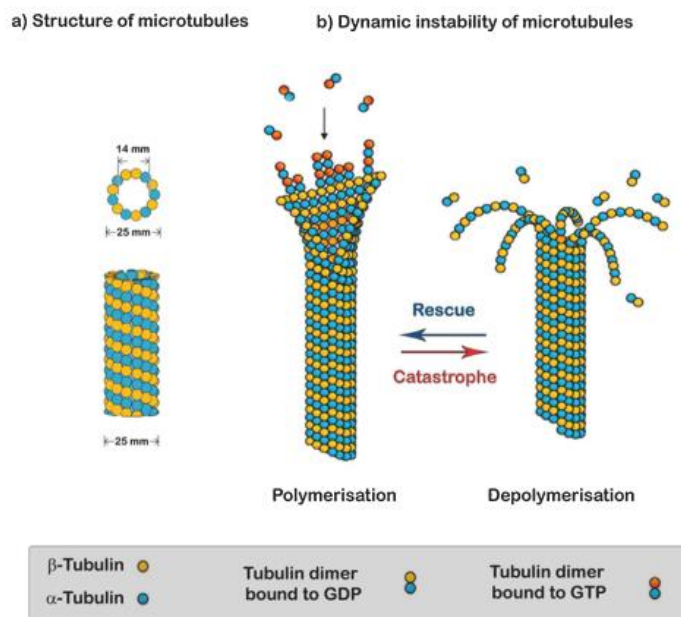
Received 2 September 2020; Received in revised form 27 October 2020; Accepted 1 November 2020

Available online 2 December 2020

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Scheme 1. a) Structure and b) dynamic instability of microtubules.

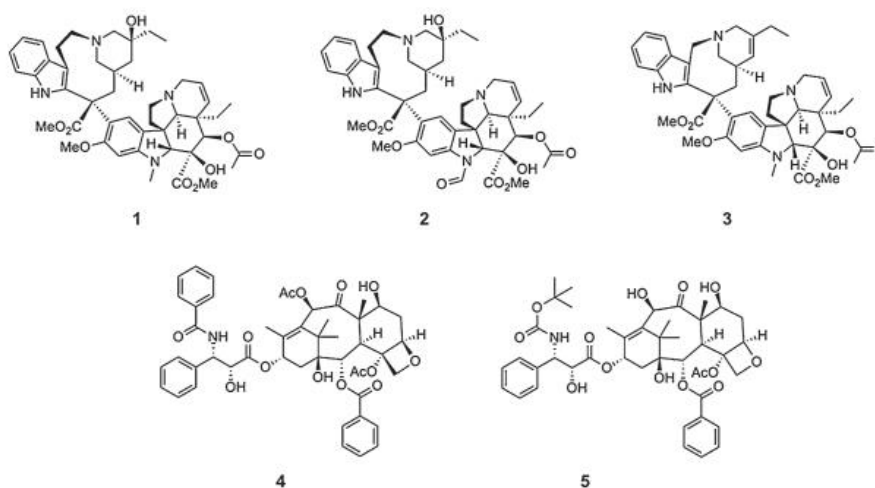


Fig. 1. The chemotherapeutic drugs: vinblastine (1), vincristine (2), vinorelbine (3), paclitaxel (4) and docetaxel (5).

activity as MTAs.

2. Targeting microtubules- tubulin-binding drugs derivatives of stilbene

Microtubule-targeting agents, constitute a various group of molecules that bind to microtubules and affect their properties. Two general classifications of substances interacting with microtubules are

distinguished. One of them was created based on the binding site of a given compound to a specific region of a heterodimer. The tubulin-binding sites for microtubule targeting agents are: taxanes site, laulimalide /peloruside site and *vinca* alkaloids site, colchicine site, maytansine site and pironetin site [9]. Considering the mechanism of action, there are two major types of microtubule targeting agents; a) microtubule stabilizers (stabilizing binders - "microtubule-stabilizing" - increasing the microtubular mass)) - compounds bonding to sites of

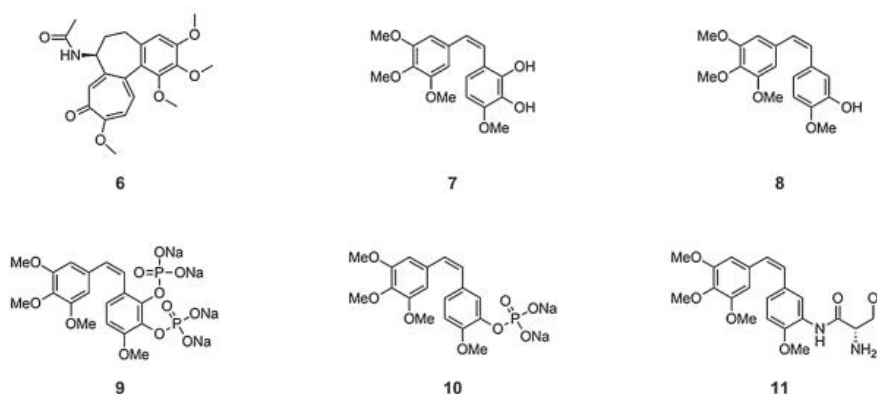


Fig. 2. The tubulin inhibitors targeting the colchicine binding site: colchicine (6), combretastatin A-1 (7), combretastatin A-4 (8), Oxi4503 (9), Fosbretabulin (10) and Ombrabulin (11).

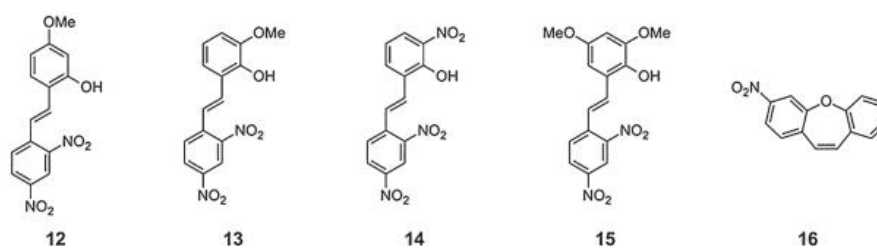


Fig. 3. The biologically active stilbene (12-15) and dibenzo[b,f]oxepin derivatives (16).

taxanes and of laulimalide/peloruside, and b) microtubule destabilizers (destabilizing binders)- compounds bonding to sites of *Vinca* alkaloids, colchicine, maytansine and pironetin [15]. It should be noted that both types of MTAs inhibit the dynamic instability of tubulin polymerization. There are some marked tubulin inhibitors - paclitaxel/taxol (4) [16], vinblastine (1) [17] and vincristine (2) [18]. They interact with tubulin with the taxane or vinca alkaloid binding sites. However, currently there have not yet been approved drugs of tubulin inhibitors targeting the

colchicine (6) binding site (Fig. 2). Such compounds e.g. combretastatins analogs (Fig. 2): combretastatin A-1 (CA1, 7) and combretastatin A-4 (8) and their three prodrugs of monosodium phosphate salt two CA-1 P (Oxi4503, 9) [19] and CA4P (Fosbretabulin, 10) [20] or amino derivative AVE8062 (Ombrabulin, 11) [21] have been extensively studied [19-22]. They can be transformed into the active components CA1 and CA4 *in vivo* [23], and have therapeutic advantages over taxanes and vinca alkaloids. They can be administered orally, have less

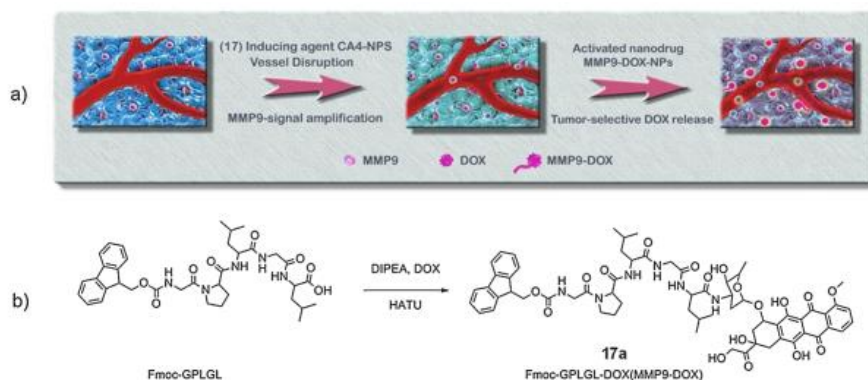


Fig. 4. a) Connecting combretastatin A4 nanodrug with MMP9-activated doxorubicin prodrug MMP9-DOX-NPs - nanodrug CA4-NPs (PLG-CA4, 17); b) Synthesis of Fmoc-GPLGL-DOX (MMP9-DOX, 17a) (according to [35]).

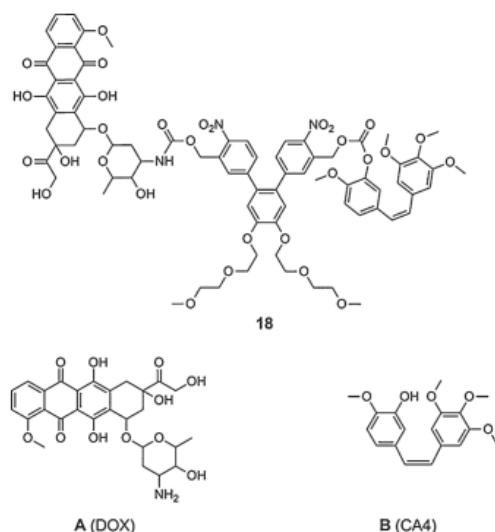


Fig. 5. The structure of photoresponsive hybrid prodrug (**18**) with which DOX part **A** and CA4 part **B** were covalently bonded.

drug-drug interaction potential, and are less prone to develop multi-drug resistance [6]. Some studies have been suspended due to lack of effects [24] or withdrawn due to the expected inability to recruit study participants in a reasonable amount of time [25] or due to limited funding [26]. Currently, only one research of OXi4503 is included in clinical trials - in phase 1. It focuses on the maximum tolerated dose of OXi4503 as a single agent and in combination with intermediate-dose cytarabine in subjects with relapsed/refractory Acute Myelogenous Leukemia or Myelodysplastic Syndromes [27].

Krawczyk et al. conducted a study concerning the synthesis and search for biologically active stilbene [28] and dibenzo[b,f]oxepin [29] derivatives. Dibenzo[b,f]oxepines have in their skeleton a (Z)-stilbene motif (like combretastatins). Moreover, dibenzo[b,f]oxepine is an important scaffold in medicinal chemistry. Its derivatives exhibit *anti*-depressive [30], *antipsychotic* [31], *anti*-estrogenic [32], *antitumor* [33] and *anti*-inflammatory [34] properties. The authors examined substituted stilbenes and discovered that compound (**12**) (Fig. 3) is effective against glioblastoma U87 cells with IC_{50} of 48.93 μ M after 48 h.

For active compounds, IC_{50} values were the lowest after 48 h of Cervical cancer HeLa treatment for four stilbenes (**12**–**15**) and one dibenzo[b,f]oxepin (**16**) and amounted 3.50, 17.18, 6.97, 17.05 and 18.54 μ M, respectively. It is currently unclear if these compounds bind tubulin, because only molecular docking studies were performed. The interaction of (**12**) - (**16**) at colchicine binding site can be observed. There are also promising studies on different forms of combretastatins e.

g. nanodrug (CA4-NPs, PLG-CA4, **17**, Fig. 4) [35]. This compound was built of a poly (L-glutamic acid) and combretastatin-A4 and its therapeutic effect was examined and compared with commercial combretastatin-A4 phosphate (CA4P) in a murine colon C26 tumor. PLG-CA4 showed significantly prolonged retention in plasma and tumor tissue.

The authors observed that the PLG-CA4 was mainly distributed around the tumor vessels and that its tissue penetration in solid tumors was low. Tumor suppression tests indicated that PLG-CA4 treatment resulted in a tumor suppression rate of 74 % and the rate of the CA4P group was 24 %. The authors also developed methods for the sequential delivery of CA4-NPs and MMP9-DOX-NPs (**17a**, matrix metalloproteinase 9 (typical tumor-associated enzyme)- activated doxorubicin prodrug, Fig. 4a) to enhance tumor-selective drug release in cancer therapy and which exhibits enhanced antitumor efficacy with reduced systemic toxicity compared with the noncooperative controls [36]. The MMP9-activated doxorubicin prodrug (Fmoc-GPLGLDOX, MMP9-DOX) was synthesized by conjugation of doxorubicin to Fmoc-GPLGL (Fig. 4b). MMP9-DOX was then encapsulated in methoxy poly (ethylene glycol)-b-poly(L-glutamic acid-co-L-phenylalanine) nanoparticles to yield MMP9-DOX-NPs. It was demonstrated that the combination of CA4-NPs MMP9-DOX-NPs exhibited superior anticancer efficacy compared with monotherapy. Recent studies of combretastatins focus on the combination of photoresponsive hybrid prodrug (**18**) that contains both doxorubicin (DOX, part **A**, Fig. 5) and combretastatin A4 (CA4, part **B**, Fig. 5) [37]. This is in order to apply photoremovable protecting groups (PPGs) in the field of combination chemotherapy.

When a sequential light stimulation strategy was applied for hybrid by 405 nm light for DOX and by 365 nm light for CA4 there was observed triggered drug release. Prodrug (**18**) improved exhibited significant cytotoxicity (cell viability assay) for MDA-MB-231 cells (human breast adenocarcinoma) compared with DOX and CA4 alone treated groups in both irradiated and nonirradiated conditions.

The search for tubulin inhibitors has also been conducted with derivatives of resveratrol, which is one of the most widely studied bioactive plant polyphenols having *anti*-cancer properties. The authors [38] showed that the derivative of resveratrol (**19**) (Fig. 6), induced cytotoxicity in several cancer cell lines *ex vivo* with an IC_{50} value of 3–5 μ M. Investigation of the effect of (**19**) in non-small cell lung cancer cell line (A549) and T-cell leukemic cell line (CEM) demonstrated that it inhibited tubulin polymerization during cell division causing cell cycle arrest at G2/M phase of the cell cycle in 12–18 h period. Besides, immunofluorescence studies proved mitosis arrest after treatment (**19**) and indicated that this molecule binds to tubulin with a dissociation constant of $0.414 \pm 0.11 \mu$ M. In turn, docking studies confirmed that (**19**) interacts with tubulin in colchicine binding site. Afifi et al. [39] presented a report regarding the development of a novel and primary model of synthesized sulfonamide compounds encompassing a chromene azo motif with the intent of becoming applicable for drug candidates in the cases of drug-resistant pathogens. The obtained molecules (**20**) and (**21**) (Fig. 6) were evaluated for their antibacterial and antifungal activities and their cytotoxic screening was performed against three cancer cell lines: human colon cancer cell line, human liver cancer

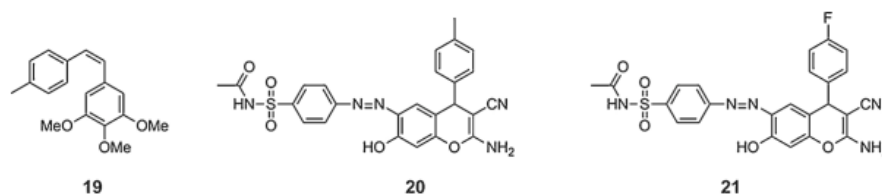


Fig. 6. The structures of derivative of resveratrol (**19**) and azosulfonamide molecules (**20**) and (**21**) (destabilizing binders- compounds bonding to sites of colchicine).

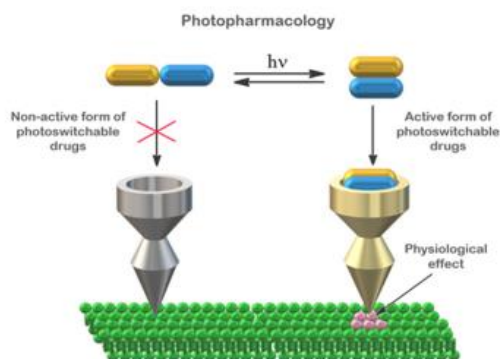


Fig. 7. The idea of the light-controlled photopharmacology- photoswitchable drugs that can be reversibly activated with light for interaction with their target receptors or enzymes.

cell line and breast cancer cell line. The incorporation of the chromene moieties into the azosulfonamide molecules improved cytotoxicity against their corresponding precursors. Some of these 4H-chromene/sulfonamide derivatives displayed potent antiproliferative activities, because they were more effective towards the examined tumor cell lines in comparison with the doxorubicin reference drug. Authors also studied the influence of two chosen derivatives (20) and (21) (Fig. 6) on the tubulin polymerization, using the HCT-116 cancer cell line. The results indicated the effect on the inhibition of the tubulin polymerization in comparison to colchicine (compound 20 7.18 μM , compound 21 8.84 μM , colchicine 0.16 μM). As we can observe from the data, the derivative (20) with an electron-donating substituent in phenyl ring (4- CH_3) demonstrated more activity in the selective comparison with (21) with an electronwithdrawing group in phenyl ring (4-F) for the inhibition of the tubulin polymerization.

3. Photoswitchable inhibitors of microtubule dynamics

Presently known inhibitors are nonspecific in the sense that their bioactivity can be neither spatially nor temporally directed, e.g. against selected cells and tissues, at defined periods. This restricts their range of application in studies because it prevents their use in spatially or temporally addressing the varied processes dependent on microtubule dynamics. In the treatment of cancer, the time and spatial nonspecificity cause severe systemic side effects such as cardiotoxicity and neurotoxicity [40], which limit the doses at which chemotherapeutics can be applied [41]. Photopharmacology [42] is a novel developing method,

not yet at the stage of clinical development. It has a potential of application in medicine because it could lead to the photo controlled, reversible and selective addressing of targets in the human body by responsive small components – drugs (by modifying their structure upon irradiation with light). Photopharmacological agents are bioactive molecules modified with photoswitches, that is, scaffolds that change (Fig. 7).

Photoswitchable inhibitors of microtubule polymerization have recently been synthesized and studied [43–46]. An interesting novel investigation of spatial and time-controlled compounds was presented by Borowiak et al. [43] They designed and synthesized the photostatins (22, Fig. 8) as reversibly photoswitchable analogs of combretastatin A-4 (CA-4). Because active combretastatins have the *Z*-(cis) configuration and *E*-(trans) CA-4 is several orders of magnitude less potent than the *Z* isomer [47,48], they replaced the bridging $\text{C}=\text{C}$ double bond of the stilbenoid combretastatins with an isosteric $\text{N}=\text{N}$ double bond to give the azobenzenes (22).

The compound (22) can be *trans*-/cis photoisomerized with full reversibility and excellent photostability over many cycles by low-intensity visible light. The switching of the (22) was demonstrated by UV-vis spectroscopy: 380–420 nm light results in approximately 90 % *Z* isomer. Longer wavelengths cause decreasing *Z* percentages until 500–530 nm gives approximately 85 % *E* isomer. Spontaneous (in the dark) isomerization leads to 100 % *E* isomer, with half-lives from 0.8 to 120 min for different compounds (22). Next, the authors studied influences of photoswitchable compounds on microtubule. Obtained molecules modulate microtubule dynamics with subsecond response time and control mitosis in living organisms with single-cell spatial precision. In conclusion, the compounds (22) are up to 250 times more cytotoxic when lit with blue light than when kept in the dark in applications in cell culture and are promising as a new class of precision chemotherapeutics whose toxicity may be spatiotemporally constrained using light. In other studies, authors [44] reported azo analog of combretastatin CA4 (22) and included data for *in vitro* and HeLa MTT assays, which showed increased cytotoxicity of (22), like the cytotoxicity observed for pure combretastatin-A4, in the presence of light. In turn, authors [45] discovered that *Z*-(22) azo compound was with ease reduced with glutathione, which can be a potent problem for biomedical applications. However, the biological results of the cytotoxicity of (22) in human umbilical vein endothelial cells and adenocarcinoma epithelial cells fit with other reported findings [43,44]. Rastogi et al. [46] have presented a study about combretastatin analogs in which an azo group was incorporated into a molecule containing the key pharmacophore of colchicine. They have performed cell assays on obtained new compounds (Fig. 8) structurally related to the previously reported azo-analog of combretastatin [43–46]. One of these compounds (23) showed enhanced potency against HeLa ($\text{IC}_{50} = 0.11 \mu\text{M}$) and H157 cells ($\text{IC}_{50} = 0.20 \mu\text{M}$) for cell studies under 400 nm irradiation and the highest photoactivity (ratio: IC_{50} with irradiation / IC_{50} in dark = 550).

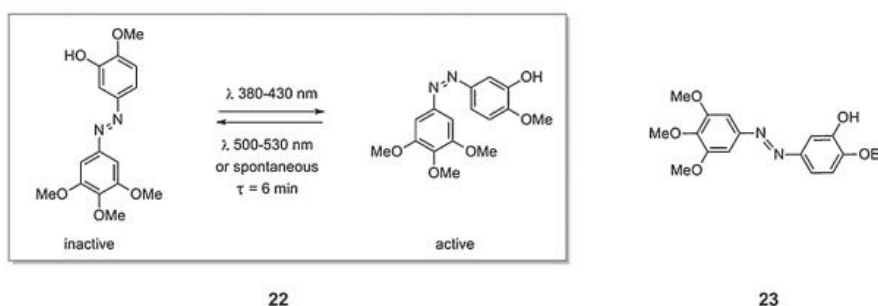


Fig. 8. The structure of analogs of combretastatin A-4 (22) and (23) (colchicine binding site).

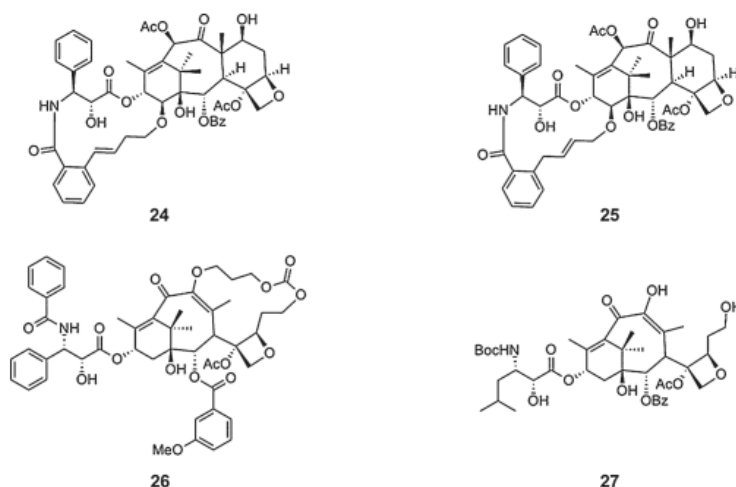


Fig. 9. Novel macrocyclic paclitaxel derivatives (24), (25) and novel 7,9-O-linked macrocyclic taxoids (26) and mimetic of IDN5390 (27).

For this compound, authors performed physicochemical and docking studies to investigate ligand binding simulations for the tubulin-combretastatin complex. Kinetic investigations in water reveal a longer half-life for the *Z* isomer of (23) which may be the factor responsible for the better IC_{50} values in cell assays and the improved photoresponsive behavior. Basing on docking, it was calculated that the azo compound (23) has nearly identical binding conformations and closely overlaps the X-ray binding structure of combretastatin. One difference between combretastatin A4 and azo (23) is the orientation of the para-methoxy group on the A-ring. The calculated binding affinity obtained for (23) was -7.6 kcal/mol and was similar to combretastatin and colchicine [46]. The docking calculation was performed for other, less active compounds and binding energies do not indicate a significant difference. In conclusion, compounds (22), (23) and synthesis of the azo-CA4 analogs represent a promising strategy for switchable potency for the treatment of cancer. However, it should be emphasized that although this novel developing method is attractive, there are still several limitations with the current photoactivatable MT targeting drugs. The main problem is that the lower energy photons from the UV/Vis range are prone to both scattering in tissue and absorption by endogenous chromophores or they contribute to the photodamage of cells. Feringa et al. [42] presented in recent excellent critical review of the different pharmacological targets (i.e. diseases/organs) in the human body, the prospects for delivering light to these organs, their specific requirements for the molecular design of a photoactivatable drug, and whether and how these challenges can be addressed.

4. Macrocyclic compounds as tubulin inhibitors

In this part, we will concentrate on macrocyclic compounds that are also tubulin-binding agents. Paclitaxel (4) [16] is a macrocyclic compound and chemotherapy medication used to treat various kinds of types of cancer. Novel macrocyclic paclitaxel derivatives were designed to imitate the bioactive conformation of paclitaxel (24, 25) [49] (Fig. 9). Studies revealed that (24) induced tubulin polymerization in the absence of G.

TP in a similar way to that of paclitaxel. The microtubules formed with (24) as well as paclitaxel were stable against Ca^{2+} -induced depolymerization. The compound (25) also exhibited the same or slightly better activity than that of paclitaxel in the same assay (Table 1).

Table 1
Cytotoxicity of the (24), of the (25) and of the paclitaxel (4) against the five cancer cell lines.

Compounds	Five Cancer line - IC_{50} (nM)				
	ovarian cancer		breast cancer		colon carcinoma
	NCI/ADR	A2780	MCF-7	LCC6-WT	HT-29
(24)	183	31	3.49	2.09	17
(25)	592	114	12.3	12.2	29.2
(4)	395	36.1	1.85	2.45	7.28

This result also supports the authors' observation that (24) and (25) are closely mimicking paclitaxel's bioactive conformation. Evaluation of the biological activities of (24, 25) novel macrocyclic taxoids has revealed that their potency is highly sensitive to the small conformational differences as well as the rigidity of the compounds. Interesting compounds of novel 7,9-O-linked macrocyclic taxoids (e.g. 26), mimetic of IDN5390 (27) (C-seco taxane more active than paclitaxel in several paclitaxel-resistant human ovarian adenocarcinoma cell lines e.g., A2780TC1, A2780TC3, and OVCAR3, expressing high β III-tubulin and P-gp levels) [50], together with modification at the C2 position were synthesized. Moreover, their cytotoxicities against drug-sensitive, P-glycoprotein and β III-tubulin overexpressed drug-resistant cancer cell lines, were evaluated. This study demonstrated that C-seco taxoids showed increased cytotoxicity on drug-resistant tumors overexpressing both III β III-tubulin and P-glycoprotein. In 1988 seventeen-member macrocyclic lactone- combretastatins D1 (28), D2 (29), D3 (30) and D4 (31) (Fig. 10) were discovered [51,52]. Cytotoxicity study (using mouse leukemia cells) which has been reported, showed modest activity (ED_{50} 3–5 μ g ml $^{-1}$) for these compounds [53].

Contrary to the cancer cell growth inhibitory activity exhibited by combretastatin D1, D2, relatively minor structural modifications also caused the elimination of those properties. These groups of cyclic combretastatins, although intensively studied by the group of Pettit in series of "Antineoplastic Agents" [50–53] are less potent as tubulin polymerization inhibitors than the parent CA-4. Another new family of macrocyclic derivatives of combretastatins B (32) (Fig. 10) connecting oxygenated substituents on the phenyl rings and indole rings is described [54]. The tubulin polymerization activity for this new class of

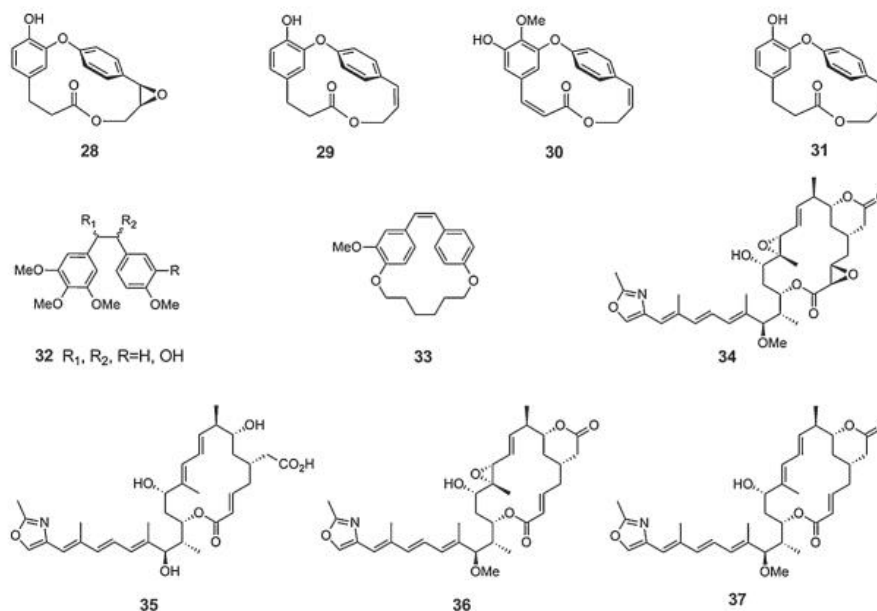


Fig. 10. The structure of combretastatins D1 (28), D2 (29), D3 (30), D4 (31), the derivative of combretastatin B (32), (33), and of rhizoxin (34) and its analogs (35), (36) and (37) (destabilizing binders- compounds bonding to sites of colchicine).

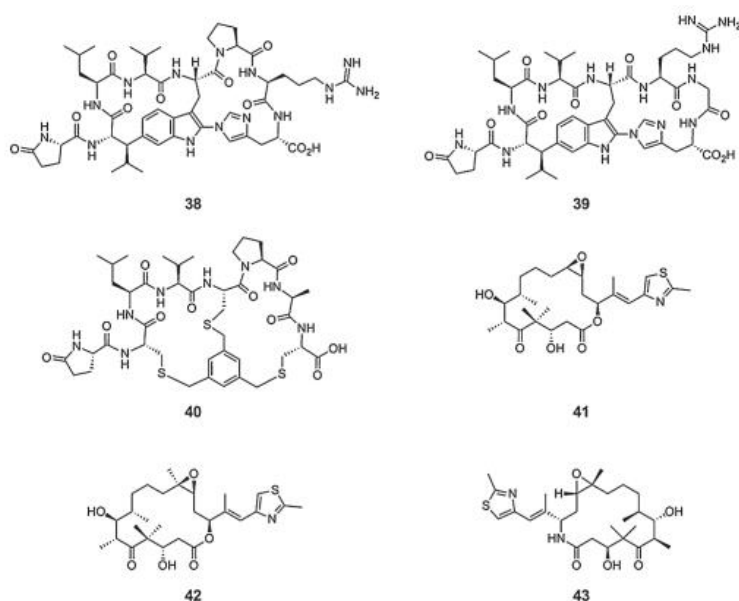


Fig. 11. The celogentin/moroidin family: (38), (39), (40) (compounds bonding probably to colchicine binding site) and the structure of epothilone A (41), epothilone B (42), and ixabepilone (43) (microtubule-stabilizing agents, compounds bonding to site of taxane).

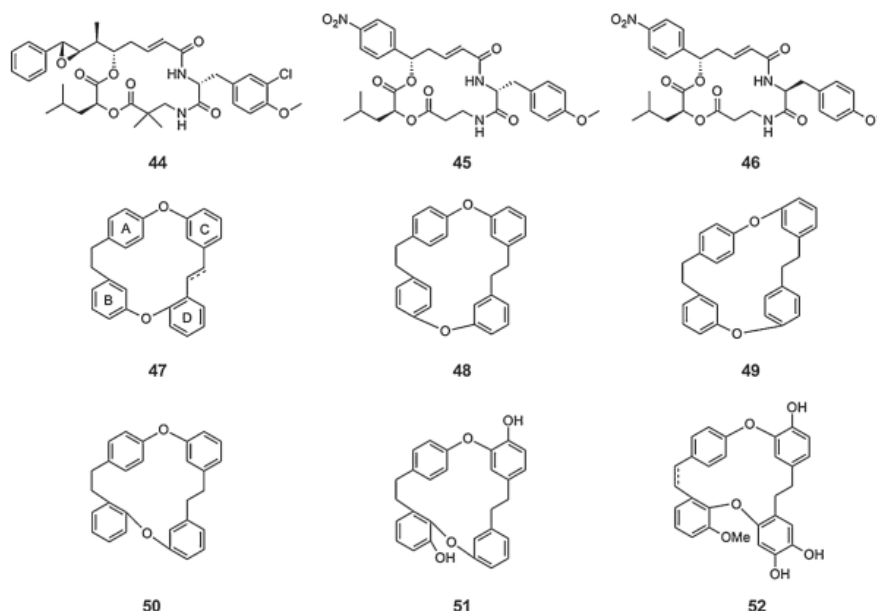


Fig. 12. The structure of (+)-Cryptophycin 52 (44), of its derivatives (45), (46) (microtubule-destabilizing agents, binding site of the *Vinca* alkaloids) and of macrocyclic bisbibenzyls: isomarchantins (47), neomarchantins (48), riccardin II (49), marchantins (50), marchantin C (51) and of (52) analog (inhibiting microtubule polymerization).

macrocycles (so-called *p,p*-dihydroxydihydrostilbenophanes) with significant differences in the structural requirements, and the impact of their conformational equilibria on the activity were analyzed. The compound (33) was most active in this group in tubulin polymerization inhibitory (TPI) (compound (33) 20 μ M for 48 % TPI compared to the most active of combretastatin CA4 3 μ M for 50 % TPI). The next studied compound was Rhizoxin (34) (Fig. 10), which is an antimetabolic agent with *anti*-tumor activity [55]. Rhizoxin binds beta-tubulin in eukaryotic cells disrupting microtubule formation [56]. This, in turn, prevents the formation of the mitotic spindle inhibiting cell division. Rhizoxin binds to tubulin in the maytansine site and depolymerizes assembled microtubules [57]. Derivative compounds of rhizoxin (35), (36) and (37) (Fig. 10) have improved biological activity [58].

The celogentin/moroidin family (38, 39) (Fig. 11) of cyclic peptides was isolated from the seeds of the cockscomb plant, *Celosia argentea*, and exhibited potent inhibition of tubulin polymerization [59]. The most potent member of the family, celogentin C (38), presented greater inhibitory activity than vinblastine [60]. Another compound - the moroidin (39), also inhibited tubulin polymerization and is a peptide composed of eight amino acids, with unusual leucine-tryptophan and tryptophan-histidine cross-links that form its two rings. Celogentin C (in the same family of compounds) is the most potent (IC_{50} 0.8 μ M) - even more potent than the *anti*-mitotic agent vinblastine (IC_{50} 3.0 μ M) [56]. Moroidin has the same potency as vinblastine [61]. Because of its biological activity, compounds in this family are still being tested as *anti*-cancer agents (compound 40, IC_{50} = 2.2 μ M [62]. The following microtubule-stabilizing macrolides, which were first isolated from the myxobacterium *Sorangium cellulosum* by Reichenbach, Höfle, et al. [63] in 1987, are epothilones A (41) and B (42) (Fig. 11). Epothilones have served as successful lead structures for the development of new *anti*-cancer drugs [64]. The inhibition of cancer cell growth by epothilones is based on the suppression of cellular microtubule dynamics, which leads

to cell-cycle arrest in G2/M and the induction of apoptosis [65]. Epothilones are interacting with β -tubulin in the taxol/epothilone binding cavity [66]. Epothilone B (42) and several of its analogs have been advanced into clinical trials on humans, and one of these compounds, ixabepilone (43) also known as BMS-247550, was approved by the US Food and Drug Administration for breast cancer treatment in 2007, under the name Ixempra [67]. It should also be emphasized that the structure-activity relationships (SAR) of epothilones have been studied extensively, including the effects of changes in the configurations of the various chiral centers, which is well described by the group of Altmann [68]. The Cryptophycins are members of the macrocyclic depsipeptide family produced by cyanobacteria of the genus *Nostoc* [69]. This group of compounds inhibits the polymerization of tubulin by blocking microtubule growth, destabilizing them in *Vinca* building domain. They are the most potent compounds known in the dynamics of tubulin and display remarkable cytotoxicity against multidrug-resistant cancer cell lines [70].

In recent years extensive research has been done on structural modifications of these molecules [71]. They led to the synthesis of (+)-Cryptophycin 52 (LY355703) (44) (Fig. 12), which reached phase 2 clinical trials [72]. The multinational pharmaceutical company Sanofi-Aventis also noticed the potential in this group of compounds and published two patents on cryptophycin antibody-drug conjugates (ADCs) for the treatment of lung cancer [73]. Studies on synthesized analogs of cryptophycin are still underway. Shah et al. [74] designed and synthesized novel cryptophycins based macrocyclic depsipeptides with a truncated epoxide chain (45) and (46) (Fig. 12). The compounds (45) and (46) exhibited good *anti*-cancer activity, induced apoptosis, blocked/delayed G2/M cell cycle as well as significantly reduced the expression of α - and β -tubulins. The next series of compounds in which tubulin inhibitors are sought for is macrocyclic bisbibenzyls [75]. This is the group of phenolic natural products that are mainly found in

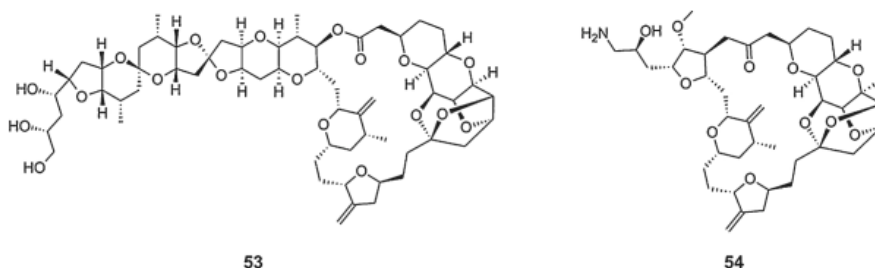


Fig. 13. Halichondrin B (53) and Eribulin (54) microtubule destabilizing agents, compounds that bind to the *Vinca* alkaloid site.

bryophytes [76]. The bisbibenzyl skeleton comprises four aromatic rings (arene A–D) and two ethano bridges (47), (48), (49) and (50) (Fig. 12). Isomarchantins (47), neomarchantins (48), riccardin II (49) and marchantins (50) are four main sub-classes of macrocyclic bisbibenzyls that possess two diaryl ether sub-units as a common structural feature. In turn, marchantin C (51) (Fig. 12), a natural macrocyclic bisbibenzyl, was discovered to be a potent tubulin inhibitor that targets the colchicine-binding site.

This compound was also found to exhibit multidrug resistance-reversing activity by inhibiting the P-glycoprotein [77]. Because its bisbibenzyl scaffold is similar to the *cis*-stilbenoid *anti*-tubulin agents, such as combretastatins (eg. 10, 11), a series of macrocyclic bisbibenzyls with novel skeletons was designed, synthesized and evaluated for anti-proliferative activity against five anthropic cancer cell lines [77]. It was discovered that molecule (52) (Fig. 12) showed excellent anticancer activity against cervical cancer cells, chronic myelogenous leukemia, breast cancer cells, Caucasian colon adenocarcinoma grade II and prostate cancer cell line, with IC_{50} values ranging from 1.51 μ M–5.51 μ M. Compound (52) also effectively inhibited tubulin polymerization in HCC1482 cells and induced HCC1482 cell cycle arrest at the G2/M phase, at the colchicine-binding site. Next important macrocycle is halichondrin B (53) (Fig. 13) isolated from sea sponges of the genus *Halichondria* and *Axinella* [78]. Lack of universal availability of a natural compound has necessitated on scientists the creation of artificial halichondrin and its analogs. Retaining similar properties to the natural product, chemical compounds are classified as non-competitive *vinca* alkaloid inhibitors, because their microtubule binding site is not identical to the above domain. An example of a simple analog halichondrin B is compound Eribulin ((53), E7389, Fig. 13), which was approved by the U.S. Food and Drug Administration on November 15, 2010 to treat patients with metastatic breast cancer who have received at least two prior chemotherapy regimens for late-stage disease, including both anthracycline- and taxane-based chemotherapies [79]. There was an interesting study of this compound in 2016. Doodhi et al. [80] demonstrated by X-ray microscopy that the binding of a single eribulin molecules to a growing microtubule end could cause MT lattice defects or induce MT catastrophe (depolymerization). The authors propound that eribulin amplifies a natural pathway toward catastrophe by promoting the arrest of protofilament elongation. The researchers proposed a general strategy to investigate and manipulate the dynamics of cytoskeletal arrays at high resolution.

In conclusion, microtubule drugs have been vital to advances both in medicine and basic sciences. Presented in this work derivative of stilbene, in particular, combretastatins, have taken the interest of the research community since the discovery of their remarkable biological activities. Such compounds as combretastatins analogs combretastatin A-1 (CA1, 7) combretastatin A-4 (8) and their three prodrugs of monosodium phosphate salt two CA-1 P (Oxi4503, 9) CA4P (Fosbretabulin, 10) amino derivative AVE8062 (Ombrabulin, 11) along with biologically (12–15) and dibenzo[b,f]oxepin derivatives have been extensively

studied and some are already in use. Macromolecular conjugates and nanocarriers of combretastatin CA4, have been developed and were successful in improving drug solubility and bioavailability. Polymer-based systems of CA4 (PLG-CA4, 17) were able to persistently disrupt the vasculature of a murine colon C26 tumor model with a tumor suppression rate of 74 % as opposed to 24 % obtained with CA4P. We presented in this work both of the already-introduced drugs e.g. paclitaxel (4) as well as potential drugs and research directions that are being conducted to obtain new drugs. The direction of creating macrocyclic compounds seems to be very attractive, as well as the newly discovered method of photopharmacology.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

This review was funded by NCHEM -1 (2020) project granted by Warsaw University of Technology, TRI-BIO-CHEM- project granted by POWR.03.02.00-00-I007/16-00 and by the Diamond Grant project (the scientific work financed from budget funds for science in the years 2019–2023).

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Article

Systematic Studies on Anti-Cancer Evaluation of Stilbene and Dibenzo[b,f]oxepine Derivatives

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Abstract: Cancer is one of the most common causes of human death worldwide; thus, numerous therapies, including chemotherapy, have been and are being continuously developed. In cancer cells, an aberrant mitotic spindle—a microtubule-based structure necessary for the equal splitting of genetic material between daughter cells—leads to genetic instability, one of the hallmarks of cancer. Thus, the building block of microtubules, tubulin, which is a heterodimer formed from α - and β -tubulin proteins, is a useful target in anti-cancer research. The surface of tubulin forms several pockets, i.e., sites that can bind factors that affect microtubules' stability. Colchicine pockets accommodate agents that induce microtubule depolymerization and, in contrast to factors that bind to other tubulin pockets, overcome multi-drug resistance. Therefore, colchicine-pocket-binding agents are of interest as anti-cancer drugs. Among the various colchicine-site-binding compounds, stilbenoids and their derivatives have been extensively studied. Herein, we report systematic studies on the antiproliferative activity of selected stilbenes and oxepine derivatives against two cancer cell lines—HCT116 and MCF-7—and two normal cell lines—HEK293 and HDF-A. The results of molecular modeling, antiproliferative activity, and immunofluorescence analyses revealed that compounds **1a**, **1c**, **1d**, **1i**, **2i**, **2j**, and **3h** were the most cytotoxic and acted by interacting with tubulin heterodimers, leading to the disruption of the microtubular cytoskeleton.

Keywords: dibenzo[b,f]oxepine; biological activity; cancer cell; microtubule; tubulin; colchicine-binding site



Citation: Borys, F.; Tobiasz, P.; Poterała, M.; Fabczak, H.; Krawczyk, H.; Joachimiak, E. Systematic Studies on Anti-Cancer Evaluation of Stilbene and Dibenzo[b,f]oxepine Derivatives. *Molecules* **2023**, *28*, 3558. <https://doi.org/10.3390/molecules28083558>

Academic Editors: Keykavous Parang and Qihua Zhu

Received: 6 March 2023

Revised: 13 April 2023

Accepted: 14 April 2023

Published: 18 April 2023



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1. Introduction

Cancer, the leading cause of human death worldwide, remains difficult to treat, despite a huge effort to develop novel therapies [1–6]. Chemotherapy, a traditional and commonly used anti-cancer treatment, targets specific proteins and, as a consequence, cellular structures or processes [7]. Among the most common targets of chemotherapy are proteins involved in cell cycle progression [8]. In cancer cells, the mitotic spindle—a structure that is indispensable for cell division—frequently shows abnormalities, as it causes the improper division of genetic material between daughter cells and, as a consequence, genomic instability [8]. The scaffold of the mitotic spindle is formed by microtubules (MTs), which are cylindrical, hollow biopolymers that are continuously polymerized and depolymerized. MTs are composed of tubulin heterodimers consisting of two ~55 kDa proteins, α - and β -tubulin. In interphase cells, MTs form a network involved in different cellular processes, including the establishment and maintenance of cell shape, intracellular transport, organelle distribution, and cell motility [9]. Thus, in both interphase and dividing cells, tubulin is among the most useful targets in chemotherapy [7].

The surface of tubulin contains so-called “pockets” or “sites” that can accommodate small molecules, which are called microtubule-targeting agents (MTAs). Intercalation of MTAs with the pockets affects MTs' polymerization/depolymerization, leading to changes

in the stability of MTs [9,10]. Of the six agent-binding pockets that have been identified, four bind microtubule-destabilizing agents (MDAs): vinca, colchicine, maytansine, and pironetin sites [9,10]. The binding of an MDA to tubulin causes MT depolymerization or the inhibition of its polymerization, thus affecting microtubule-dependent processes and, as a consequence, leading to cell death. The constant search for new and more specific agents targeting MDA-accommodating pockets has driven many studies [11–16]. As a result, several new compounds have been approved for cancer therapy [17,18].

Colchicine pockets, which are the largest MDA-accommodating sites in tubulin [9,10], bind structurally diverse molecules, which usually contain one phenyl ring and another aromatic ring—phenyl, indole, thiophene, or imidazole, which are separated by a linker [18]. Colchicine-site-binding agents (CSBAs) have drawn attention because, in contrast to other MTAs, they overcome multidrug resistance [19]. Additionally, it was shown that CSBAs may target not only the microtubular cytoskeletons of cancer cells, but also those of endothelial cells that line the tumor vasculature (so-called vascular disrupting agents—VDAs); thus, by disrupting existing vessels, they can reduce the feeding of a tumor and lead to its necrosis [19,20]. Of the different types of double-target CSBAs, a large group, that of natural stilbenoids and their derivatives, such as the combretastatin family (CA1P, CA4P, OXI4503 (Figure 1) [21,22]), have been studied as potential anti-cancer drugs [23,24]. In fact, currently, a methoxy stilbenoid, combretastatin A-4 phosphate CA4P/fosbretabulin (Figure 1), is the only CSBA that is currently approved by FDA for cancer treatment as a VDA [18,21].

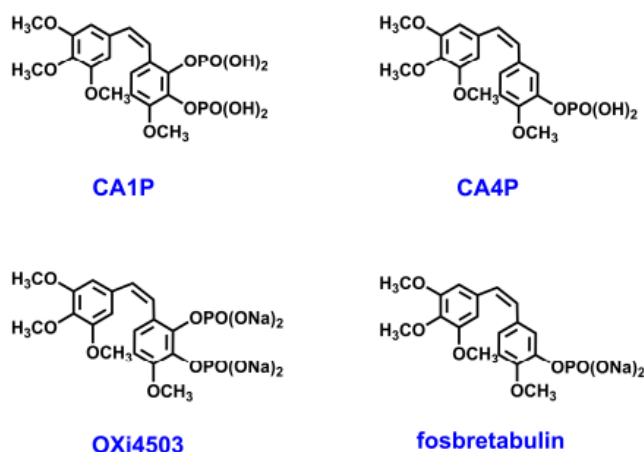


Figure 1. Structure of natural stilbenoids with anti-cancer activity: combretastatin CA1 and CA4 phosphate (CA1P and CA4P, respectively), as well as their disodium salts (OXI4503 and fosbretabulin, respectively).

On the other hand, CSBAs that include stilbenoids are poorly soluble in water, and they cause strong side effects [19]. For example, CA4P/fosbretabulin, which is generally well tolerated, causes acute but transient hypertension [25]. Therefore, efforts are being made to develop new stilbenoid derivatives in the search for more potent anti-cancer agents with improved features [26–31].

It was shown that the addition of functional groups, e.g., methyl, prenyl, O-allyl, or methoxy groups, can enhance the biological activity of natural stilbenoids [32–38].

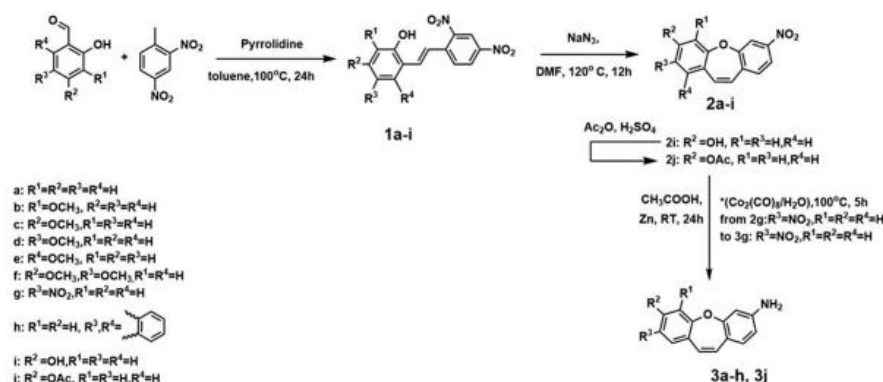
In our previous study [39,40], we synthesized and evaluated the cytotoxicity of five stilbenes (1a–1e) and six dibenzo[*b,f*]oxepines (2a–2e, 2h) (the latter are frequently used as scaffolds in medicinal chemistry, they have a (*Z*)-stilbene motif in their skeleton, and their aromatic rings are connected by oxygen). We showed that one substituted dibenzo[*b,f*]oxepine and two stilbene derivatives, while operating through tubulin binding, acted more selec-

tively toward cancer cell lines (HeLa and U87) than toward normal cell lines. In search of novel compounds with higher cytotoxicity and selectivity toward cancer cells, we studied previously obtained compounds and synthesized new derivatives of hydroxy, methoxy, nitro, and amine groups with 4 stilbenes (**1f–1i**) and 13 dibenzo[*b,f*]oxepines (**2f, 2g, 2i, 2j, 3a–3j**). For the new compounds, we determined their structure by using NMR and theoretical calculations. For all 28 compounds, we evaluated cytotoxicity for the HCT116 and MCF-7 cell lines. We collected these compounds to conduct a systematic study of stilbenes with methoxy and nitro groups and of dibenzo[*b,f*]oxepines with nitro, methoxy, and amino groups. Our results indicated that the addition of a methoxy group could maintain or abolish the cytotoxicity of stilbene derivatives in a position-dependent manner, while, with some exceptions, the restriction of the double bonds that connected phenyl rings to create a (*Z*)-stilbene motif with an oxygen bridge decreased the compounds' activity. Moreover, we demonstrated that three of the most active agents were selective toward cancer cell lines and that they targeted tubulin, thus disrupting the microtubular cytoskeleton.

2. Results and Discussion

2.1. Chemistry and NMR Spectra

We synthesized a series of compounds **3a–h, 3j** in three synthetic steps. The synthetic routes are summarized in Scheme 1. Various stilbene moieties **1a–i** were obtained by starting from the condensation of suitable 2-hydroxyaldehyde and 2,4-dinitrotoluene with pyrrolidine as a catalyst to obtain the substituted stilbene. The next step was the reaction between a derivative of 2,4-dinitrostilbene **1a–i** and sodium azide. In the reactions, the corresponding nitrodibenzo[*b,f*]oxepine **2a–i** appeared. Nitrodibenzo[*b,f*]oxepine bearing a hydroxyl substituent **2i** was protected in the reaction with acetic anhydride and catalytic amounts of concentrated sulfuric acid, which yielded an acetox derivative of dibenzo[*b,f*]oxepine **2j**. Nitrodibenzo[*b,f*]oxepines **2a–f, 2h, 2j** were converted into amino derivatives **3a–f, 3h, 3j** in the presence of Zn in acetic acid. Nitrodibenzo[*b,f*]oxepine with two nitro groups **2g** was selectively reduced to **3g** by using dicobalt octacarbonyl in water.



Scheme 1. Synthesis of stilbene **1a–i** and dibenzo[*b,f*]oxepine **2a–j, 3a–h, 3j** derivatives.

In order to determine the structures of the reaction products of the derivatives of stilbenes **1a–i** and dibenzo[*b,f*]oxepines **2a–j, 3a–h, 3j** in solution, the 1H and ^{13}C NMR spectra of all of the products were measured (see Figures S8–S61 in the Supplementary Materials). The coupling constants ($^1H-^1H$) were measured directly by using the resolution-enhanced 1D spectra and were confirmed, when necessary, through homo-decoupling. The coupling constants ($^1H-^1H$) for olefin protons α and β in compounds **1a–i** were about 16 Hz (*E* configuration) and changed to about 11 Hz for molecules **2a–j, 3a–h, 3j** (*Z* configuration). In summary, we developed a method with mild conditions and operational simplicity for

the versatile synthesis of derivatives of stilbenes or dibenzo[*b,f*]oxepines **1a–i**, **2a–2j**, **3a–h**, **3j** from substituted benzaldehydes and 2,4-dinitrotoluene.

2.2. Anti-Cancer Potential of Stilbenes and Oxepines

To verify if the synthesized compounds showed cytotoxic effects in cancer cells, we determined the viability of compound-treated HTC116 cells by performing an MTT colorimetric assay (Figure 2) [41]. Besides compound **1a**, in which only the hydroxy group was present at position 2, the most active stilbenes were **1c**, **1d**, **1i**; e.g., compounds that were also at position 4 substituted with methoxy or hydroxy groups in **1c**, **1i** or in the compound **1d** containing a methoxy group at position 5. Generally, we observed that the stilbene derivatives were more cytotoxic than the dibenzo[*b,f*]oxepine derivatives and that the substitution of $-\text{NO}_2$ with $-\text{NH}_2$ decreased oxepine activity, but often improved its solubility in organic solvents (such as dimethyl sulfoxide). The opposite tendency was observed in the **2h**, **3h** series, but these were the only derivatives in which the phenyl ring was substituted by the naphthalene ring. In two series of derivatives, **1b**, **2b**, **3b** and **1f**, **2f**, **3f**, cell viability was only minimally affected. These agents had a methoxy group in the *ortho* position or two methoxy groups in the *meta* and *para* positions. Therefore, these substitutions seemed to abolish the anti-cancer activity of stilbene and oxepine derivatives.

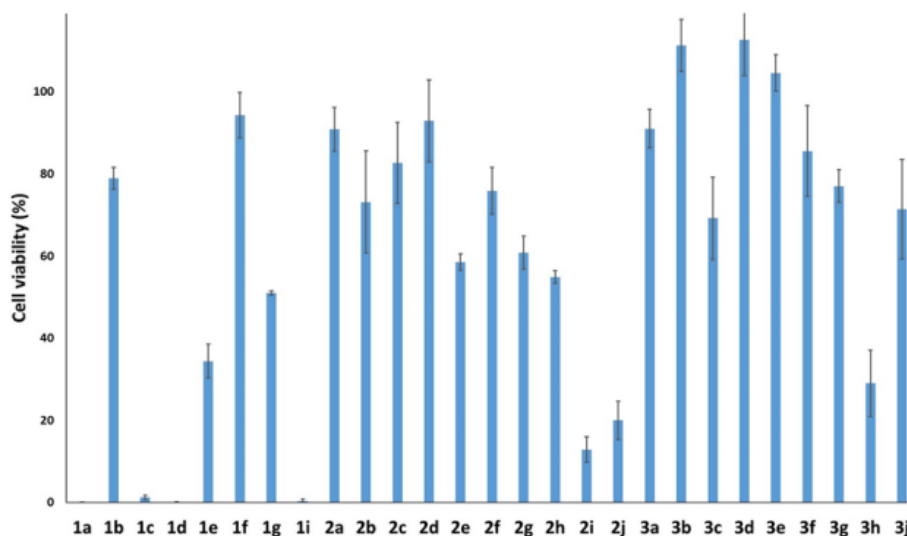


Figure 2. The cytotoxicity effect of tested compounds **1a–g**, **1i**, **2a–2j**, **3a–3h**, **3j** at 100 μM on cancer HCT116 cells based on MTT assay after 48h of treatment.

Interestingly, a previous report on the anti-cancer activity of compound **2h** in U87 (glioblastoma) and HeLa (cervical cancer) cell lines [39] showed that it was significantly more active than in the HTC116 (colon carcinoma) cells used in this study. On the other hand, **2h** also showed severe activity in normal cell lines [39].

Among the tested compounds, four stilbenes **1a**, **1c**, **1d**, **1i** and three dibenzo[*b,f*]oxepine derivatives **2i**, **2j**, **3h** showed high potency against cancer cell lines (cell viability < 30%); hence, these molecules were selected for the next part of the study.

To determine the selectivity of the chosen stilbenes and dibenzo[*b,f*]oxepine derivatives **1a**, **1c**, **1d**, **1i**, **2i**, **2j**, **3h** toward cancer cells, we estimated the cell viability in a series of increasing concentrations of these compounds by using two cancer cell lines (human colon carcinoma (HCT116) and human breast adenocarcinoma (MCF-7)) and two normal cell lines (human embryonic kidney (HEK293) and human dermal fibroblasts (HDF-A)).

(Figure 3). The tested compounds inhibited cell growth dose-dependently, and generally, a stronger effect was observed for HCT116 cells than for MCF-7 cells and for HEK293 cells than for HDF-A cells.

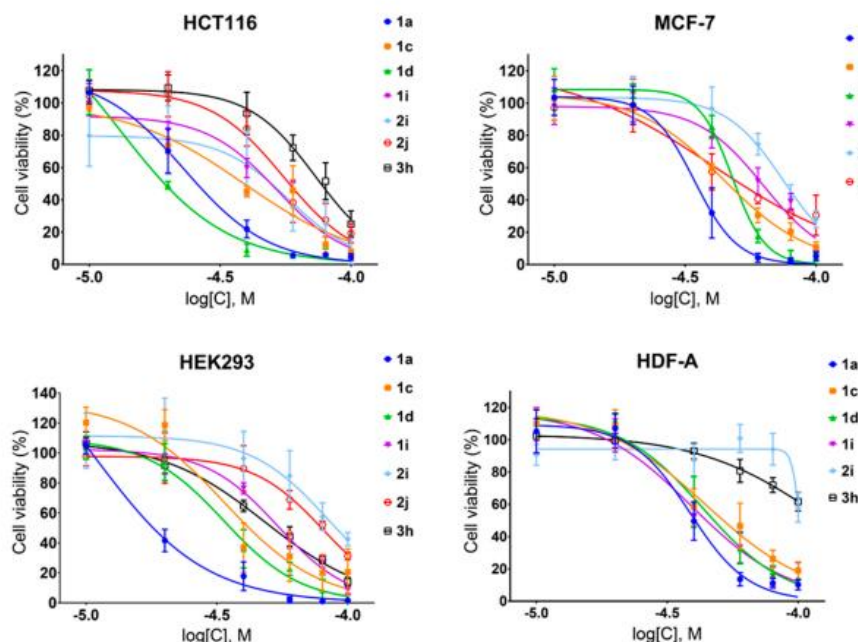


Figure 3. Activity of compounds **1a**, **1c**, **1d**, **1i**, **2i**, **2j**, and **3h**, in cancer cell lines (HCT116 and MCF-7) and normal cell lines (HEK293 and HDF-A) as determined with an MTT assay after 48 h of treatment. Experiments were performed in triplicate (N = 3). Data are presented as the mean $\pm$ SD.

Next, based on the data presented above, we calculated the half-maximal inhibitory concentration (IC_{50}) and selectivity index (SI). As observed here (Figure 2) and in previous studies [39], in most cases, stilbenes exhibited stronger cytotoxicity than dibenzo[*b,f*]oxepines did (Figure 3), but, with the exception of compound **1d**, they showed no selectivity toward cancer cells (Table 1). The highest selectivity indexes were obtained for stilbene **1d** and dibenzo[*b,f*]oxepine derivatives **2i**, **2j**, which varied between 1.4 and 2.4 (Table 1).

Table 1. Half-maximal inhibitory concentration (IC_{50}) and selectivity index (SI) calculated for tested compounds. Data are presented as the mean $\pm$ standard error of the mean (SEM).

Compound	HEK293	HDF-A	HCT116	SI_1	SI_2	MCF-7	SI_3	SI_4
	IC_{50} (μ M)	IC_{50} (μ M)	IC_{50} (μ M)			IC_{50} (μ M)		
1a	18 $\pm$ 1	38 $\pm$ 1	23 $\pm$ 1	0.8	0.8	36 $\pm$ 2	0.5	1.1
1c	34 $\pm$ 4	40 $\pm$ 4	38 $\pm$ 5	0.9	1.1	43 $\pm$ 2	0.7	1.0
1d	33 $\pm$ 2	40 $\pm$ 2	18 $\pm$ 3	1.8	2.2	47 $\pm$ 1	0.7	0.9
1i	54 $\pm$ 2	41 $\pm$ 2	52 $\pm$ 2	1.0	0.8	69 $\pm$ 3	0.8	0.6
2i	88 $\pm$ 5	93 $\pm$ 7	55 $\pm$ 4	1.6	1.7	67 $\pm$ 6	1.2	1.4
2j	85 $\pm$ 4	ND	53 $\pm$ 3	1.6	ND	36 $\pm$ 3	2.4	ND
3h	48 $\pm$ 3	127 *	66 $\pm$ 2	0.7	1.9 *	ND	ND	ND

$SI_1 = IC_{50}(\text{HEK-293})/IC_{50}(\text{HCT116})$; $SI_2 = IC_{50}(\text{HDF-A})/IC_{50}(\text{HCT116})$; $SI_3 = IC_{50}(\text{HEK-293})/IC_{50}(\text{MCF-7})$; $SI_4 = IC_{50}(\text{HDF-A})/IC_{50}(\text{MCF-7})$; “*” —The predicted IC_{50} (above the maximum concentration tested—100 μ M); “ND”—the IC_{50} could not be calculated.

2.3. The Structure of Microtubular Cytoskeleton in HT116 Cells Treated with Compound (1d)

The IC_{50} value of stilbene **1d** obtained for HTC116 cells (18 μ M) was the lowest obtained in cancer cells for all tested compounds, while the selectivity index was among the highest reached for HTC116 cells with respect to both types of control cells. Therefore, we decided to visualize the microtubular network within the control and **1d**-treated HTC116 cells. Until now, the arrangement of microtubules during treatment with dibenzo[*b,f*]oxepine derivatives has not been analyzed, but several results exist for stilbenes [42–44]. Immunostaining of α -tubulin showed that the control cells contained a well-developed microtubular cytoskeleton with dense microtubules that were arranged along the long axis and on the cells' borders in elongated cells (the arrow and arrowhead, respectively, in Figure 4a).

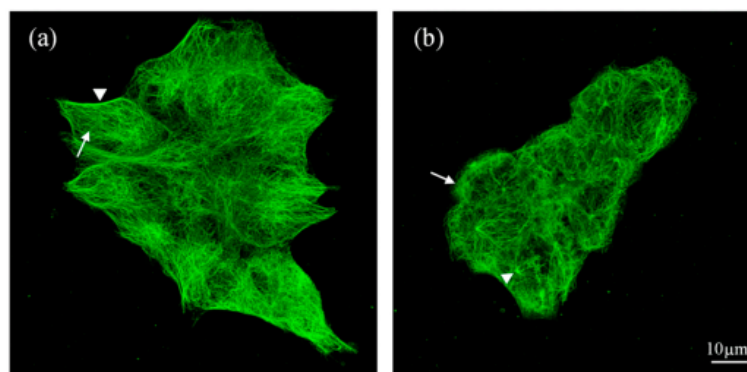


Figure 4. Microtubular networks in the control and **1d**-treated HTC116 cells visualized by staining with anti- α -tubulin 12g10 Ab. (a) Control cells—arrow: MTs arranged along the long cell axis; arrowhead: MTs lining the cell border. (b) Cells treated with compound **1d**—arrow: amorphous material; arrowhead: center of microtubule organization with the arising microtubules.

In contrast, the HTC116 cells treated with 60 μ M of compound **1d** were rounded, and the density of the microtubular network was decreased (Figure 4b). Moreover, in many cells, anti- α -tubulin stained an amorphous material, which was probably depolymerized tubulin (the arrow in Figure 4b). In contrast to the control cells, in the **1d**-treated cells, the point of the organization of MTs, which was presumably a centrosome, was well defined, with several MTs (probably the most stable) arising from it (the arrowhead in Figure 4b). This observation confirmed that compound **1d** affected the microtubular cytoskeleton, which, in turn, could lead to the cells' death.

2.4. Computational Analysis and Molecular Docking Simulations

The combination of experimental and computational methodologies has been of considerable importance in the discovery and further improvement of new candidates for potent anti-cancer compounds [45,46]. Molecular docking methods rely on the exploration of the ligand conformation within the active site of a protein or macromolecule until the minimum energy is achieved. Notably, this technique allows the computation of the binding free energy by including processes that are responsible for molecular recognition [47,48]. As mentioned earlier, in the Z derivatives of the stilbenes combretastatin CA1 and CA4 phosphate (**CA1P** and **CA4P**, respectively) and their disodium salts (**Oxi4503** and **fos-bretabulin**, respectively), as well as dibenzo[*b,f*]oxepines, which have a (Z)-stilbene in their skeleton, the motif docks at the colchicine-binding site [9,49]. Based on the available literature and our cytotoxicity results, we expected that selected stilbene and dibenzo[*b,f*]oxepine derivatives could be potent tubulin inhibitors that bind to colchicine pockets [39,50–53]. Therefore, the interactions between the most active compounds **1a**, **1c**, **1d**, **1i**, **2i**, **2j**, **3h** and tubulin heterodimer (crystal structure from PDB: 1SA0) were analyzed with molecu-

lar modeling. The optimal structures of the compounds with the best biological activity were calculated by using the DFT B3LYP/6-311G 6-311++g (2d,p) method (and with a polarizable continuum model (PCM), Gaussian 03W) [54,55] (Figures S1–S7, Tables S1–S7). Molecular docking was performed by simulating the incorporation of compounds **1a**, **1c**, **1d**, **1i**, **2i**, **2j**, **3h** into the colchicine-binding site in tubulin (Figure 5, Table S8). The docking protocol was validated by re-docking the DAMA–colchicine that was extracted from PDB crystal structure. In the crystal structure, the tropolone ring of colchicine is stabilized by van der Waals contacts with Val181, Ser178, and Val315. The carbonyl group is stabilized by a hydrogen bond with Val181. The ring with trimethoxy substituents is buried in the aliphatic part confined by Lys352, Asn350, Leu378, Ala316, Leu255, Lys254, Ala250, and Leu242. The methoxy group at position 3 participated in a hydrogen bond with the -SH group of Cys241 [56]. The algorithm that was applied correctly reproduced the binding mode of the native ligand. The binding poses of all tested compounds exhibited a high affinity for the target protein. The estimated binding free energy varied from -7.7 to -9.3 kcal/mol, in contrast to the -8.9 kcal/mol of redocked DAMA–colchicine (for a detailed list of the estimated binding free energies and the predicted interactions, see the supporting information). Analysis of the binding model of the most active compounds and tubulin allowed the discovery of several interactions with the protein residues in the colchicine-binding site (Table S8). Compounds **1a**, **1c**, **1d**, **1i**, **2i**, **2j**, **3h** were stabilized in their binding poses by hydrophobic interactions with β -tubulin residues (Leu242, Leu248, Ala250, Lys254, Leu255, Lys352, Ile378, Val318). Of the active compounds, four ligands (**1d**, **1i**, **2i**, **3h**) were stabilized in their binding poses by hydrogen bond interactions with the tubulin heterodimer (Figure 5 and Figure S3). However, no hydrogen bonds with Cys241 were predicted, though these are considered to be the most crucial for high potency. This may partially justify the high micromolar cytotoxicity of the tested compounds [57,58].

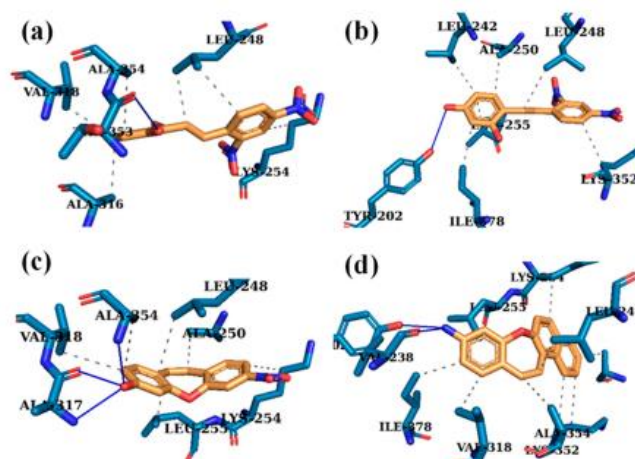


Figure 5. Stick representation of the top-ranked docking poses (orange) and predicted interactions of compounds (a) **1d**, (b) **1i**, (c) **2i**, and (d) **3h** with $\alpha\beta$ -tubulin (PDB code: 1SA0). For clarity, only amino acids interacting with ligands are shown. Blue lines represent hydrogen bond interactions; dashed gray lines represent hydrophobic interactions.

3. Conclusions

In this study, we report the synthesis and cytotoxic effects of nine stilbenoids **1a–i** and nineteen dibenzo[*b,f*]oxepine derivatives **2a–i**, **3a–h**, **3j**. The compounds were obtained by substituting a methoxy group in various positions of the 2-hydroxy-2',4'-dinitrostilbene skeleton or nitro, hydroxy, acetoxy, or benzo groups of stilbene or dibenzo[*b,f*]oxepine. For

the most promising compounds, we performed molecular docking, anti-cancer activity measurements, and MT visualization through immunofluorescence. Our results showed that the stilbene derivatives were more active than the dibenzo[*b,f*]oxepine derivatives. The most active compounds were **1a**, **1c**, **1d**, **1i**, **2i**, **2j**, and **3h**.

It is worth noting that for both stilbenes and the nitro or amine dibenzo[*b,f*]oxepines, the molecular skeletons were the same, and the compounds differed in their substituents and the positions that they took. Some regularities could be observed: The introduction of electron-donating (EDG; enriched electron density in the aromatic ring) hydroxy, methoxy, or acetyloxy groups into the ring in the *para* or *ortho* position in **1c**, **1i**, **1a** and in the *meta* position in **1d** had a positive effect on the anticancer activity of both stilbene and nitro dibenzo[*b,f*]oxepine **2i**, **2j**. The last set of molecules consisted of amine dibenzo[*b,f*]oxepines **3a–3h**, **3j**. In this case, only compound **3h** showed an effect, and there were little data for investigating the effect of the electron density of the substituent on anticancer activity.

The selectivity toward cancer cells (HCT116 and MCF-7) was investigated in comparison with the selectivity toward normal cells (HEK293 and HDF-A) for stilbenes **1a**, **1c**, **1d**, **1i** and dibenzo[*b,f*]oxepines **2i**, **2j**, **3h**; this indicated that the three most promising anti-cancer agents were compounds **1d**, **2i**, and **2j**. The molecular docking and visualization of the microtubular cytoskeleton in the stilbene and/or dibenzo[*b,f*]oxepine derivatives confirmed that the cytotoxicity was related to their tubulin-targeting abilities and, as a consequence, depolymerization of cytoplasmic MTs.

4. Materials and Methods

4.1. Chemistry

4.1.1. PROCEDURE A—Obtained Compounds **1a–1i** (Figure 6)

To a stirred mixture of 2,4-dinitrotoluene (12.00 mmol), appropriate aldehyde (12.00 mmol), and toluene (20 mL) under argon, dry pyrrolidine (0.9 mL, 0.782 g, 11.00 mmol) was added. After 24 h of heating at 100 °C, the solvent was distilled off on a rotary evaporator. Then, ethyl acetate (150 mL) was added to the residue, and the resulting mixture was washed with 0.5 M hydrochloric acid (2 × 40 mL) and water to neutral pH. Next, the mixture was dried by the addition of anhydrous MgSO₄ and the solvent was removed in vacuo. The crude product was purified by crystallization from methanol or ethanol with a small amount of the activated charcoal (in order to absorb colored impurities).

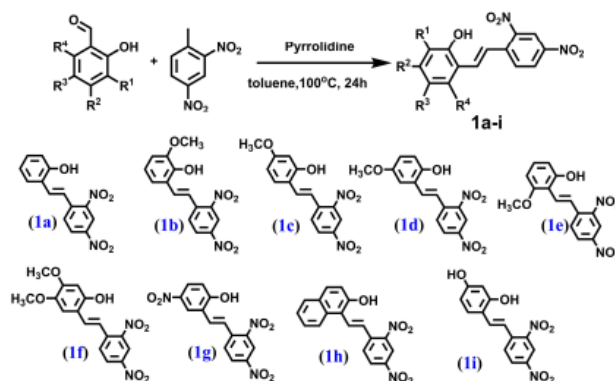


Figure 6. The structure of: (E)-2-hydroxy-2',4'-dinitrostilbene (**1a**), (E)-2-hydroxy-3-methoxy-2',4'-dinitrostilbene (**1b**), (E)-2-hydroxy-4-methoxy-2',4'-dinitrostilbene (**1c**), (E)-2-hydroxy-5-methoxy-2',4'-dinitrostilbene (**1d**), (E)-2-hydroxy-6-methoxy-2',4'-dinitrostilbene (**1e**), (E)-2-hydroxy-4,5-dimethoxy-2',4'-dinitrostilbene (**1f**), (E)-2-hydroxy-5,2',4'-trinitrostilbene (**1g**), (E)-1-(2,4-dinitrophenyl)naphthalen-2-ol (**1h**) and (E)-2,4-dihydroxy-2',4'-dinitrostilbene (**1i**).

4.1.2. PROCEDURE B—Obtained Compounds 2a–2i (Figure 7)

To the solution of appropriate stilbene (5.00 mol) in DMF (15 mL/mmol) NaN_3 (9.50 mmol) was sequentially added and the reaction flask was fitted with a condenser. The mixture was stirred at 120 °C overnight and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with DCM as the mobile phase.

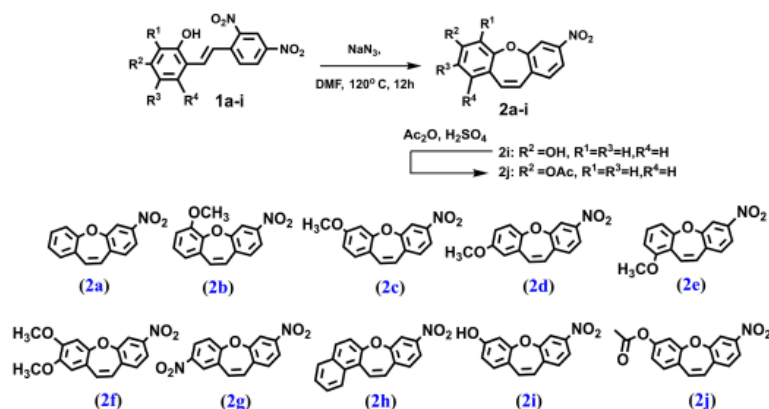


Figure 7. The structure of: 3-nitrodibenzo[*b,f*]oxepine (2a), 6-methoxy-3-nitrodibenzo[*b,f*]oxepine (2b), 3-methoxy-7-nitrodibenzo[*b,f*]oxepine (2c), 2-methoxy-7-nitrodibenzo[*b,f*]oxepine (2d), 1-methoxy-7-nitrodibenzo[*b,f*]oxepine (2e), 2,3-dimethoxy-7-nitrodibenzo[*b,f*]oxepine (2f), 2,7-dinitrodibenzo[*b,f*]oxepine (2g), 9-nitrobenzo[*b*]naphtho[1,2-*f*]oxepine (2h), 7-nitrodibenzo[*b,f*]oxepin-3-ol (2i) and 7-nitrodibenzo[*b,f*]oxepin-3-yl acetate (2j).

4.1.3. PROCEDURE C—Obtained Compound 2j (Figure 7)

The compound 2i (1.00 mmol) was dissolved in excess of acetic anhydride. A few drops of concentrated sulfuric acid were added to the obtained solution and the mixture was stirred at room temperature overnight. Next, the reaction was quenched by dropwise addition of a concentrated aqueous solution of sodium bicarbonate (1 mL) and evaporated under reduced pressure. The residue was re-dissolved in ethyl acetate and transferred to a separatory funnel, washed two times with water and with brine. After solvent evaporation, the residue was purified by flash column chromatography on silica gel with a 10% mixture of ethyl acetate in hexane as a mobile phase.

4.1.4. PROCEDURE D—Obtained Compound 3a–3f and 3j (Figure 8)

To the solution of corresponding nitrodibenzo[*b,f*]oxepine (1.00 mmol) in acetic acid (20.0 mL/mmol) activated zinc dust was added (10 mmol) and the resulting slurry was allowed to stir overnight at room temperature. Next, the mixture was filtrated through a pad of celite and a solvent was evaporated under reduced pressure. The residue was subjected to column chromatography on silica gel with hexane: ethyl acetate 1:1 mixture (*v:v*) as the eluent.

4.1.5. PROCEDURE E—Obtained Compound 3g (Figure 8)

Dicobalt octacarbonyl (1.00 g, 3.00 mmol) was placed in a 50 mL three-necked flask equipped with a reflux condenser. The vessel was purged with argon, and (1.76 mmol) of 2,7-dinitrodibenzo[*b,f*]oxepine 2g and 20 mL of dimethoxyethane were added and the solution was stirred under an argon atmosphere. After the precipitate had dissolved, 2 mL of water was added and it was heated at 100 °C for 5 h. Next, the mixture was cooled down to room temperature and concentrated on a rotary evaporator. The resulting precipitate was

dissolved in chloroform and then purified by column chromatography using chloroform as the eluent.

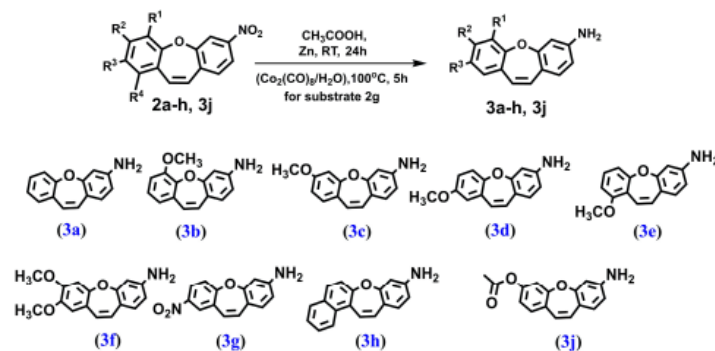


Figure 8. The structure of: dibenzo[*b,f*]oxepin-3-amine (**3a**), 6-methoxydibenzo[*b,f*]oxepin-3-amine (**3b**), 7-methoxydibenzo[*b,f*]oxepin-3-amine (**3c**), 8-methoxydibenzo[*b,f*]oxepin-3-amine (**3d**), 9-methoxydibenzo[*b,f*]oxepin-3-amine (**3e**), 7,8-dimethoxydibenzo[*b,f*]oxepin-3-amine (**3f**), 8-nitrodibenzo[*b,f*]oxepin-3-amine (**3g**), benzo[*b*]naphtho[1,2-*f*]oxepin-9-amine (**3h**) and 7-aminodibenzo[*b,f*]oxepin-3-yl acetate (**3j**).

(*E*)-2-hydroxy-2',4'-dinitrostilbene (1a), Yield 12% (0.412 g). mp. 192 °C. ^1H NMR (500 MHz, DMSO- d_6 , 298 K): δ (ppm): 10.18 (br. s, 1H), 8.70 (d, $J = 2.4$ Hz, 1H), 8.44 (dd, $J = 8.8$ Hz, 1H), 8.20 (d, 1H), 7.66 (d, $J = 16$ Hz, 1H), 7.59 (d, $J = 16$ Hz, 1H), 7.54 (dd, $J = 7.5$ Hz, $J = 1.5$ Hz, 1H), 7.20 (t, $J = 8.5$ Hz, 1H), 6.90 (dd, $J = 1$ Hz, 1H), 6.85 (td, 1H). ^{13}C NMR (125 MHz, DMSO- d_6 , 298 K): δ (ppm): 156.3, 147.2, 145.5, 138.3, 133.5, 130.7, 128.8, 128.5, 127.2, 122.5, 120.7, 120.3, 119.5, 116.2.

(*E*)-2-hydroxy-3-methoxy-2',4'-dinitrostilbene (1b), Yield 50% (1.896 g). mp. 182 °C. ^1H NMR (500 MHz, CDCl_3 , 298 K): δ (ppm): 8.79 (d, $J = 2$ Hz, 1H), 8.40 (dd, $J = 8.8$ Hz, 1H), 8.03 (d, $J = 8.8$ Hz, 1H), 7.72 (d, $J = 16$ Hz, 1H), 7.62 (d, $J = 16$ Hz, 1H), 7.19 (dd, $J = 7.5$ Hz, $J = 2$ Hz, 1H), 6.90 (dd, $J = 8$ Hz, 1H), 6.87 (dd, 1H), 3.94 (s, 3H). ^{13}C NMR (125 MHz, CDCl_3 , 298 K): δ (ppm): 146.8, 144.7, 139.4, 132.7, 128.9, 126.9, 121.9, 121.7, 120.6, 120.0, 119.6, 111.3, 56.2. HRMS (ESI-) calc. for $[\text{M}-\text{H}]^-$ ($\text{C}_{15}\text{H}_{11}\text{N}_2\text{O}_6$): 315.0623, found 315.0624.

(*E*)-2-hydroxy-4-methoxy-2',4'-dinitrostilbene (1c), Yield 53% (2.01 g). mp. 175 °C. ^1H NMR (500 MHz, CDCl_3 , 298 K): δ (ppm): 8.75 (d, $J = 2.4$ Hz, 1H), 8.35 (dd, $J = 8.8$ Hz, $J = 2.4$ Hz, 1H), 7.88 (d, $J = 8.8$ Hz, 1H), 7.57 (s, 2H), 7.50 (d, $J = 8.8$ Hz, 1H), 6.54 (dd, $J = 7.7$ Hz, $J = 2.4$ Hz, 1H), 3.81 (s, 3H). ^{13}C NMR (125 MHz, CDCl_3 , 298 K): δ (ppm): 162.0, 155.6, 147.0, 145.4, 139.7, 133.0, 129.4, 128.4, 126.8, 120.7, 118.9, 116.25, 107.3, 102.0, 55.5. HRMS (ESI-) calc. for $[\text{M}-\text{H}]^-$ ($\text{C}_{15}\text{H}_{11}\text{N}_2\text{O}_6$): 315.0623, found 315.0622.

(*E*)-2-hydroxy-5-methoxy-2',4'-dinitrostilbene (1d), Yield 56% (2.123 g). mp. 168 °C. ^1H NMR (500 MHz, DMSO- d_6 , 298 K): δ (ppm): 9.75 (br. s, 1H), 8.73 (d, $J = 2.5$ Hz, 1H), 8.47 (dd, $J = 8.5$ Hz, 1H), 8.21 (d, 1H), 7.65 (d, $J = 11.5$ Hz, 1H), 7.61 (d, 1H), 7.12 (t, $J = 2$ Hz, 1H), 6.84 (d, 2H), 3.72 (s, 3H). ^{13}C NMR (125 MHz, DMSO- d_6 , 298 K): δ (ppm): 152.3, 150.4, 147.2, 145.6, 138.3, 133.4, 128.9, 127.2, 122.8, 121.1, 120.3, 117.19, 117.02, 112.27, 30.70. HRMS (ESI-) calc. for $[\text{M}-\text{H}]^-$ ($\text{C}_{15}\text{H}_{11}\text{N}_2\text{O}_6$): 315.0623, found: 315.0624.

(*E*)-2-hydroxy-6-methoxy-2',4'-dinitrostilbene (1e), Yield 69% (2.616 g). mp. 212 °C. ^1H NMR (500 MHz, DMSO- d_6 , 298 K): δ (ppm): 10.33 (s, 1H), 8.72 (d, $J = 2.5$ Hz, 1H), 8.44 (dd, $J = 9$ Hz, 1H), 8.14 (d, 1H), 7.96 (d, $J = 16.5$ Hz, 1H), 7.71 (d, 1H), 7.16 (t, $J = 8.5$ Hz, 1H), 6.56 (dd, $J = 0.5$ Hz, 1H), 6.54 (dd, 1H), 3.84 (s, 3H). ^{13}C NMR (125 MHz, DMSO- d_6 , 298 K): δ (ppm): 159.1, 157.7, 147.1, 145.2, 139.2, 130.8, 129.6, 128.1, 127.1, 122.2, 120.1, 111.3, 108.7, 102.2, 55.8. HRMS (ESI-) calc. for $[\text{M}-\text{H}]^-$ ($\text{C}_{15}\text{H}_{11}\text{N}_2\text{O}_6$): 315.0623, found: 315.0625.

(*E*)-2-hydroxy-4,5-dimethoxy-2',4'-dinitrostilbene (1f), Yield 75% (3.114 g). mp. 221 °C. ^1H NMR (500 MHz, DMSO- d_6 , 298 K): δ (ppm): 9.91 (s, 1H), 8.69 (d, $J = 2.4$ Hz, 1H), 8.42

(dd, $J = 8.8$ Hz, $J = 2.4$ Hz, 1H), 8.17 (d, $J = 8.9$ Hz, 1H), 7.67 (d, $J = 16.1$ Hz, 1H), 7.46 (d, $J = 16.1$ Hz, 1H), 7.11 (s, 1H), 6.53 (s, 1H), 3.76 (s, 3H), 3.73 (s, 3H). ^{13}C NMR (125 MHz, DMSO- d_6 , 298 K): δ (ppm): 151.8, 151.6, 146.8, 144.8, 142.3, 138.8, 133.7, 128.1, 127.0, 120.4, 117.5, 113.8, 111.3, 100.5, 56.2, 55.4, 40.0, 39.9, 39.7, 39.5, 39.4, 39.2, 39.0. HRMS (ESI+) calc. for $[\text{M}+\text{H}]^+$ ($\text{C}_{16}\text{H}_{15}\text{N}_2\text{O}_7$): 347.0874, found 347.0867.

(E)-2-hydroxy-5,2',4'-tridinitrostilbene (1g), Yield 8% (0.317 g). mp. 241 °C. ^1H NMR (500 MHz, DMSO- d_6 , 298 K): δ (ppm): 8.73 (d, $J = 2.3$ Hz, 1H), 8.54 – 8.44 (m, 2H), 8.24 (d, $J = 8.8$ Hz, 1H), 8.11 (dd, $J = 9.0$ Hz, $J = 2.8$ Hz, 1H), 7.84 (d, $J = 16.3$ Hz, 1H), 7.64 (d, $J = 16.3$ Hz, 1H), 7.07 (d, $J = 9.0$ Hz, 1H). ^{13}C NMR (125 MHz, DMSO- d_6 , 298 K): δ (ppm): 162.3, 147.5, 146.0, 139.8, 137.8, 131.3, 129.2, 127.3, 126.0, 124.7, 124.0, 123.2, 120.2, 116.6, 40.0, 39.9, 39.7, 39.5, 39.4, 39.2, 39.0. HRMS (ESI+) calc. for $[\text{M}+\text{H}]^+$ ($\text{C}_{14}\text{H}_9\text{N}_3\text{O}_7$): 331.0440, found 331.0438.

(E)-1-(2,4-dinitrostyryl)naphthalen-2-ol (1h), Not isolated. Crude product mixture was used as a substrate in a subsequent reaction.

(E)-2,4-dihydroxy-2',4'-dinitrostilbene (1i), Yield 12% (0.434 g). mp. 201 °C. ^1H NMR (500 MHz, DMSO- d_6 , 298 K): δ (ppm): 10.13 (s, 1H), 9.83 (s, 1H), 8.69 (d, $J = 2.4$ Hz, 1H), 8.40 (dd, $J = 8.9$ Hz, $J = 2.4$ Hz, 1H), 8.17 (d, $J = 8.9$ Hz, 1H), 7.63 (d, $J = 16.1$ Hz, 1H), 7.42 (d, $J = 16.1$ Hz, 1H), 7.38 (d, $J = 8.6$ Hz, 1H), 6.37 (d, $J = 2.3$ Hz, 1H), 6.31 (dd, $J = 8.5$ Hz, $J = 2.3$ Hz, 1H). ^{13}C NMR (125 MHz, DMSO- d_6 , 298 K): δ (ppm): 160.3, 158.0, 146.7, 144.6, 138.9, 134.5, 130.1, 127.9, 126.9, 120.3, 116.4, 114.4, 107.9, 102.6. HRMS (ESI+) calc. for $[\text{M}+\text{H}]^+$ ($\text{C}_{16}\text{H}_{15}\text{N}_2\text{O}_7$): 302.0539, found 302.0543.

3-nitrodibenzo[*b,f*]oxepine (2a), Yield 95% (1.135 g). mp. 156 °C. ^1H NMR (500 MHz, DMSO- d_6 , 298 K): δ (ppm): 8.10 (d, $J = 2.5$ Hz, 1H), 8.04 (dd, $J = 8.5$ Hz, $J = 2.4$ Hz, 1H), 7.57 (d, $J = 8.5$ Hz, 1H), 7.44 (ddd, $J = 7.5$ Hz, $J = 7$ Hz, $J = 1.5$ Hz, 1H), 7.40 (dd, $J = 1.5$ Hz, 1H), 7.36 (dd, $J = 8$ Hz, 1H), 7.24 (ddd, 1H), 7.03 (d, $J = 11.5$ Hz, 1H), 6.92 (d, $J = 11.5$ Hz, 1H). ^{13}C NMR (125 MHz, DMSO- d_6 , 298 K): δ (ppm): 156.0, 155.9, 148.2, 137.1, 133.5, 131.2, 130.2, 129.9, 129.5, 128.2, 125.8, 121.4, 120.4, 116.5.

6-methoxy-3-nitrodibenzo[*b,f*]oxepine (2b), Yield 99% (1.331 g). mp. 184 °C. ^1H NMR (500 MHz, CDCl_3 , 298 K): δ (ppm): 8.16 (d, $J = 2.3$ Hz, 1H), 7.98 (dd, $J = 8.5$ Hz, $J = 2.3$ Hz, 1H), 7.30 (d, $J = 8.5$ Hz, 1H), 7.10 (dd, $J = 8.1$ Hz, $J = 7.7$ Hz, 1H), 6.99 (dd, $J = 8.2$ Hz, $J = 1.5$ Hz, 1H), 6.90 (d, $J = 11.4$ Hz, 1H), 6.80 (1H, dd, H_a), 6.77 (d, $J = 11.4$ Hz, 1H), 3.96 (s, 3H). ^{13}C NMR (125 MHz, CDCl_3 , 298 K): δ (ppm): 156.9, 151.8, 148.4, 144.7, 137.5, 133.5, 131.0, 129.1, 128.2, 125.5, 120.8, 112.0, 117.6, 113.3, 56.2.

3-methoxy-7-nitrodibenzo[*b,f*]oxepine (2c), Yield 98% (1.318 g). mp. 189 °C. ^1H NMR (500 MHz, CDCl_3 , 298 K): δ (ppm): 8.00 (d, $J = 2.3$ Hz, 1H), 7.97 (dd, $J = 8.4$ Hz, $J = 2.3$ Hz, 1H), 7.26 (d, $J = 8.5$ Hz, 1H), 7.09 (d, $J = 8.5$ Hz, 1H), 6.79 (d, $J = 11.4$ Hz, 1H), 6.76 (d, $J = 2.5$ Hz, 2H), 6.72 (dd, $J = 8.4$ Hz, $J = 2.5$ Hz, 1H), 6.58 (d, $J = 11.3$ Hz, 2H), 3.84 (s, 3H). ^{13}C NMR (125 MHz, CDCl_3 , 298 K): δ (ppm): 162.4, 157.8, 156.3, 148.2, 137.8, 133.5, 130.6, 129.3, 125.7, 122.4, 120.1, 117.1, 111.7, 106.9, 55.6.

2-methoxy-7-nitrodibenzo[*b,f*]oxepine (2d), Yield 98% (1.318 g). mp. 167 °C. ^1H NMR (500 MHz, CDCl_3 , 298 K): δ (ppm): 7.03 (d, $J = 8.5$ Hz, 1H), 6.93 (d, $J = 8$ Hz, 1H), 6.77 (dd, $J = 3$ Hz, 1H), 6.63 (d, 1H), 6.59 (d, $J = 11$ Hz, 1H), 6.49 (d, 1H), 6.45 (d, 1H), 6.41 (dd, 1H), 3.76 (s, 3H). ^{13}C NMR (125 MHz, CDCl_3 , 298 K): δ (ppm): 158.9, 156.4, 150.5, 148.7, 131.7, 130.6, 130.5, 126.4, 121.9, 120.9, 114.5, 113.4, 111.3, 107.5, 55.6. HRMS (ESI+) calc. for $[\text{M}]^+$ ($\text{C}_{15}\text{H}_{11}\text{NO}_4$) 269.0688, found: 269.0690.

1-methoxy-7-nitrodibenzo[*b,f*]oxepine (2e), Yield 96% (1.291 g). mp. 178 °C. ^1H NMR (500 MHz, CDCl_3 , 298 K): δ (ppm): 8.00 (d, $J = 2.5$ Hz, 1H), 7.97 (dd, $J = 8.5$ Hz, 1H), 7.31 (t, $J = 8.5$ Hz, 1H), 7.27 (d, 1H), 7.21 (d, $J = 11.5$ Hz, 1H), 6.85 (dd, $J = 0.5$ Hz, 1H), 6.74 (d, 1H), 6.49 (dd, 1H), 3.86 (s, 3H). ^{13}C NMR (125 MHz, CDCl_3 , 298 K): δ (ppm): 158.5, 157.4, 156.9, 148.3, 138.0, 131.2, 129.2, 128.6, 127.0, 120.1, 119.0, 117.0, 113.6, 107.6, 56.0. HRMS (ESI+) calc. for $[\text{M}]^+$ ($\text{C}_{15}\text{H}_{11}\text{NO}_4$) 269.0688, found: 269.0683.

2,3-dimethoxy-7-nitrodibenzo[*b,f*]oxepine (2f), Yield 96% (1.435 g). mp. 165 °C. ^1H NMR (500 MHz, CDCl_3 , 298 K): δ (ppm): 8.00 (d, $J = 2.3$ Hz, 1H), 7.96 (dd, $J = 8.4$ Hz, $J = 2.3$ Hz, 1H), 7.25 (d, $J = 8.4$ Hz, 1H), 6.78 (s, 1H), 6.77 (d, $J = 11.2$ Hz, 1H), 6.65 (d,

$J = 11.2$ Hz, 1H), 6.63 (s, 1H), 3.93 (s, 3H), 3.85 (s, 3H). ^{13}C NMR (125 MHz, CDCl_3 , 298 K): δ (ppm): 156.7, 151.3, 150.4, 148.3, 146.5, 137.6, 133.5, 129.3, 126.5, 121.3, 120.0, 116.9, 111.4, 105.2, 56.3, 56.2. HRMS (ESI+) calc. for $[\text{M}]^+$ ($\text{C}_{16}\text{H}_{13}\text{NO}_5$): 299.0794, found 299.0786.

2,7-dinitrobenzo[*b*]floxepine (2g), Yield 80% (1.136 g). mp. 216 °C. ^1H NMR (500 MHz, $\text{DMSO}-d_6$, 298 K): δ (ppm): 8.32 (d, $J = 2.8$ Hz, 1H), 8.29 (dd, $J = 8.8$ Hz, $J = 2.9$ Hz, 1H), 8.23 (d, $J = 2.4$ Hz, 1H), 8.10 (dd, $J = 8.5$ Hz, $J = 2.4$ Hz, 1H), 7.68 (d, $J = 8.8$ Hz, 1H), 7.63 (d, $J = 8.5$ Hz, 1H), 7.17 (d, $J = 11.5$ Hz, 1H), 7.08 (d, $J = 11.5$ Hz, 1H). ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$, 298 K): δ (ppm): 160.4, 155.2, 148.6, 145.1, 136.3, 131.7, 130.7, 130.6, 130.2, 126.2, 125.3, 123.0, 121.0, 117.0. HRMS (ESI+) calc. for $[\text{M}]^+$ ($\text{C}_{14}\text{H}_8\text{N}_2\text{O}_5$): 284.0433, found 284.0439.

9-nitrobenzo[*b*]naphtho [1,2-*f*]oxepine (2h), Yield 98% (1.416 g). mp. 197 °C. ^1H NMR (500 MHz, CDCl_3 , 298 K): δ (ppm): 8.09 (d, $J = 2.3$ Hz, 1H), 8.07 – 8.03 (m, 1H), 8.00 (dd, $J = 8.4$, 2.3 Hz, 1H), 7.89 (d, $J = 8.8$ Hz, 1H), 7.86 – 7.81 (m, 1H), 7.65 (d, $J = 11.6$ Hz, 1H), 7.57 (ddd, $J = 8.4$ Hz, $J = 6.9$ Hz, $J = 1.4$ Hz, 1H), 7.48 (ddd, $J = 8.0$ Hz, $J = 6.9$ Hz, $J = 1.1$ Hz, 1H), 7.42 (d, $J = 8.8$ Hz, 1H), 7.35 (d, $J = 8.5$ Hz, 1H), 7.04 (d, $J = 11.6$ Hz, 1H). ^{13}C NMR (125 MHz, CDCl_3 , 298 K): δ (ppm): 157.6, 155.9, 148.6, 137.8, 131.8, 131.5, 131.4, 130.0, 129.1, 129.0, 128.6, 127.4, 125.6, 123.3, 123.2, 120.9, 120.2, 116.9. HRMS (ESI+) calc. for $[\text{M}+\text{H}]^+$ ($\text{C}_{16}\text{H}_{15}\text{N}_2\text{O}_7$): 289.0739, found 289.0728.

7-nitrobenzo[*b*]floxepin-3-ol (2i), Yield 30% (0.382 g). mp. 164 °C. ^1H NMR (500 MHz, $\text{DMSO}-d_6$, 298 K): δ (ppm): 10.10 (s, 1H), 8.02 (d, $J = 2.4$ Hz, 1H), 7.99 (dd, $J = 8.4$ Hz, $J = 2.4$ Hz, 1H), 7.47 (d, $J = 8.5$ Hz, 1H), 7.13 (d, $J = 8.4$ Hz, 1H), 6.86 (d, $J = 11.3$ Hz, 1H), 6.75 (d, $J = 2.4$ Hz, 1H), 6.67 (d, $J = 11.3$ Hz, 1H), 6.63 (dd, $J = 8.4$ Hz, $J = 2.4$ Hz, 1H). ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$, 298 K): δ (ppm): 160.8, 157.2, 155.3, 147.7, 137.8, 133.7, 130.9, 129.8, 124.8, 120.7, 120.3, 116.6, 112.9, 108.4. HRMS (ESI-) calc. for $[\text{M}-\text{H}]^-$ ($\text{C}_{14}\text{H}_8\text{NO}_4$): 254.0459, found 254.0456.

7-nitrobenzo[*b*]floxepin-3-yl acetate (2j), Yield 63% (0.187 g). mp. 133 °C. ^1H NMR (500 MHz, $\text{DMSO}-d_6$, 298 K): δ (ppm): 7.96 – 7.93 (m, 2H), 7.25 (d, $J = 9.1$ Hz, 1H), 7.17 (d, $J = 8.4$ Hz, 1H), 7.00 (d, $J = 2.2$ Hz, 1H), 6.93 (dd, $J = 8.3$ Hz, $J = 2.3$ Hz, 1H), 6.80 (d, $J = 11.4$ Hz, 1H), 6.66 (d, $J = 11.4$ Hz, 1H), 2.31 (s, 3H). ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$, 298 K): δ (ppm): 169.0, 156.9, 156.4, 152.6, 148.3, 137.0, 132.7, 130.1, 129.5, 127.8, 127.4, 120.2, 118.9, 117.0, 115.2, 21.02. HRMS (ESI+) calc. for $[\text{M}+\text{H}]^+$ ($\text{C}_{16}\text{H}_{12}\text{NO}_5$): 298.0710, found 298.0709.

Dibenzo[*b*]floxepin-3-amine (3a), Yield 59% (0.123 g). mp. 155 °C. ^1H NMR (500 MHz, CDCl_3 , 298 K): δ (ppm): 7.25 – 7.22 (m, 1H), 7.14 – 7.04 (m, 3H), 6.93 (d, $J = 8.2$ Hz, 1H), 6.58 (d, $J = 11.3$ Hz, 1H), 6.51 (d, $J = 2.5$ Hz, 1H), 6.50 (d, $J = 11.3$ Hz, 1H), 6.42 (dd, $J = 8.2$ Hz, $J = 2.3$ Hz, 1H). ^{13}C NMR (125 MHz, CDCl_3 , 298 K): δ (ppm): 158.6, 156.7, 148.6, 131.1, 130.4, 130.1, 129.2, 129.1, 126.5, 124.8, 121.3, 121.1, 111.4, 107.7. HRMS (ESI+) calc. for $[\text{M}+\text{H}]^+$ ($\text{C}_{14}\text{H}_{12}\text{NO}$): 210.0913, found 210.0910.

6-methoxydibenzo[*b*]floxepin-3-amine (3b), Yield 85% (0.203 g). mp. 179 °C. ^1H NMR (400 MHz, CDCl_3 , 298 K): δ (ppm): 7.02 (t, $J = 7.9$ Hz, 1H), 6.95 (d, $J = 8.1$ Hz, 1H), 6.87 (dd, $J = 8.1$ Hz, $J = 1.2$ Hz, 1H), 6.73 (dd, $J = 7.7$ Hz, $J = 1.1$ Hz, 1H), 6.66 (d, $J = 2.0$ Hz, 1H), 6.64 (d, $J = 11.2$ Hz, 1H), 6.53 (d, $J = 11.2$ Hz, 1H), 6.43 (dd, $J = 8.2$ Hz, $J = 2.3$ Hz, 1H), 3.91 (s, 3H), 3.80 (br. s, 2H). ^{13}C NMR (100 MHz, CDCl_3 , 298 K): δ (ppm): 158.4, 151.7, 148.6, 144.7, 132.4, 130.3, 130.0, 126.4, 124.6, 121.3, 120.5, 111.7, 111.4, 108.3, 56.1. HRMS (ESI+) calc. for $[\text{M}+\text{H}]^+$ ($\text{C}_{15}\text{H}_{14}\text{NO}_2$): 240.1019, found 240.1016.

7-methoxydibenzo[*b*]floxepin-3-amine (3c), Yield 84% (0.200 g). mp. 156 °C. ^1H NMR (400 MHz, CDCl_3 , 298 K): δ (ppm): 7.02 (d, $J = 8.4$ Hz, 1H), 6.92 (d, $J = 8.2$ Hz, 1H), 6.69 (d, $J = 2.6$ Hz, 1H), 6.65 (dd, $J = 8.4$ Hz, $J = 2.6$ Hz, 1H), 6.52 (d, $J = 2.3$ Hz, 1H), 6.49–6.41 (m, 3H), 3.80 (s, 3H). ^{13}C NMR (100 MHz, CDCl_3 , 298 K): δ (ppm): 160.9, 157.9, 157.6, 147.8, 130.2, 129.7, 127.8, 126.4, 123.7, 121.5, 111.7, 110.8, 107.9, 106.8, 55.5. HRMS (ESI+) calc. for $[\text{M}+\text{H}]^+$ ($\text{C}_{15}\text{H}_{14}\text{NO}_2$): 240.1019, found 240.1015.

8-methoxydibenzo[*b*]floxepin-3-amine (3d), Yield 84% (0.200 g). mp. 158 °C. ^1H NMR (500 MHz, CDCl_3 , 298 K): δ (ppm): 7.03 (d, $J = 8.5$ Hz, 1H), 6.93 (d, $J = 8$ Hz, 1H), 6.77 (dd, $J = 3$ Hz, 1H), 6.63 (d, 1H), 6.59 (d, $J = 11$ Hz, 1H), 6.49 (d, 1H), 6.45 (d, 1H), 6.41 (dd,

1H), 3.76 (s, 3H). ¹³C NMR (125 MHz, CDCl₃, 298 K): δ (ppm): 158.9, 156.4, 150.5, 148.7, 131.7, 130.6, 130.5, 126.4, 121.9, 120.9, 114.5, 113.4, 111.3, 107.5, 55.6. HRMS (ESI+) calc. for [M+H]⁺ (C₁₅H₁₃NO₂+H) 240.1019, found: 240.1019.

9-methoxydibenzo[b]floxepin-3-amine (3e), Yield 71% (0.170 g). mp. 170 °C. ¹H NMR (500 MHz, CDCl₃, 298 K): δ (ppm): 7.19 (t, *J* = 8.5 Hz, 1H), 6.94 (d, *J* = 8 Hz, 1H), 6.84 (d, *J* = 11.5 Hz, 1H), 6.77 (dd, *J* = 0.5 Hz, 1H), 6.65 (dd, 1H), 6.64 (d, 1H), 6.49 (1H, d, *J* = 2.5 Hz, H₆), 6.42 (dd, 1H), 3.83 (s, 3H). ¹³C NMR (125 MHz, CDCl₃, 298 K): δ (ppm): 158.6, 158.5, 157.0, 148.5, 130.1, 129.3, 129.3, 121.6, 121.3, 120.2, 113.8, 111.5, 107.6, 106.9, 55.9. HRMS (ESI+) calc. for [M+H]⁺ (C₁₅H₁₃NO₂+H) 240.1019, found: 240.1019.

7,8-dimethoxydibenzo[b]floxepin-3-amine (3f), Yield 29% (0.078 g). mp. 223 °C. ¹H NMR (400 MHz, CDCl₃, 298 K): δ (ppm): 6.93 (d, *J* = 8.2 Hz, 1H), 6.69 (s, 1H), 6.59 (s, 1H), 6.56 – 6.48 (m, 2H), 6.48 – 6.38 (m, 2H), 3.88 (s, 3H), 3.83 (s, 3H). ¹³C NMR (100 MHz, CDCl₃, 298 K): δ (ppm): 158.2, 150.1, 149.7, 147.7, 145.8, 130.3, 128.6, 126.4, 122.5, 121.5, 111.8, 110.8, 107.8, 105.1, 56.1, 56.0. HRMS (ESI+) calc. for [M+H]⁺ (C₁₆H₁₆NO₃): 270.1125, found 270.1121.

8-nitrodibenzo[b]floxepin-3-amine (3g), Yield 72% (0.322 g). m.p. 138 °C. ¹H NMR (500 MHz, DMSO-d₆, 298 K): δ (ppm): 8.14 – 8.10 (m, 2H), 7.33 (d, *J* = 9.7 Hz, 1H), 6.94 (d, *J* = 8.3 Hz, 1H), 6.68 (d, *J* = 11.3 Hz, 1H), 6.53 (d, *J* = 11.3 Hz, 1H), 6.41 (d, *J* = 2.2 Hz, 1H), 6.38 (dd, *J* = 8.2 Hz, *J* = 2.2 Hz, 1H). ¹³C NMR (100 MHz, DMSO-d₆, 298 K): δ (ppm): 160.3, 157.0, 152.3, 144.4, 132.7, 132.4, 130.8, 124.3, 124.2, 122.6, 122.6, 116.9, 110.8, 105.7. HRMS (ESI+) calc. for [M+H]⁺ (C₁₄H₁₁N₂O₃): 255.0764, found 255.0763.

benzo[b]naphtho [1,2-*f*]floxepin-9-amine (3h), Yield 25% (0.065 g). mp. 172 °C. ¹H NMR (500 MHz, CDCl₃, 298 K): δ (ppm): 8.09 (d, *J* = 8.5 Hz, 1H), 7.83 – 7.75 (m, 2H), 7.52 (ddd, *J* = 8.4 Hz, *J* = 6.8 Hz, *J* = 1.4 Hz, 1H), 7.43 (ddd, *J* = 8.0 Hz, *J* = 6.8 Hz, *J* = 1.1 Hz, 1H), 7.34 (d, *J* = 8.8 Hz, 1H), 7.29 (d, *J* = 11.4 Hz, 1H), 7.01 (d, *J* = 8.2 Hz, 1H), 6.93 (d, *J* = 11.4 Hz, 1H), 6.57 (d, *J* = 2.3 Hz, 1H), 6.44 (dd, *J* = 8.2 Hz, *J* = 2.3 Hz, 1H), 3.79 (s, 2H). ¹³C NMR (125 MHz, CDCl₃, 298 K): δ (ppm): 159.2, 155.9, 148.8, 131.5, 131.3, 131.1, 130.0, 129.9, 128.3, 126.5, 124.9, 124.4, 123.4, 122.9, 121.5, 121.4, 111.6, 107.3. HRMS (ESI+) calc. for [M+H]⁺ (C₁₆H₁₄NO₃): 259.0997, found 259.0993.

7-aminodibenzo[b]floxepin-3-yl acetate (3j), Yield 38% (0.101 g). mp. 145 °C. ¹H NMR (500 MHz, CDCl₃, 298 K): δ (ppm): 7.09 (d, *J* = 8.4 Hz, 1H), 6.91 (d, *J* = 8.2 Hz, 1H), 6.90 (d, *J* = 2.3 Hz, 1H), 6.84 (dd, *J* = 8.3 Hz, *J* = 2.3 Hz, 1H), 6.55 (d, *J* = 11.3 Hz, 1H), 6.46–6.44 (m, 2H), 6.42 (dd, *J* = 8.1 Hz, *J* = 2.3 Hz, 1H). ¹³C NMR (125 MHz, CDCl₃, 298 K): δ (ppm): 169.1, 158.1, 157.0, 151.3, 148.7, 130.4, 130.0, 129.3, 129.0, 125.6, 120.8, 118.0, 115.0, 111.6, 107.7, 21.1. HRMS (ESI+) calc. for [M+H]⁺ (C₁₆H₁₄NO₃): 268.0968, found 268.0964.

4.2. NMR Measurements

All the spectra were recorded using a Varian VNMRs spectrometer operating at an 11.7 T magnetic field. Measurements were performed for ca. 1.0M solutions of all the compounds in DMSO-d₆ or CDCl₃. The residual signals of DMSO-d₆ (2.54 ppm) and CDCl₃ (7.26 ppm) in ¹H NMR and of the DMSO-d₆ signal (40.45 ppm) and of CDCl₃ (77.0 ppm) in ¹³C NMR spectra were used as the chemical shift references. Spin multiplicities are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and dd (double doublet). Coupling constants are reported in hertz. All the proton spectra were recorded using the standard spectrometer software and parameters set: acquisition time 3 s, pulse angle 30°. The standard measurement parameter set for ¹³C NMR spectra was: pulse width 7 μs (the 90° pulse width was 12.5 μs), acquisition time 1 s, spectral width 200 ppm, 1000 scans of 32 K data point were accumulated and after zero-filling to 64 K; and the FID signals were subjected to Fourier transformation after applying a 1Hz line broadening.

4.3. Biological Evaluation

4.3.1. Cell Culturing

Cells were cultured in a humidified incubator at 37 °C under 5% CO₂. Human colorectal carcinoma (HCT116) cells were grown in McCoy's 5A Medium (ATCC, Catalog

No. 30-2007), human breast adenocarcinoma (MCF-7) and human dermal fibroblasts (HDF-A) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma D5546) supplemented with 2 mM L-glutamine. Human embryonic kidney (HEK293) cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco 31966-021). All culture media were supplemented with 1% Penicillin-Streptomycin (Sigma-Aldrich, P4333) and 10% fetal bovine serum (Gibco, 10270-106). Cells were sub-cultured at approximately 80–90% confluency to maintain the culture in the logarithmic growth phase.

4.3.2. MTT Cytotoxicity Assay

Cells were seeded at a 96-well plate at the density of 7×10^3 cells per well and allowed to grow for 24 h. Afterward, the medium was aspirated and fresh medium was added (200 μ L) with serial dilutions (100 – 10 μ M) of tested compounds or DMSO at corresponding concentrations as a control. Following 48 h of incubation, the medium was replaced with phenol red-free medium (100 μ L) containing MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, 0.5 mg/mL) and replaced in the incubator for 4 h. The formed formazan was dissolved in DMSO (100 μ L) followed by incubation of the mixture at 37 °C for 10 min. The absorbance was measured at 540 nm. After blank subtraction, the half maximal effective concentration (EC_{50}) was calculated by GraphPad Prism software (GraphPad Software Inc, San Diego, CA, USA).

4.3.3. Immunofluorescence

Cells were seeded on coverslips at a 24-well plate at a density of 5×10^4 cells per well and allowed to grow overnight. Next medium was replaced with a medium containing 60 μ M solution of the selected compound and incubated for 24 h. Then, cells were fixed and permeabilized with 100% methanol at –20 °C for 15 min and subsequently washed three times with PBS at room temperature. After 1 h blocking with 3% BSA/PBS at 4 °C, slides were incubated with anti- α -tubulin 12G10 primary antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA) (diluted to 2 μ g/mL in 3% BSA/PBS) overnight at 4 °C. After 3×10 min washing with PBS, slides were incubated with AlexaFluor 555-conjugated secondary antibody (diluted to 50 ng/mL in 3% BSA/PBS) (Thermo Fisher Scientific, Waltham, MA, USA, A31570) for 1 h at room temperature. After washing (3×10 min with PBS), slides were mounted in Fluoromount-G (Southern Biotech., Birmingham, AL, USA). Cells were recorded using Leica TCS SP8 (Leica Microsystems, Wetzlar, Germany) confocal microscope.

4.4. Computational Aspects

The optimum ground-state geometry for selected compounds **1a**, **1c**, **1d**, **1i**, **2i**, **2j**, and **3h** was calculated using density functional theory (DFT) [54]. The B3LYP functional and 6-311++g (2d,p) basis set and the continuum model (PCM; Gaussian 03W) [55] were used to simulate the effects of the solvent -DMSO. All calculations were performed on a server equipped with a 16 quad-core XEON (R) CPU E7310 processor operating at 1.60 GHz. The operating system was Open SUSE 10.3.

Selected compounds were docked into the 3D X-ray structure of $\alpha\beta$ -tubulin heterodimer (PDB code: 1SA0) [59] using the Auto-Dock Vina software (the Broyden-Fletcher-Goldfarb-Shanno (BFGS) method) [60]. The PyMOL graphical user interface and python scripts provided by AutodockTools [61] were employed to set up the $\alpha\beta$ -tubulin structure: chains C, D, and E were removed as well as small molecules (magnesium ion, GDT, GTP, and DAMA-colchicine), and all hydrogens were added. Next python script `prepare_receptor4.py` was used for .pdbqt file generation. The 3D structures of ligand molecules were built, optimized (B3LYP functional and 6-311G 6-311++g (2d,p) basis set level), and saved in .pdb format. The python script `prepare_ligand4.py` was employed to set up the ligand and the .pdbqt file was saved. Auto-Dock Vina software was employed for all docking calculations. The AutoDockTools program was used to generate the docking input files. In docking, a grid box size of $56 \times 56 \times 56$ points in x, y, and z directions was built,

and the maps were center located (39.82, 53.24, −8.21) in the binding site of the protein. A grid spacing of 0.375 Å (approximately one-fourth of the length of a carbon-carbon covalent bond) was used for the calculation of the energetic map. All computations were performed on an Intel® Core™ and 7-4702MQ 3.2 GHz processor running Ubuntu 18.04 Workstation Linux distribution. PyMOL software (www.pymol.org/ (accessed on 1 January 2021)) was used to analyze the docking results [62]. The Protein-Ligand Interaction Profiler (PLIP) was used to predict protein-docked ligand interactions (the default threshold was used for detection steps: 4.0 Å max. distance of carbon atoms for a hydrophobic interaction; 4.1 Å max. distance between acceptor and donor in hydrogen bonds) [63].

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/molecules28083558/s1>. Figures S1–S54. NMR spectra of obtained compounds. Tables S1–S7. The calculated coordinates of compounds (1a, 1c, 1d, 1i, 2i, 2j and 3h). Figures S55–S61. Visualization of calculated geometry of compounds (1a, 1c, 1d, 1i, 2i, 2j, 3h). Table S8. Estimated binding free energy, visualization of binding pose and predicted interactions with $\alpha\beta$ -tubulin heterodimer for compounds (1a, 1c, 1d, 1i, 2i, 2j, 3h).

Author Contributions: Conceptualization, F.B., H.F., H.K. and E.J.; Data curation, F.B. and H.K.; Formal analysis, F.B., P.T. and M.P.; Funding acquisition, P.T., H.K. and E.J.; Investigation, F.B., P.T., M.P., H.F., H.K. and E.J.; Methodology, F.B. and H.K.; Project administration, H.K. and E.J.; Resources, H.F., H.K. and E.J.; Supervision, H.K. and E.J.; Validation, F.B., P.T., M.P. and E.J.; Visualization, F.B. and E.J.; Writing—original draft, F.B., H.K. and E.J.; Writing—review and editing, F.B., P.T., M.P., H.F., H.K. and E.J. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Warsaw University of Technology within the Excellence Initiative: Research University (IDUB) program (NChem 3) and by National Science Centre, Poland grant no. OPUS15 2018/29/B/NZ3/02443 to E.J. This work was implemented as a part of Operational Project Knowledge Education Development 2014–2020 co-financed by the European Social Fund, Project No POWR.03.02.00-00-1007/16-00 (POWER 2014–2020) and by the Diamond Grant project (the scientific work financed from budget funds for science in the years 2019–2023).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in Supplementary Materials.

Acknowledgments: The monoclonal anti- α -tubulin 12G10 antibody developed by J. Frankel and E. M. Nelsen was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biology, University of Iowa, Iowa City, IA. The immunofluorescence confocal imaging was performed in the Laboratory of Imaging Tissue Structure and Function, Nencki Institute of Experimental Biology, PAS.

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

Sample Availability: Samples of the compounds are available from the authors.

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Article

First-in-Class Colchicine-Based Visible Light Photoswitchable Microtubule Dynamics Disrupting Agent

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Abstract: Compounds that disrupt microtubule dynamics, such as colchicine, paclitaxel, or Vinca alkaloids, have been broadly used in biological studies and have found application in clinical anti-cancer medications. However, their main disadvantage is the lack of specificity towards cancerous cells, leading to severe side effects. In this paper, we report the first synthesis of 12 new visible light photoswitchable colchicine-based microtubule inhibitors **AzoCols**. Among the obtained compounds, two photoswitches showed light-dependent cytotoxicity in cancerous cell lines (HCT116 and MCF-7). The most promising compound displayed a nearly twofold increase in potency. Moreover, dissimilar inhibition of purified tubulin polymerisation in cell-free assay and light-dependent disruption of microtubule organisation visualised by immunofluorescence imaging sheds light on the mechanism of action as microtubule photoswitchable destabilisers. The presented results provide a foundation towards the synthesis and development of a novel class of photoswitchable colchicine-based microtubule polymerisation inhibitors.

Keywords: photopharmacology; colchicine; tubulin; photoswitches



Citation: Borys, F.; Tobiasz, P.; Fabczak, H.; Joachimiak, E.; Krawczyk, H. First-in-Class Colchicine-Based Visible Light Photoswitchable Microtubule Dynamics Disrupting Agent. *Cells* **2023**, *12*, 1866. <https://doi.org/10.3390/cells12141866>

Academic Editors: Armando Varela-Ramirez, Elisa Robles-Escajeda, Blanca E. Ruiz-Medina, Patricia Talamás-Rohana and Rachid Skouta

Received: 23 May 2023

Revised: 10 July 2023

Accepted: 11 July 2023

Published: 16 July 2023



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1. Introduction

Photopharmacology is an emerging method based on incorporation of photoswitchable component—molecular switches into the skeleton of a parent compound with expected biological activity. The goal of photopharmacology is to reduce the effects of drug substances apart from the cellular target and severe systemic/environmental side effects by establishing an external and selective means of controlling the activity of these compounds with time and spatial precision. It involves the design, synthesis, research, and application of drugs in the form of photochromic molecular switches which can be regulated by light [1–4]. Although photopharmacology is a relatively new technique that has not yet found clinical application, recent years have abounded with outstanding research towards the development of novel photoresponsive bioactive compounds including G protein-coupled receptors (GPCRs) agonists [5–7], ion channels activity modulators [8–10], and enzyme inhibitors [11]. Azobenzenes are photoswitches that can have many applications in photopharmacology. They can be switched between the (*E*) and (*Z*) configurations by light [12,13]. Due to their small size, high quantum efficiency, high extinction coefficients, low photobleaching factor, and easy synthesis, they are perfect structural elements for creating complex optical tools—they require low-intensity light and, because of their stability, they can be switched many times in a large number of cycles [14–16]. For example, the action of photopharmaceuticals containing skeletal fragments of azobenzene as a functionalizing unit allows for control of biological functions with precision in space and time [17–20]. Classical azobenzenes also have disadvantages that limit their practical use in biological sciences. The first is the necessity to use destructive and harmful-to-cells UV

light necessary to induce (*E*) → (*Z*) isomerisation by excitation of $\pi \rightarrow \pi^*$. The second is incomplete reverse (*Z*) → (*E*) photoisomerisation, caused by radiation with the maximum absorption in the visible region, in which the $n \rightarrow \pi^*$ bands of (*E*) and (*Z*) isomers overlap, which makes it impossible to selectively analyse each of the geometric isomers and select excitation. Therefore, azobenzenes should be modified by introducing various substituents, e.g., halogen, alkoxy, etc. [21–23]. Implementation of ortho-fluorine atoms renders the separation of the $n \rightarrow \pi^*$ absorption bands in the UV-VIS spectrum possible. This enables selective addressing of each geometric isomer and its selective activation [24–27]. Microtubules (MT) are vital cytoskeletal constituent present in eukaryotic cells which are involved in many cellular processes. MT are hollow cylindrical polymers composed of $\alpha\beta$ -tubulin heterodimers noncovalently bounded longitudinally and laterally. Their ability to rapidly reorganise from growing (by incorporating new $\alpha\beta$ -tubulin subunits at the (+) end of MT) to shrinking (by removal of $\alpha\beta$ -tubulin subunits) and vice versa, known as “dynamic instability”, is crucial for MT bioactivity and can be modified by small molecules known as microtubule-targeting agents (MTAs). These compounds can be divided into two main groups, depending on their influence on polymerised tubulin mass at high concentrations, namely microtubule-stabilising agents (e.g., taxanes and epothilones) and microtubule-destabilising agents (e.g., vinca alkaloids, maytansines, and colchicine derivatives) [28,29]. In turn, when low concentrations are applied, both classes suppress microtubule dynamics [30–32]. Importantly, microtubule-targeting agents disrupt formation and proper activity of mitotic spindle, leading to impaired chromosome segregation during mitosis and, consequently, cell death [33]. Over the past few decades, hundreds of MTAs have been synthesised and evaluated for their bioactivity [34–36]. However, there are only a handful of classes of photoswitchable microtubule-targeting agents (PMTAs) that are currently known [19]. Studies from three independent groups have described the first potent photoswitchable analogues of combretastatin A-4 **CA4**, namely photostatins **PTS-1**, in which the isosteric nitrogen–nitrogen double bond replaces the carbon–carbon double bond of **CA4** (Figure 1a) [37–39]. Since then, other photoswitchable microtubule-destabilising agents based on **CA4** analogues have been developed, e.g., hemithioindigos **HOTub-31**, **PHTub-7** [40–42], spiropyran [43,44], and styrylbenzothiazoles (**SBTub-A4**) [45,46] (Figure 1a). Recently, photoswitchable plinabulin-based microtubule inhibitors have been developed [47]. Despite extensive research towards novel PMTAs, no colchicine-based photoswitchable microtubule-destabilising agents have yet been described. In contrast, only two classes of photoswitchable microtubule-stabilising agents have been published: paclitaxel-based [48] and epothilone-based photoswitchable microtubule stabilisers [49] (Figure 1b). Each set of compounds has its own disadvantages and advantages. In cases where UV light is used to induce photoisomerization in a biological context, several inherent limitations have to be taken into account. UV light has low tissue penetration ability [50], and hard UV light might lead to DNA mutations; thus, it is toxic to normal cells. Furthermore, high-energy UV light can cause irreversible photolysis (e.g., photooxidation, photoisomerisation, free radical formation) and thus cannot be applied in some pharmacophore structures. It was shown that exposure to UV light irradiation of colchicine causes the formation of β -lumicolchicine, γ -lumicolchicine, α -lumicolchicine, and loss of bioactivity [51,52].

In this study, our goal was to develop novel visible light photoswitchable colchicine-based microtubule disrupting agents and assess their antiproliferative activity against selected tumorous cell lines.

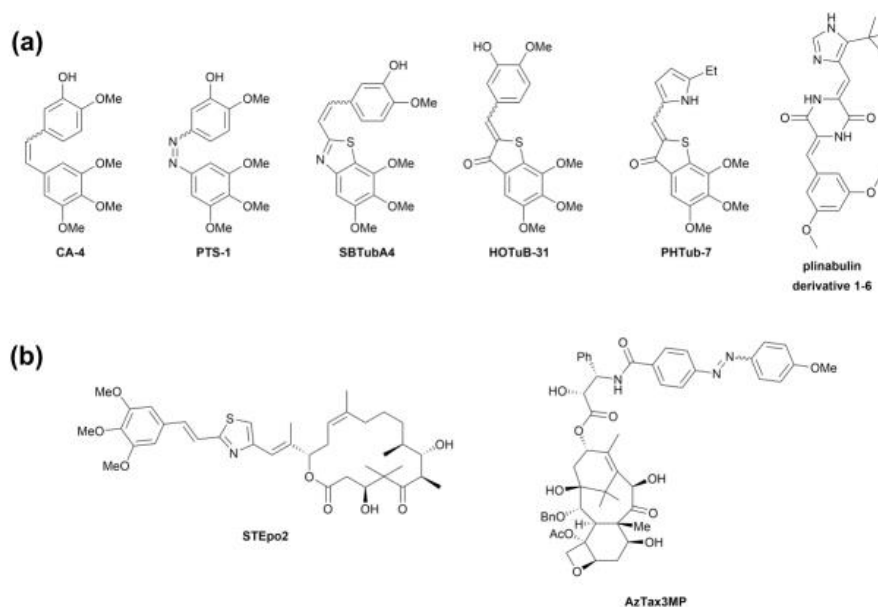


Figure 1. Structures of (a) combretastatin A-4 CA4 and known photoswitchable microtubule destabilisers and (b) stabilisers.

2. Materials and Methods

2.1. Synthesis

Compound characterisation and copies of NMR spectra are provided in the Supporting Information.

(*R*)-*N*-deacetyl colchicine was synthesised according to a protocol previously published [53].

General procedure A: the aqueous solution (35 mL) of oxone® (3.5 mmol) was added, dropwise, to the solution of aniline derivative **1a–d** (1 mmol) in dichloromethane (20 mL). The reaction mixture was vigorously stirred at room temperature. After disappearance of the starting material (analysed by TLC), the reaction was quenched by addition of NaHCO₃. After separation, the aqueous phase was extracted twice with DCM. The combined organic layers were dried over MgSO₄ and concentrated in a vacuum. The residue containing nitrosoarene **2a–d** was dissolved in acetic acid (50 mL) and appropriate isomer of aminobenzoic acid (1 mmol) was added. The reaction was stirred at room temperature for 24 h and then poured into water. The crude product was collected by filtration and recrystallized from ethyl acetate to afford the analytically pure product. For soluble products, the solvent was evaporated, and the residue was subjected to column chromatography with 1% of acetic acid in DCM used as eluent.

General procedure B: a solution of appropriate azobenzene *m*-, *p*-**3a–d** obtained from procedure A (0.3 mmol) in DMF (5 mL) was added to (*R*)-*N*-deacetyl colchicine (0.15 mmol), HATU (0.15 mmol), and DIPEA (0.9 mmol) under argon atmosphere. The mixture was stirred at room temperature for 4 h and then diluted with ice-cooled water and extracted with ethyl acetate (2 × 40 mL). Combined organic layers were washed with brine, dried over MgSO₄ and evaporated under reduced pressure. The residue was purified by silica gel column chromatography (DCM/MeOH 9:1).

General procedure C: thionyl chloride (5.0 mmol) was added to a solution of 2-aminobenzoic acid (1.05 mmol) in toluene (5 mL), and the mixture was refluxed for 4 h.

Next, the solvent was evaporated under reduced pressure to obtain the crude acid chloride as yellow oil, which was used immediately in the next step without any purification. Et₃N (1.05 mmol) was added to a solution of (*R*)-*N*-deacetyl colchicine (1.0 mmol) in DCM (10 mL) at 0 °C and stirred for 15 min. The solution of acid chloride in methylene chloride (5 mL) was added dropwise to the latter mixture at 0 °C and stirred overnight. Thereafter, the solvent was removed under reduced pressure and the residue was purified by column chromatography (ethyl acetate/acetone 4:1) to afford intermediate **S1**. In subsequent reactions, nitrosobenzenes **2a–d** (obtained as in general procedure A, 0.2 mmol) was dissolved in acetic acid (5 mL) and intermediate **S1** (0.15 mmol) was added in DCM (5 mL). The reaction was stirred at room temperature for 24 h and then the solvent was evaporated. The residue was dissolved in ethyl acetate (10 mL), washed with NaHCO₃ (2 × 2 mL) water (2 mL), dried over MgSO₄, and concentrated in a vacuum. The product was purified by silica gel column chromatography (DCM/MeOH 95:5).

2.2. Tubulin Polymerisation Assay

Tubulin from porcine brain was purified according to a protocol published previously [54]. The tubulin polymerisation reaction was conducted at 3.5 mg/mL tubulin, in a tubulin polymerisation buffer (80 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) pH = 6.9; 0.5 mM EGTA; 2 mM MgCl₂), in a 96-well plate (100 µL), in a EnSpire[®] multimode plate reader (PerkinElmer, Turku, Finland) with temperature maintained at 37 °C. Tubulin was initially preincubated for 30 min at room temperature with (*Z*) enriched isomer (green light pre-illuminated) or with all (*E*) isomer (thermally adapted in dark) of *o*-AzoCol26DF (10 µM) in buffer with 1% DMSO, without GTP. A sample with 1% DMSO alone was used as a control. GTP was added to the concentration 1 mM, and the change in absorbance at 340 nm was monitored at 15 s intervals for 20 min. A solution of colchicine (5 µM) or cosolvent (DMSO) was used as a control.

2.3. Cell Culturing

Cells were cultured in a humidified incubator at 37 °C under 5% CO₂. Human breast adenocarcinoma (MCF-7) cells were maintained in phenol red-free Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, Waltham, MA, USA, 11054020) supplemented with 2 mM L-glutamine, and human colorectal carcinoma (HCT116) cells were maintained in the same medium but supplemented with 4.5 g/L glucose and 4 mM L-glutamine. All culture media were supplemented with 1% Penicillin-Streptomycin (Sigma-Aldrich, Burlington, MA, USA, P4333) and 10% fetal bovine serum (Gibco, Billings, MT, USA, 10270-106). Cells were sub-cultured at approximately 70–90% confluency to maintain the culture in the logarithmic growth phase.

2.4. MTT Cytotoxicity Assay with Green and Blue Light Irradiation

Cells were seeded in a 96-well plate at the density of 7×10^3 cells per well and allowed to grow for 24 h. Afterwards, the medium was aspirated, and a fresh medium was added (200 µL) with serial dilutions of tested compounds or DMSO at corresponding concentrations as a control. Following 48 h of incubation under 500 ms pulsed green or blue light irradiation every 15 s, the medium was replaced with a medium (100 µL) containing MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, 0.5 mg/mL) and incubated at 37 °C for 4 h. The formed formazan was dissolved in DMSO (100 µL) and incubated at 37 °C for 10 min. The absorbance was measured at 540 nm. After blank subtraction, the half maximal effective concentration (IC₅₀) was calculated by GraphPad Prism software version 7 (GraphPad Software Inc., San Diego, CA, USA). Each independent experiment was performed in triplicate.

2.5. Immunofluorescence

Cells were seeded on coverslips on a 24-well plate at a density of 5×10^4 cells per well and allowed to grow overnight. Next, the medium was replaced with a medium

containing the *o*-AzoCol26DF or the colchicine (without illumination) or DMSO as control and incubated for 24 h under 500 ms pulsed green or blue light irradiation every 15 s. Then, cells were fixed and permeabilised with 100% methanol at -20°C for 15 min and subsequently washed three times with PBS at room temperature. After 1 h blocking with 3% BSA/PBS at 4°C , slides were incubated with anti- α tubulin 12G10 antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA) (diluted 1:300 in 3% BSA/PBS) and anti-acetylated α - tubulin antibody (Cell Signalling, Danvers, MA, USA) (diluted 1:1000 in 3% BSA/PBS) overnight at 4°C . After 3×10 min washing with PBS, slides were incubated with AlexaFluor555-conjugated anti-rabbit and AlexaFluor488-conjugated anti-mouse secondary antibodies (diluted 1:400 in 3% BSA/PBS) (Thermo Fisher Scientific, Waltham, MA, USA, A31570) and with DAPI (50 ng/mL) for 1 h at room temperature. After washing (3×10 min with PBS), slides were mounted in Fluoromount-G (Southern Biotech., Birmingham, AL, USA). Images were recorded using Leica TCS SP8 (Leica Microsystems, Wetzlar, Germany) confocal microscope and analysed using ImageJ 1.53t software.

2.6. Cell Cycle Stages Analysis

Approximately 10^6 cells were plated and treated with DMSO, colchicine, or *o*-AzoCol26DF. Next, the medium was replaced with a medium containing solution of the *o*-AzoCol26DF or solution of the colchicine (without illumination) or DMSO as control and incubated for 24 h under 500 ms pulsed green or blue light irradiation every 15 s, as described above. After 24 h, cells were harvested, fixed with 70% ethanol, and stained with 50 $\mu\text{g/mL}$ propidium iodide with presence of 50 $\mu\text{g/mL}$ RNase A. Stained cells were immediately analysed in Cytometer BD FACSCalibur (BD, Franklin Lakes, NJ, USA).

3. Results and Discussion

3.1. The Computational Study

As mentioned in the introduction, the implementation of fluorine atoms makes it possible to separate the $n \rightarrow \pi^*$ absorption bands in the UV-VIS spectrum and to separate them from the $\pi \rightarrow \pi^*$ band. This is why we first computed and analysed the geometry proposed by using compounds, *o*-, *m*-, *p*-3a–d for (*E*) and (*Z*) isomers, using the density functional theory (DFT). In the calculations, the B3LYP functional, 6-31G*, and basis set was employed and the continuum model (PCM; Gaussian 03W, see Supporting Information) was used to simulate the effects of the solvent, DMSO [55,56]. This method successfully reproduces the relative energies of the isomers of many azobenzene derivatives, including bridged azobenzenes [57,58]. The SCF energy for the (*E*) and (*Z*) isomers of *o*-, *m*-, *p*-3a–d is presented in Table S1 in the Supporting Information. For all compounds, the (*E*) isomer has a lower energy (mostly 55.8–59.8 kJ/mol). The smallest differences were observed for (*E*)-*p*-3d/(*Z*)-*p*-3d and (*E*)-*m*-3d/(*Z*)-*m*-3d at 43.38 kJ/mol and 45.98 kJ/mol, respectively. Moreover, for compounds (*E*)-*o*-3b/(*Z*)-*o*-3b and (*E*)-*o*-3a/(*Z*)-*o*-3a, due to the large spherical hindrance, differences are the largest at 81.08 kJ/mol and 80.61 kJ/mol, respectively. In the case of (*E*)-*o*-3d/(*Z*)-*o*-3d and (*E*)-*o*-3c/(*Z*)-*o*-3c, the hydrogen bonds between fluorine of first ring and hydrogen of second ring can be observed, such bonds reducing the energy difference between the (*E*) and (*Z*) isomers (56.13 kJ/mol and 56.78 kJ/mol, respectively, see Supporting Information). The geometry of the respective photoswitch has a strong influence on the $n \rightarrow \pi^*$ excitation energies. To determine whether the conformational changes of the photoswitch structures provide shifts in the excitation energies, we calculated the energy of (*E*) and (*Z*) orbitals HOMO and LUMO for switches *o*-, *m*-, *p*-3a–d (Table S2). It is known from the framework of MO theory that the lowest excited states of azobenzenes can be quite well described using singly excited $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ configurations [59]. The separation of n and π orbitals by symmetry is easy for the planar (*E*) isomers, and the relevant orbitals, i.e., π , n , and π^* , are readily recognised in MO calculations also for the differently shaped (*Z*) isomer. Analysing the obtained data, we can observe that, for all compounds, the π^* orbital level is much higher in the (*Z*) isomer relative to the (*E*) isomer.

This relates to the fact that in the (Z) isomers the π -electron delocalisation is reduced due to the large dihedral angles about the N–C single bonds. The n-orbital energy level in the (Z) isomer is also much higher than in the E-isomer. This effect is connected with the linearity of the (E) isomer and the interaction of the lone pair orbitals on the two neighboring N atoms through bonds. In the case of the nonlinear (Z) isomer, the lone pair orbitals interact much more strongly through space. As described by Hecht [24,26], the repulsive interaction of the nitrogen lone pairs increases the n-level in azobenzenes and the introduction of a fluorine atom (σ -electron withdrawing groups) to aromatic ring; especially in ortho position, it should lower the n-orbital energy. It is worth noting that the $n \rightarrow \pi^*$ excitation energies are very similar for both (E) and (Z) isomers. This conclusion is consistent with the results of Ali et al. and Hecht et al. obtained for other fluorinated compounds [24,59]. The energy differences of the (E) and (Z) orbitals HOMO and LUMO are small, ranging from 3.597 eV to 3.935 eV. The information obtained theoretically was verified by synthesis of *o*-, *m*-, *p*-**3a–d** compounds (Figure 2) and by measuring their UV-Vis spectra (see Supporting Information).

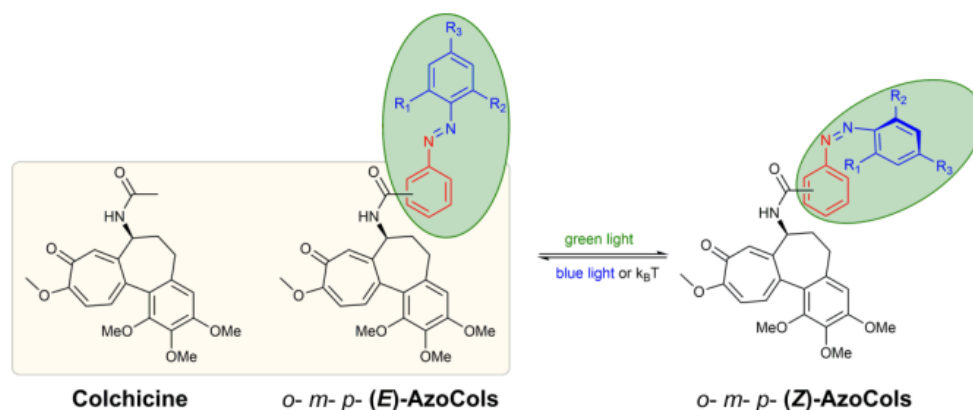
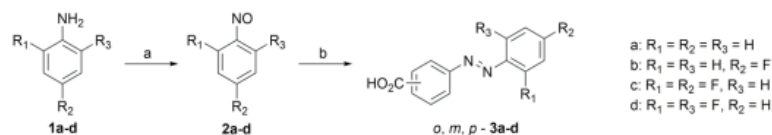


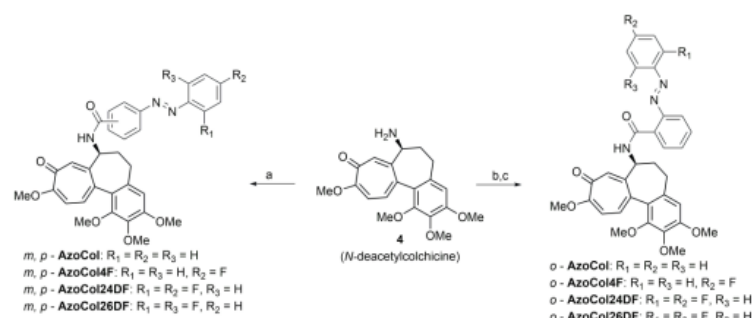
Figure 2. Structure of colchicine and rationale design of azobenzamides-colchicines **AzoCols**.

3.2. Design and Synthesis of Azocolchicines

Our synthetic strategy towards photoswitchable azobenzamides-colchicines **AzoCols** is based on the replacement of the acetoamide group of a well-known and potent microtubule disrupting agent, colchicine, with an azobenzene unit (Figure 2). Previous data suggest that substitutions at this position are well tolerated and do not lead to loss of bioactivity [60,61]. Hence, we anticipated that this approach would maintain the antiproliferative activity and simultaneously allow for precise spatiotemporal control of its activity with light irradiation. Recently, we explored the various synthetic methods to obtain azo compounds. Utilising the optimised conditions, we focused on a one-step method with oxone synthesis [62]. We started from aniline derivatives substituted with a fluorine atom at various positions **1a–d** and oxidised them to corresponding nitroso compounds **2a–d** by reaction with potassium peroxydisulfate in biphasic dichloromethane/water solution. Obtained nitroso derivatives were used in Baeyer–Mills reactions with *ortho*-, *meta*-, and *para*-aminobenzoic acid affording azobenzenes *o*-, *m*-, *p*-**3a–d** (Scheme 1) [14]. A one-pot condensation reaction between *N*-deacetylcolchicine **4** and *meta*- or *para*-**3a–d** isomers allowed for a straightforward synthesis of photoswitchable azobenzamides-colchicines *m*-, *p*-**AzoCols**. Unfortunately, reactions with *ortho*-**3a–d** resulted in a complicated, inseparable products mixture. Thus, we decided to react **4** with 2-aminobenzoyl chloride in the presence of triethylamine and, in the following step, with nitrosobenzene **2a–d**. This synthetic route resulted in the desired target compounds *ortho*-**AzoCols** (Scheme 2).



Scheme 1. Synthetic route towards azobenzenes *o*-, *m*-, *p*-**3a–d**. Reagents and conditions: (a) potassium peroxydisulfate, DCM/H₂O (1:1 v/v); (b) *ortho*-, *meta*-, or *para*- isomer of aminobenzoic acid, AcOH, RT, 24 h.



Scheme 2. Synthesis strategy towards photoswitchable azobenzamides-colchicines *o*-, *m*-, *p*-**AzoCols**. Reagents and conditions: (a) *m*-, *p*-**3a–d**, HATU, DIPEA, DMF, RT, Ar, 4 h; (b) 2-aminobenzoic acid chloride, NEt₃, DCM, 0 °C to RT, 12; (c) **2a–d**, AcOH/DCM (1:1 v/v), 24 h.

3.3. Photochemical Characterisation

Photochemical properties of **AzoCols** photoswitches are attributed to azobenzene **3a–d** moiety incorporated into the parent pharmacophore structure; therefore, we evaluated the photochemical properties of obtained azobenzenes by NMR spectroscopy and UV-Vis spectrophotometry. As a solvent, we chose DMSO due to its ability to dissolve polar and nonpolar molecules, which is crucial for the analytical methods used in this study. Additionally, its intermediate polarity allows for good approximation of organic and aqueous solvents. Furthermore, in the context of photopharmacology, DMSO is used for stock solution preparation which is illuminated and then diluted into aqueous systems for biological activity assessment [63–65]. The photoisomerisation of azobenzenes **3a–d** is not altered upon condensation with colchicine (see Supporting Information Figures S2 and S3). We assumed that ultraviolet light would cause photocatalyzed degradation of azobenzamides-colchicines **AzoCols**. Indeed, irradiation of *p*-**AzoCol4F** with UV light (365 nm) led to the formation of a complex mixture of products (see Supporting Information Figure S4). This result confirms that ultraviolet light is incompatible with photoswitchable ligands based on the colchicine structure. Therefore, we determined the distribution of (*E*) and (*Z*) isomers for azobenzenes *o*-, *m*-, *p*-**3a–d** at the photostationary state (PSS) under constant illumination with selected wavelengths of the visible spectrum (390–610 nm) by ¹H or ¹⁹F NMR analysis (see Supporting Information Figures S6–S17). The obtained results are summarised in Figure 3a. The green light (505–535 nm) induced (*E*) → (*Z*) photoconversions, affording the highest PSS ratios. On the other hand, blue light (390–430 nm) induced reverse (*Z*) → (*E*) photoconversion, affording low PSS compositions. The lowest PSS percentages were obtained for compounds without a fluorine substituent, i.e., *o*-, *m*-, *p*-**3a** (46, 35, and 35%, respectively), while introducing a fluorine atom at 4- and 2,4-positions in compounds *o*-, *m*-, *p*-**3b–c** caused only a slight increase in PSS ratios. The azobenzenes bearing an ortho-fluorine substituent, i.e., *o*-, *m*-, *p*-**3d**, displayed the highest PSS compositions (88, 77, and 71%, respectively). After selection of optimal wavelengths, we acquired UV-Vis absorption spectra for azobenzenes *o*-, *m*-, *p*-**3a–d** in dimethyl sulfoxide at 500 μM concentrations for visualisation and analysis of weak n-π* bands. Spectroscopic data are summarised in

Table 1. In general, the strong π - π^* transition band was observed at 315–330 nm and the weaker n - π^* band at approximately 445 nm for (*E*) isomers. After illumination with green light (causing photoconversion to the (*Z*) isomer), a decrease in π - π^* band intensity and an increase in n - π^* bands were observed. Most importantly, for compounds *o*-, *m*-, *p*-**3d**, due to the introduction of an *ortho*-fluoro substituent, causing stabilisation of nonbonding electron pairs of the azo-bond, significant separation of the $n \rightarrow \pi^*$ transition band of the (*E*) and (*Z*) isomers (around 30 nm) was observed (Figure 3b), allowing for selective addressing of both isomers with visible light. Multiple cycles of photoreversible switching under alternating green and blue light irradiation without noticeable photobleaching or degradation confirmed repeatable and robust photochromic conversion of the obtained azobenzenes (Figure 3c). Moreover, we checked photochemical stability of *o*-AzoCol26DF and corresponding azobenzene *o*-**3d** in cell growth media at conditions similar to photopharmacological assays (high glucose medium supplemented with 4 mM L-glutamine, 500 ms pulses of green or blue light every 15 s for 48 h, 10% of DMSO). No changes in spectrum indicating degradation were observed. The only change in spectrum was attributed to (*E*) $\rightarrow$ (*Z*) photoconversions (see Supporting Information Figure S5). All photoswitches displayed substantially slower spontaneous (*Z*) $\rightarrow$ (*E*) relaxation (the half-life at 37 °C varied from 5 h to >48 h) than the biological assays timescale. Stability in organic and aqueous media, near-ideal photochemical characteristics, and bidirectional photoswitching showed that the obtained molecular switches can be used in photopharmacological assays or in vivo.

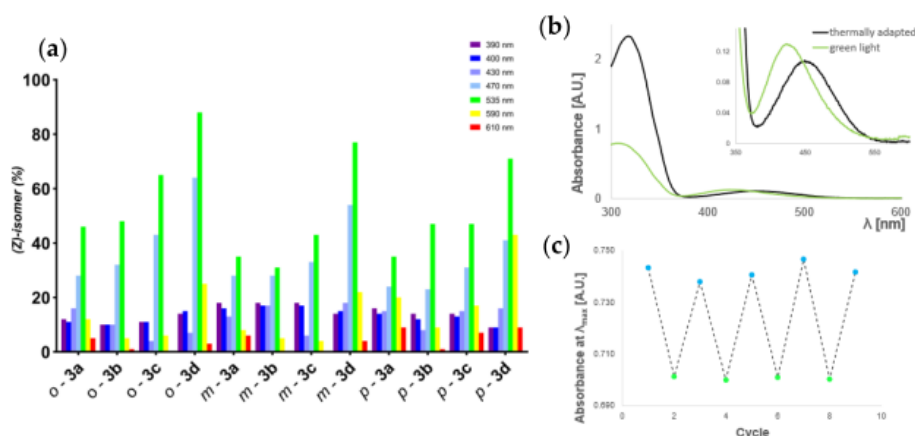


Figure 3. (a) Photostationary state (PSS) compositions for azobenzenes *o*-, *m*-, *p*-**3a–d** determined by ^1H or ^{19}F NMR analysis ($c \approx 10$ mM in $\text{DMSO-}d_6$); (b) representative UV-Vis absorption spectra of thermally adapted and green light (535 nm) irradiated 500 μM *o*-**3d** in DMSO; (c) representative multiple photoswitching rounds of 50 μM *o*-**3d** in DMSO by pulsed green (535 nm) and blue (430 nm) light illumination.

3.4. Photocontrollable in Cellulo Studies

Colchicine is a potent microtubule polymerisation inhibitor leading to mitotic arrests and, as a consequence, cell death [66]. We expected photoswitchable azobenzamides-colchicines **AzoCols** to show similar antiproliferative activity. Therefore, we decided to evaluate in cellulo cytotoxicity for twelve obtained colchicine analogues at the most favourable illuminating conditions (430 nm for predominantly (*E*) and 535 nm for predominantly (*Z*) isomer, respectively). For irradiation of cell cultures, we used self-built arrays of 24 low-power light-emitting diodes (LED) controlled by the Adurino board (see Supporting Information). Such an automated system allowed for precise pulsed illumination (500 ms

pulses of light every 15 s in our experiments) during long-term assays. Most importantly, it has been proven to be compatible with cell culturing conditions [37,40,42,45,46]. We first screened for bioactivity on an MCF-7 cell line as a model for human breast adenocarcinoma (N = 1). For lead compound exhibiting light-dependent cytotoxicity, we further expanded our research to include a HCT116 cell line (human colorectal carcinoma) and a HEK293 cell line (human embryonic kidney). The obtained results are summarised in Table 2. Notably, all **AzoCols** showed potent antiproliferative activity (IC₅₀ ranging from 28 to 187 nM), clearly demonstrating that incorporation of azobenzene moiety neither suppress binding to tubulin nor disrupt permeation through the cytoplasmic membrane. The *meta*-**AzoCols** set of compounds displayed equipotent bioactivity under illumination with green or blue light. In contrast, throughout *para*- and *ortho*- isomers, *p*-**AzoCol24DF** and *o*-**AzoCol26DF** showed dissimilar cytotoxicity, dependent on the irradiation conditions. However, for *o*-**AzoCol** and *o*-**AzoCol4F**, we obtained somewhat inconsistent results. In the predominantly (Z) isomer state (green light illumination), *p*-**AzoCol24DF** and *o*-**AzoCol26DF** showed higher potency than in the predominantly (E) isomer state (blue light illumination). The most promising compound, *o*-**AzoCol26DF**, displayed c.a. a double potency shift on HCT116 cells upon illumination with green light.

Table 1. Spectroscopic data for 500 μ M solution of *o*-, *m*-, *p*-**3a-d** in DMSO at 25 °C. The photostationary state (PSS) composition was determined by ¹⁹F or ¹H NMR (c $\approx$ 10 mM in DMSO-*d*₆). Thermal relaxation half-life (t_{1/2}) was measured for 100 μ M DMSO solutions at 37 °C.

Compound	(E)-isomer ^a		(Z)-isomer ^b		t _{1/2} [h]	PSS _(E) ^c (%)	PSS _(Z) ^d (%)
	$\lambda_{\max}$ ($\pi-\pi^*$) [nm]	$\lambda_{\max}$ (n- π^*) [nm]	$\lambda_{\max}$ (n- π^*) [nm]	$\Delta\lambda$ (n- π^*) [nm]			
<i>o</i> - 3a	324	448	440	8	6	95	46
<i>o</i> - 3b	326	445	437	8	5	99	48
<i>o</i> - 3c	330	444	433	11	23	96	66
<i>o</i> - 3d	315	450	420	30	>48	97	88
<i>m</i> - 3a	321	437	429	8	>48	94	35
<i>m</i> - 3b	322	431	423	8	>48	95	32
<i>m</i> - 3c	326	440	423	17	>48	96	46
<i>m</i> - 3d	315	448	417	31	>48	96	77
<i>p</i> - 3a	330	447	440	7	17	91	35
<i>p</i> - 3b	330	446	440	6	16	99	47
<i>p</i> - 3c	334	448	437	11	23	93	47
<i>p</i> - 3d	322	454	421	33	>48	91	71

^a Thermally adapted; ^b PSS after irradiation with green light (535 nm); ^c Percentage of (E) isomer at 430 nm PSS;

^d Percentage of (Z) isomer at 535 nm PSS.

Table 2. Light-dependent cytotoxicity of **AzoCols** from MTT assay.

Compound	Cell Line	IC ₅₀ Blue Light (nM)	IC ₅₀ Green Light (nM)	IC Ratio
<i>p</i> - AzoCol	MCF-7 (N = 1)	48 $\pm$ 2	44 $\pm$ 2	1.1
<i>p</i> - AzoCol4F	MCF-7 (N = 1)	44 $\pm$ 1	43 $\pm$ 1	1.0
<i>p</i> - AzoCol24DF	MCF-7 (N = 3)	50 $\pm$ 1	36 $\pm$ 1	1.4
<i>p</i> - AzoCol26DF	MCF-7 (N = 1)	55 $\pm$ 1	54 $\pm$ 1	1.0
<i>m</i> - AzoCol	MCF-7 (N = 1)	31 $\pm$ 2	27 $\pm$ 1	1.1
<i>m</i> - AzoCol4F	MCF-7 (N = 1)	42 $\pm$ 1	40 $\pm$ 1	1.1
<i>m</i> - AzoCol24DF	MCF-7 (N = 1)	49 $\pm$ 1	47 $\pm$ 1	1.0
<i>m</i> - AzoCol26DF	MCF-7 (N = 1)	45 $\pm$ 2	46 $\pm$ 4	1.0
<i>o</i> - AzoCol	MCF-7 (N = 1)	nc	nc	nc
<i>o</i> - AzoCol4F	MCF-7 (N = 1)	nc	nc	nc
<i>o</i> - AzoCol24DF	MCF-7 (N = 1)	183	174	1.1
<i>o</i> - AzoCol26DF	MCF-7 (N = 3)	184 $\pm$ 4	126 $\pm$ 2	1.5
<i>o</i> - AzoCol26DF	HCT116 (N = 2)	187 $\pm$ 9	97 $\pm$ 2	1.9
<i>o</i> - AzoCol26DF	HEK293 (N = 3)	>250	>250	-
Colchicine	MCF-7	12 ^a	-	-
Colchicine	HCT116	11 ^b	-	-

^a without illumination ref. [67]; ^b without illumination ref. [68]; nc—not calculated.

3.5. *o*-AzoCol26DF Disrupt Tubulin Polymerisation and Cellular Microtubule Organisation in Light-Dependent Manner

To further explore the molecular mechanism of *o*-AzoCols light-dependent cellular activity, we examined the influence of *o*-AzoCol26DF on tubulin polymerisation in cell-free assays with purified tubulin. In the mainly (Z) isomer state (green light), 10 μ M solution of *o*-AzoCol26DF resulted in ca. 40% inhibition of polymerisation over control (referenced to DMSO as 0%), while the thermally adapted state yielded only a 30% polymerisation inhibition (Figure 4b). In comparison, 5 μ M solution of colchicine caused ca. 70% of tubulin polymerisation inhibition (Figure 4b). It is worth noticing that this is a highly nonlinear assay in an environment far from cellular conditions. Thus, these results cannot be used for evaluating potencies, but rather to shed light on the mechanism of action as microtubule destabilisers, as the parent colchicine is. To verify the effect of *o*-AzoCol26DF on the microtubular cytoskeleton in vivo, we incubated HTC116 cells for 24 h with either DMSO (control) or *o*-AzoCol26DF. During the incubation, all cell samples were illuminated with green or blue light as described in the Material and Methods section. As an additional positive control, we used cells treated with 20 nM colchicine without light illumination. In both control samples, non-dividing cells had a dense, well-expanded network of interphase microtubules, while dividing cells formed a bipolar mitotic spindle (Figure 5a,b). A similar cell phenotype was observed in *o*-AzoCol26DF-treated, blue-light-illuminated cells (Figure 5c). In cells treated with 100 nM *o*-AzoCol26DF and activated by the green light, the interphase microtubular cytoskeleton was only slightly less prominent with respect to the control. However, we observed more dividing cells, and the mitotic spindle structure was frequently abnormal, with more than two spindle poles or misaligned microtubules (Figure 5d). This phenotype was even more pronounced in 120 nM *o*-AzoCol26DF-treated and green-light-illuminated cells (Figure 5f). Importantly, similar changes were observed in cells treated with 20 nM colchicine (Figure 5g).

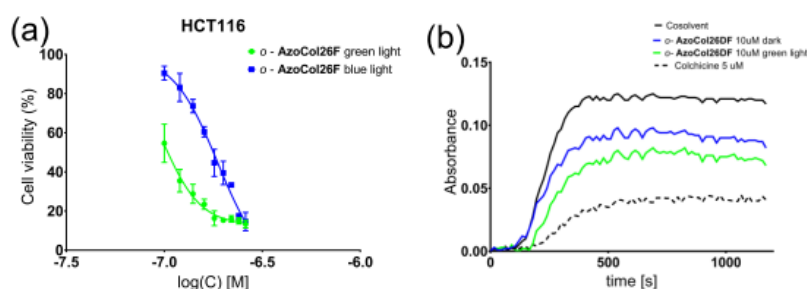


Figure 4. (a) Cell viability curves for *o*-AzoCol26DF under green (535 nm) or blue (430 nm) light irradiation displayed approximately a double shift in potency on HCT116 cells (MTT assay, N = 2); (b) light-dependent influence of *o*-AzoCol26DF (10 μ M) on tubulin polymerisation in cell-free assays. Solution of colchicine (5 μ M) or cosolvent (DMSO) used as a control (curves represent mean from two replicates).

To verify these observations, we determined the distribution of cell cycle stages using FACS analysis (Figure 6). Under the control treatment, within the HTC116 samples, ~31–34% cells were in G1 phase, 22–26% in S phase, and 25–26% in G2/M phases. We also observed some polyploid and apoptotic cells (Figure 6). In 100 nM *o*-AzoCol26DF-treated and blue-light-illuminated cells, the distribution of cell cycle stages was similar to that in control cells; however, we observed a higher number of apoptotic cells. In contrast, in green-light-illuminated 100 nM *o*-AzoCol26DF-treated cells, the number of G2/M cells increased to ~40%, while the number of apoptotic cells was the same as that in blue-light-illuminated cells. Treatment of cells with 20 nM colchicine raised the number of G2/M cells to ~45%, indicating that, likely, 100 nM *o*-AzoCol26DF has similar activity to 20 nM

colchicine. Importantly, the increase in the concentration of *o*-AzoCol26DF to 120 nM elevated the number of G2/M cells to ~60% in green-light-illuminated cells, while in the corresponding blue-light-treated cells G2/M cell numbers remained at 29%.

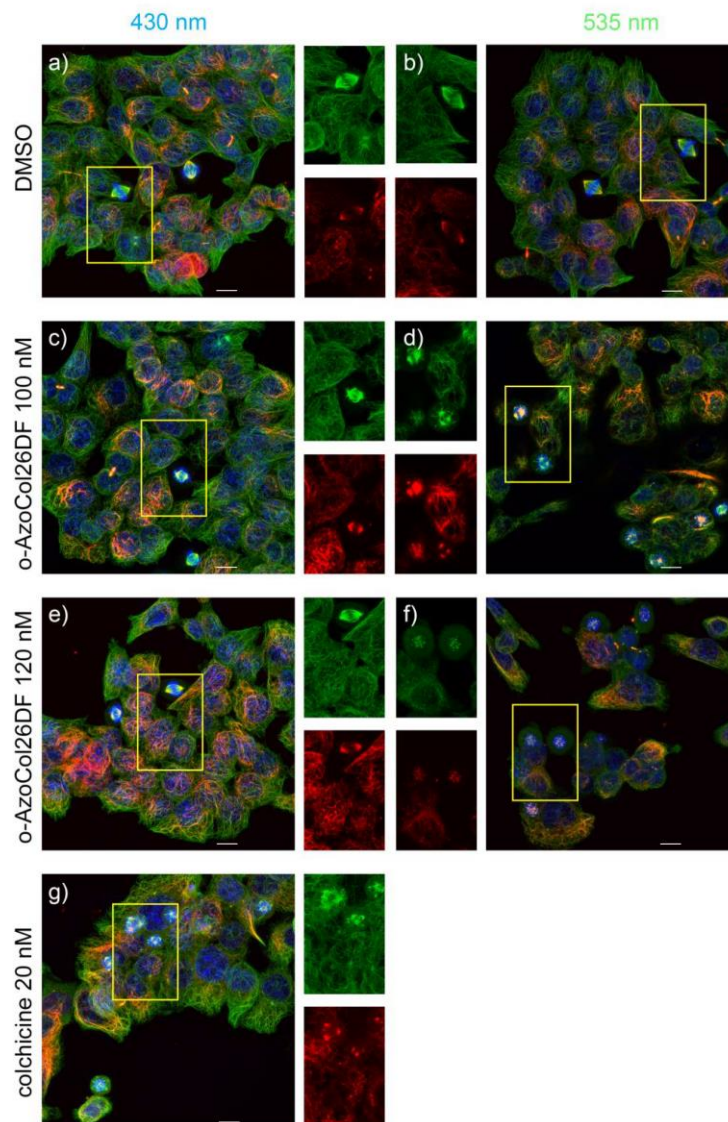


Figure 5. Analysis of HTC116 cells upon treatment with DMSO, *o*-AzoCol26DF, or colchicine. Cells were stained with 12G10 monoclonal antibody against total α -tubulin (green) and with anti-acetylated α -tubulin antibody (red). Nuclei stained with DAPI (blue). Insets show magnification of the mitotic spindle structure. Bars in all subfigures = 10 μ m. Control cells (DMSO) illuminated with blue (a) or green (b) light, respectively. Cells show dense microtubular cytoskeleton and normal cell shape. (c) Cells incubated with 100 nM *o*-AzoCol26DF illuminated with blue light. Notice that microtubular

cytoskeleton resembles control. (d) Cells incubated with 100 nM *o*-AzoCol26DF illuminated with green light. Mitotic spindles frequently are aberrant, showing multipolarity and misarrangement. (e) Cells incubated with 120 nM *o*-AzoCol26DF illuminated with blue light. (f) Cells incubated with 120 nM *o*-AzoCol26DF illuminated with green light. Many mitotic cells with disarranged and/or nearly completely depolymerized mitotic spindle are present. (g) Cells incubated with 20 nM colchicine. The arrangement of mitotic spindles is similar as in cell treated with 100 nM *o*-AzoCol26DF illuminated with green light.

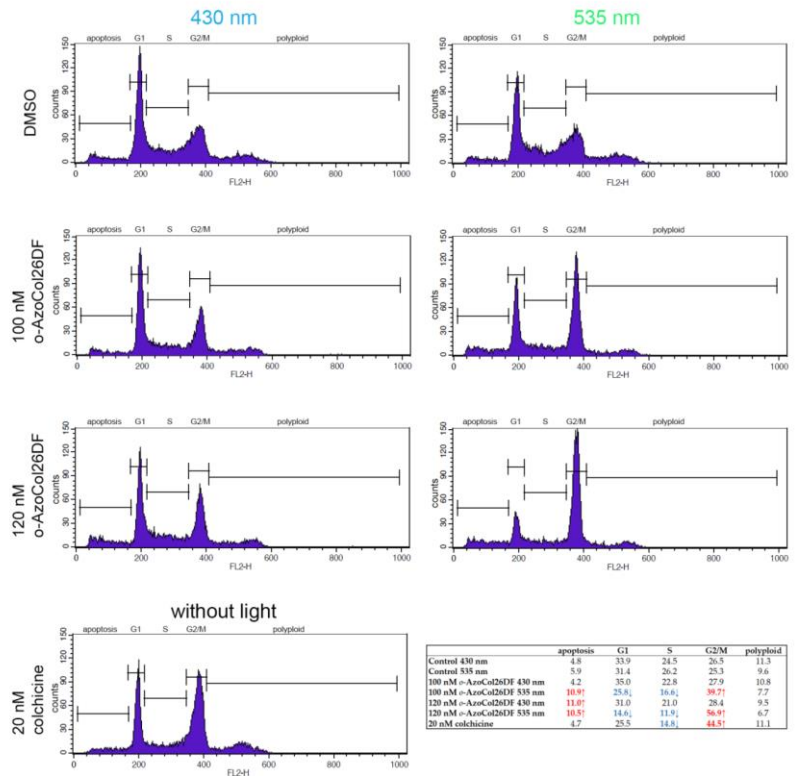


Figure 6. Analysis of cell cycle stages of HTC116 cells upon treatment with DMSO, *o*-AzoCol26DF, or colchicine and blue or green light exposure. Graphs show representative flow cytometry analysis for each sample. In the table, the mean of at least two experiments for each sample are included. The increase with respect to the control is marked by the red color and upwards arrow, while the decrease is marked by the blue color and downwards arrow.

To summarise, under used conditions, we were able to control the activity of *o*-AzoCol26DF and, consequently, disrupt microtubule cytoskeleton leading to mitotic arrest and, eventually, cell death.

4. Conclusions

In this study, we successfully designed and synthesised a set of novel photoswitchable colchicine-based microtubule dynamics disrupting agents. The developed photoswitches can be photoisomerised by visible light instead of UV light, which is used in classical photopharmaceutical reagents. This is crucial due to colchicine instability under UV light

irradiation. For the lead compound *o*-AzoCol26DF, we demonstrated light-dependent cytotoxicity in both the HCT116 and the MCF-7 cancerous cell lines. Inhibition of purified tubulin polymerization, as well as disruption of microtubule organization, support proof-of-concept of using AzoCols as photoswitchable microtubule dynamics disrupting agents. In summary, we have proven that AzoCols provide the basis for further improvement and development of the novel class of photoswitchable colchicine-based microtubule polymerisation inhibitors that could be used in future studies and applications.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cells12141866/s1>, Chemistry: General information and general procedures; Characterization of obtained compounds; NMR & UV-VIS Data; Photostationary State (PSS) Analysis; Photoswitching and Half-Life Determination.; Biological assays; Copies of NMR spectra and Computational calculations.

Author Contributions: Conceptualization, F.B., H.F., H.K. and E.J.; Data curation, F.B. and H.K.; Formal analysis, F.B. and P.T.; Funding acquisition, H.K. and E.J.; Investigation, F.B., P.T., H.F., H.K. and E.J.; Methodology, F.B. and H.K.; Project administration, H.K. and F.B.; Resources, H.F., H.K. and E.J.; Supervision, H.K. and E.J.; Validation, F.B., P.T. and E.J.; Visualization, F.B. and E.J.; Writing—original draft, F.B. and H.K.; Writing—review and editing, F.B., H.F., H.K. and E.J. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by National Science Centre, Poland grant no. OPUS15 2018/29/B/NZ3/02443 to E.J. This work was implemented as a part of Operational Project Knowledge Education Development 2014–2020 co-financed by the European Social Fund, Project No POWR.03.02.00-00-1007/16-00 (POWER 2014–2020).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in Supplementary Materials.

Acknowledgments: The monoclonal anti- α -tubulin 12G10 antibody developed by J. Frankel and E. M. Nelsen was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biology, University of Iowa, Iowa City, IA. The immunofluorescence confocal imaging was performed in the Laboratory of Imaging Tissue Structure and Function, Nencki Institute of Experimental Biology, PAS. Cell cycle analysis was performed in the Laboratory of Cytometry of the Nencki Institute of Experimental Biology.

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

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