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Karbokrzemowe dendrymery z atomami miedzi jako nośniki leków i materiału genetycznego w terapii przeciwnowotworowej

Carbosilane copper dendrimers as drug and gene carriers in anticancer therapy

Praca doktorska

wykonana w Katedrze Biofizyki Ogólnej
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SPIS TREŚCI

Załączniki:

1. Publikacje wchodzące w skład rozprawy doktorskiej
2. Oświadczenia współautorów publikacji wchodzących w skład rozprawy doktorskiej

LISTA UŻYWANYCH SKRÓTÓW

CCD - Carbosialne Copper Dendrimers (Karbokrzemowe dendrymery zawierające atomy miedzi),

CD - Dichroizm kołowy,

DLS - Metoda hydrodynamicznego rozpraszania światła,

LDE - Laserowa elektroforeza Dopplera,

TEM - Transmisyjna mikroskopia elektronowa,

PDI - Indeks polidyspersyjności,

RFT - Reaktywne formy tlenu,

DPH - 1,6-Diphenyl-1,3,5-hexatriene,

TMA-DPH - 1-(4-Trimethylammoniumphenyl)-6-Phenyl-1,3,5-Hexatriene *p*-Toluene sulfonate,

HSA - Albumin surowicy ludzkiej,

siRNA - Małe interferujące RNA,

HL-60 - Komórki ostrej białaczki promielocytowej,

HepG2 - Komórki ludzkiego raka wątroby,

MCF7 - Komórki ludzkiego raka piersi,

PBMC - Jednojądrzaste komórki krwi obwodowej,

1301 - Komórki ludzkiej białaczki limfoblastycznej,

5-Fu - 5-flurouracyl,

DOX - Doksorubicyna,

MTX - Metotreksat

WPROWADZENIE

W ostatnich latach liczba wykrywanych nowotworów znacząco wzrasta. Szacuje się, że w 2020 roku były one powodem ponad 10 milionów zgonów. Mimo znaczącego rozwoju medycyny lekarze wciąż zmagają się z niepowodzeniem terapii nowotworów, a w konsekwencji z wysoką śmiertelnością pacjentów [1–3]. Obecnie najczęściej stosowanymi metodami leczenia tych poważnych chorób są chemioterapia, radioterapia oraz chirurgiczna resekcja tkanek zmienionych nowotworowo. Jednak stosowanie tych metod nie zawsze prowadzi do całkowitej eliminacji tkanek bądź komórek nowotworowych. Ponadto, wspomniane techniki obarczone są wieloma skutkami ubocznymi, które negatywnie wpływają na kondycję pacjentów. To wszystko powoduje, że wciąż poszukuje się nowych podejść terapeutycznych, które podwyższyłyby efektywność stosowanego leczenia, a jednocześnie poprawiły jakość życia pacjentów [1,4,5].

Powstawanie tkanki nowotworowej związane jest z różnego rodzaju mutacjami w obrębie genomu i zaburzeniami procesów naprawczych. Wynikiem tego może być niekontrolowana proliferacja zmutowanych komórek. To prowadzi do tworzenia się ognisk rakowych, a następnie do rozprzestrzeniania się patologicznych komórek i powstawania przerzutów [6–10].

W warunkach fizjologicznych procesem, który chroni organizm przed namnażaniem się nieprawidłowych komórek jest apoptoza, czyli programowana śmierć komórki. Zadaniem apoptozy jest zapewnienie wewnętrznej równowagi w organizmie [11,12]. Apoptoza indukowana jest różnego rodzaju sygnałami, na przykład stresem oksydacyjnym. W regulacji apoptozy uczestniczą m.in. białka z rodziny Bcl-2, wśród których rozróżnia się białka proapoptotyczne i antyapoptotyczne. Według dostępnej literatury komórki nowotworowe charakteryzują się nadekspresją genów kodujących białka antyapoptotyczne. To powoduje zablokowanie mechanizmów indukujących wejście komórek rakowych na drogę apoptozy. W konsekwencji komórki te mogą swobodnie proliferować, tworząc tkankę guza [13–17].

Obecnie jednym z kierunków mogących prowadzić do zwalczenia nowotworów jest terapia genowa z wykorzystaniem siRNA (z ang. *small interfering RNA*). Polega ona na wyciszeniu genów na drodze interferencji. Efektem wprowadzenia terapeutycznego siRNA do komórki rakowej jest degradacja niepożądanego mRNA, co ogranicza syntezę białek odpowiedzialnych za blokowanie apoptozy. W wyniku powodzenia takiej terapii komórki rakowe ulegałyby naturalnej eliminacji bez uszkadzania zdrowych tkanek [11,18–21]. Jednak wprowadzenie siRNA do komórek jest obarczone wieloma trudnościami. Główną z nich jest ujemny ładunek siRNA uniemożliwiający interakcję z ujemnie naładowaną błoną komórkową. Kolejna bariera to wrażliwość na działanie nukleaz. Dlatego też poszukuje się nośnika, który umożliwiłby skuteczną internalizację siRNA do wnętrza komórki, i chroniłby go przed degradacją [15,22–25].

Kolejną przyczyną niepowodzeń w leczeniu nowotworów i wysokiej śmiertelności u pacjentów jest wykształcenie przez zmienione komórki mechanizmu oporności na chemioterapię [26]. Jednym z potencjalnych sposobów poprawy efektywności terapeutycznej chemioterapii jest wykorzystanie odpowiednich nanonośników substancji czynnej. Obecnie sugeruje się, że leki bazujące na nanonośnikach mogą być bardziej skuteczne i mniej toksyczne niż konwencjonalne chemioterapeutyki. Wykorzystanie nanocząstek może poprawić stabilność, biokombatybilność, bioaktywność, bioprzyswajalność i rozpuszczalność skoniugowanych z nimi leków [27–29].

Spośród wielu obecnie testowanych nanocząstek dendrymery wydają się być najbardziej obiecującą platformą transportującą siRNA oraz leki. Dendrymery to nanocząstki zsyntezowane po raz pierwszy w latach 70. XX wieku [25,30,31]. Charakteryzują się unikalnymi właściwościami, takimi jak: rozgałęziona struktura, monodyspersyjność, stabilność termiczna oraz chemiczna. Ponadto, z wcześniej przeprowadzonych badań wynika, że dendrymery zdolne są do tworzenia stabilnych kompleksów z siRNA, w których kwas nukleinowy jest chroniony przed działaniem nukleaz [32–35]. W ostatnich latach bada się też dendrymery pod kątem ich wykorzystania jako nośników leków [31,36]. Klasyczna chemioterapia wymaga stosowania niskich dawek ze względu na jej toksyczne działanie na cały organizm [28]. Terapia z wykorzystaniem dendrymerów pozwala na minimalizację dawek chemioterapeutyku i maksymalizację efektu leczniczego. Według literatury wydaję się być prawdopodobne, że opóźnione uwalnianie leku z dendrypleksu mogłoby rozwiązać też problem lekooporności komórek nowotworowych [29,31]. Od chwili, kiedy po raz pierwszy zsyntezowano dendrymery, nieustannie podejmowane były próby udoskonalania ich struktury i poprawy właściwości. Wiele z nich zakończyło się sukcesem. Niemniej wciąż wydaje się możliwe wyprodukowanie jeszcze lepszych dendrymerów, które poza rolą efektywnego nośnika, same posiadałyby potencjał terapeutyczny. Jedną z takich modyfikacji szkieletu dendrytycznego jest dołączanie do grup powierzchniowych atomów metali wykazujących działanie przeciwnowotworowe. Do metali wykazujących tego rodzaju aktywność należą mi.in. platyna, złoto, srebro, ruten czy miedź [30,37,38]. Miedź jest pierwiastkiem, który odgrywa istotną rolę w procesach zachodzących w żywych organizmach, biorąc udział w ich wzroście i rozwoju. Jest on kluczowym mikroelementem niezbędnym do funkcjonowania niektórych enzymów oraz białek, biorących udział przede wszystkim w metabolizmie energetycznym czy syntezie DNA. Dokładny mechanizm przeciwnowotworowego działania miedzi nie jest jeszcze do końca poznany. Niemniej jednak wiadomo, że jej aktywność przeciwnowotworowa jest związana ze zdolnością do generowania RFT (reaktywnych form tlenu), a związki miedzi indukują apoptozę w komórkach nowotworowych [39–42].

W niniejszej pracy doktorskiej scharakteryzowano kationowe, karbokrzemowe dendrymery zawierające atomy miedzi z ligandami azotowymi i chlorkowymi, zsyntezowane przez zespół Prof. de la Maty z Uniwersytetu w Alcalá de Henares (Hiszpania) i rozważono je jako potencjalne nośniki

proapoptotycznego siRNA oraz chemioterapeutyków (doksorubicyny, metotreksatu i 5-flurouracylu) do komórek [29,38,43].

CEL PRACY

Celem niniejszej pracy doktorskiej była ocena możliwości wykorzystania karbokrzemowych dendrymerów z atomami miedzi jako nośników proapoptotycznego siRNA (siBcl-2 i siMcl-1) oraz leków przeciwnowotworowych (doksorubicyna, metotreksat i 5-flurouracyl) do komórek nowotworowych. Realizacja celu głównego wymagała wyznaczenia następujących etapów:

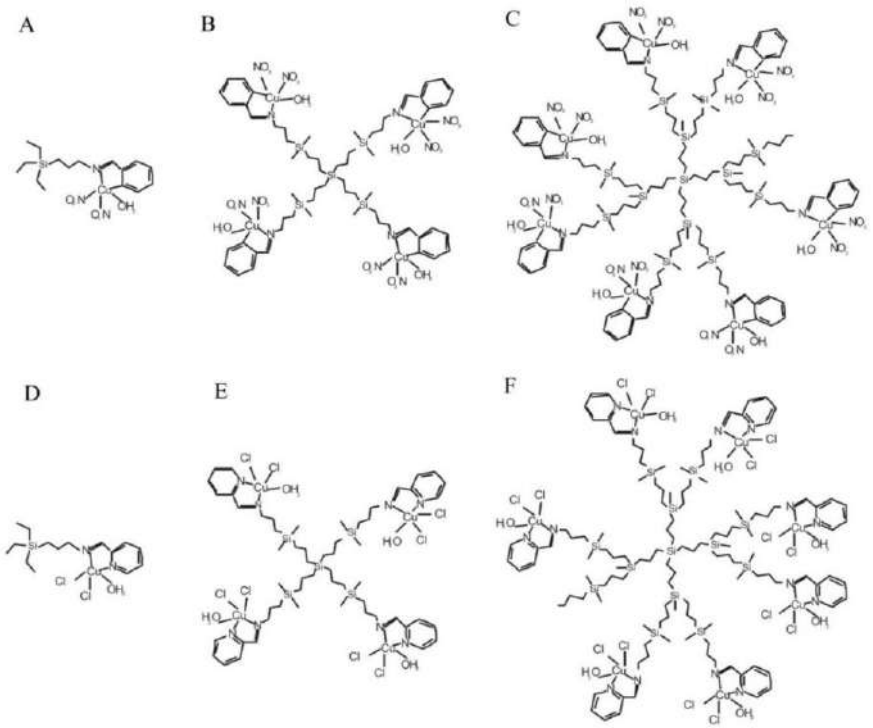
1. Biofizyczna charakterystyka karbokrzemowych metalodendrymerów zawierających atomy miedzi (CCD-NO_n oraz CCD-Cl_n) oraz określenie ich właściwości hemolitycznych i cytotoksycznych.
2. Ocena zdolności Cu(II) metalodendrymerów do tworzenia kompleksów z kwasami nukleinowymi oraz lekami przeciwnowotworowymi, doksorubicyną (DOX), metotreksatem (MTX) i 5-flurouracylem (5-Fu).
3. Określenie aktywności przeciwnowotworowej metalodendrymerów z miedzią skompleksowanych z przeciwnowotworowymi siRNA (siBcl-2 i siMcl-1) lub lekami (DOX, MTX oraz 5-Fu).
4. Ocena terapeutycznego działania kompleksów dendrymer/lek *in vitro*.

HIPOTEZA BADAWCZA

Karbokrzemowe metalodendrymery zawierające atomy miedzi mogą być stosowane jako nośniki leków oraz siRNA do komórek nowotworowych.

PRZEDMIOT BADAŃ

Przedmiotem badań były dwie grupy karbokrzemowych dendrymerów zawierających atomy miedzi -CCD (Carbosilane Copper Dendrimers) różniące się ligandem powierzchniowym. W pierwszej z nich jest to ligand chlorkowy, natomiast w drugiej azotanowy. W skład obu grup wchodzą dendrymery zerowej, pierwszej oraz drugiej generacji (Ryc.1, Tab.1).



Ryc. 1. Struktura karbokrzemowych dendrymerów z atomami miedzi z ligandem azotanowym (A) – CCD-NO-0, (B) – CCD-NO-1, (C) – CCD-NO-2; oraz z ligandem chlorkowym: (D) – CCD-Cl-0, (E) – CCD-Cl-1, (F) – CCD-Cl-2 (Hołota i wsp. 2019).

Tabela 1. Charakterystyka karbokrzemowych dendrymerów z atomami miedzi.

	Generacja	Liczba grup funkcyjnych	Rozpuszczalność	Masa molowa [g/mol]
CCD-NO-0	0	1		468,04
CCD-NO-1	1	4	MeOH/DMF/DMSO	1840,10
CCD-NO-2	2	8		3992,90
CCD-Cl-0	0	1		414,93
CCD-Cl-1	1	4	DMF/DMSO/$CHCl_3$/CH_2Cl_2	1627,68
CCD-Cl-2	2	8		3696,01

MATERIAŁY I METODY

Materiały

W pracy zostały wykorzystane:

- Komercyjnie dostępne leki przeciwnowotworowe, Doksorubicyna (DOX), Metotreksat (MTX) i 5-flurouracyl (5-Fu), (Sigma-Aldrich Sp. Z O.O., Poznań, Polska).
- Proapoptotyczne siRNA Mcl-1 Sense: 5'-GGACUUUUAUACCUGUUAUtt 3'; Antisense: 5'-AUAACAGGUAUAAAAGUCCtg 3; Bcl-2 Sense: 5'-G CUG CAC CUG ACG CCC UUCtt 3'; Antisense: 5'-GAA GGG CGU CAG GUG CAG Ctt 3', oraz znakowane siRNA skoniugowane z FITC, (Darmacon, Inc., Lafayette, CO, USA).
- Albumina surowicy ludzkiej (Sigma-Aldrich Sp. Z.O.O., Poznań, Polska).
- Erytrocyty wyizolowane z krwi obwodowej zdrowych dawców uzyskanej z Regionalnego Centrum Krwiodawstwa i Krwiolecznictwa w Łodzi.
- Błony erytrocytarne wyizolowane z erytrocytów krwi obwodowej zdrowych dawców uzyskanej z Regionalnego Centrum Krwiodawstwa i Krwiolecznictwa w Łodzi.
- Komórki prawidłowe: jednojądrzaste komórki krwi obwodowej (PBMC) wyizolowane z krwi zdrowych dawców uzyskanej z Regionalnego Centrum Krwiodawstwa i Krwiolecznictwa w Łodzi.
- Nowotworowe linie komórkowe: 1301 (komórki ludzkiej białaczki limfoblastycznej), HL-60 (komórki ostrej białaczki promielocytowej), MCF-7 (komórki ludzkiego raka piersi), HepG2 (komórki ludzkiego raka wątroby), (ATCC, Manassas, Virginia, USA).

Metody

Podczas realizacji badań zastosowano następujące techniki badawcze:

- Technika dynamicznego rozpraszania światła (DLS) oraz laserowa elektroforeza Dopplera (LDE). Za pomocą tych metod wykonano pomiary zeta potencjału oraz określono średnicę hydrodynamiczną analizowanych dendrymerów. Scharakteryzowano też właściwości powstałych kompleksów dendrymer/siRNA, a także dendrymer/lek.
- Transmisyjna mikroskopia elektronowa (TEM) – technika ta została wykorzystana do określenia morfologii oraz do oszacowania rozmiarów metalodendrymerów oraz ich kompleksów z siRNA.
- Spektrofluorymetria – pomiary anizotropii fluorescencji dwóch sond fluorescencyjnych: DPH oraz TMA-DPH zostały wykorzystane do określenia zdolności oddziaływania dendrymerów z błonami biologicznymi. Pomiary polaryzacji fluorescencji znakowanego siRNA posłużyły do oceny oddziaływań pomiędzy dendrymerami, a siRNA.
- Spektropolarymetria dichroizmu kołowego (CD) – pomiary widm CD pozwoliły określić wpływ metalodendrymerów na drugorzędową strukturę albuminy ludzkiej i siRNA.

- Agarozowa elektroforezę żelowa – zastosowanie tej metody pozwoliło wyznaczyć stosunek molowy dendrymer/siRNA, w jakim tworzone są kompleksy oraz ocenić zdolność metalodendrymerów do ochrony siRNA przed degradacją w obecności nukleaz.
- Test AlamarBlue – posłużył do określenia cytotoksyczności dendrymerów wobec komórek zawiesinowych (PBMC oraz 1301 i HL-60).
- Test MTT – wykorzystano w celu określenia cytotoksyczności metalodendrymerów, leków, oraz kompleksów dendrymer/siRNA, a także dendrymer/lek wobec komórek adherentnych (MCF-7 i HEPG2).
- Oznaczanie zmian poziomu reaktywnych form tlenu w komórkach pod wpływem metalodendrymerów, leków oraz kompleksów dendrymer/lek przy użyciu sondy H_2DCFDA.
- Oznaczanie zmian błonowego potencjału mitochondrialnego pod wpływem metalodendrymerów, leków oraz kompleksów dendrymer/lek przy użyciu sondy JC-1.
- Cytometria przepływowa – posłużyła do określenia internalizacji kompleksów dendrymer/siRNA-FITC. Podwójne barwienie aneksyną V-FITC/jodkiem propidyny wykorzystano do oszacowania odsetka komórek apoptotycznych oraz nekrotycznych.
- Mikroskopia konfokalna – została zastosowana do określenia internalizacji dendrypleksów do komórek nowotworowych MCF-7. Wykorzystując podwójne barwienie oranżem akrydyny /bromkiem etydyny za pomocą tej techniki, oceniono zdolności indukowania apoptozy oraz nekrozy w komórkach po inkubacji z kompleksami dendrymer/lek.

OMÓWIENIE PRAC WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ

W pierwszej pracy wchodzącej w skład rozprawy doktorskiej, **Hołota, M.; Magiera, J.; Michlewska, S.; Kubczak, M.; Sanz del Olmo, N.; García-Gallego, S.; Ortega, P.; de la Mata, F.J.; Ionov, M.; Bryszewska, M. In Vitro Anticancer Properties of Copper Metallodendrimers. Biomolecules 2019, 9, 155**, przeprowadzono biofizyczną charakterystykę dwóch grup karbokrzemowych metalodendrymerów z atomami miedzi (CCD - Copper Carbosilane Dendrimers). W pierwszej z tych grup przy atomie miedzi usytuowany jest ligand chlorkowy, natomiast w drugiej azotanowy. W skład obu grup wchodzą dendrymery zerowej, pierwszej oraz drugiej generacji (G0, G1, G2). W pierwszej kolejności dzięki zastosowaniu techniki laserowej elektroforezy Dopplera (LDE) oraz techniki dynamicznego rozpraszania światła (DLS) wyznaczono wielkość i potencjał zeta badanych metalodendrymerów. Dodatkowo do analizy morfologii nanocząstek zastosowano technikę

transmisyjnej mikroskopii elektronowej (TEM). Wszystkie badane dendrymery wykazywały dodatni potencjał zeta, a jego wartość była zależna od generacji nanoczastki. Dla dendrymerów drugiej generacji wartość wyznaczonego potencjału zeta wynosiła blisko 40 mV. Dzięki wykonaniu pomiarów średnicy hydrodynamicznej wykazano, że najwyższe wartości tego parametru wynoszące ponad 100 nm odnotowano dla dendrymerów zerowej generacji. Dodatkowo w przypadku tych nanocząstek obserwowano najwyższy wskaźnik polidyspersyjności (Polydispersity index - PDI). Wyniki analizy morfologii dendrymerów wykonanej za pomocą TEM wskazały, że nanocząstki generacji zerowej były najmniejszego rozmiaru. Odmienne wyniki uzyskane tymi dwoma metodami mogą wynikać z różnic w przygotowaniu próbek. Uzyskane rezultaty mogą wskazywać na istnienie oddziaływań pomiędzy nanoczastkami, które mogą skutkować powstawaniem agregatów. W celu poznania dokładniejszej specyfiki działania badanych nanocząstek określono ich interakcję z albuminą ludzką (HSA) oraz błonami erytrocytarnymi.

Analiza widm uzyskanych metodą dichroizmu kołowego (CD) wykazała, że wszystkie badane dendrymery oddziaływały z albuminą. Efekt ten nasilał się wraz ze wzrostem generacji nanocząstki. Największe zmiany eliptyczności powodowały dendrymery drugiej generacji. Stopień i sposób oddziaływania dendrymerów z błonami erytrocytarnymi określono, wykonując pomiary anizotropii fluorescencji znaczników DPH i TMA-DPH wprowadzonych do dwuwarstwy lipidowej błon. Zmiany anizotropii fluorescencji sondy DPH odzwierciedlają stopień płynności dwuwarstwy w regionie hydrofobowym, natomiast anizotropia sondy TMA-DPH wskazuje na zmiany płynności błony przy powierzchni, w rejonie hydrofilowym. Wszystkie dendrymery silnie oddziaływały z błonami erytrocytarnymi. Najniższe wartości anizotropii fluorescencji DPH zaobserwowano dla dendrymerów zerowej generacji obu badanych grup, podczas gdy spowodowały one największy wzrost anizotropii fluorescencji TMA-DPH.

Kolejnym krokiem do poznania natury dendrymerów CCD było określnie ich właściwości hemolitycznych. Wszystkie badane dendrymery powodowały hemolizę erytrocytów, zależną od generacji i zastosowanego stężenia nanocząstki. Następnie określono cytotoksyczność badanych dendrymerów wobec komórek nowotworowych (1301 i HL-60) oraz prawidłowych (PBMC). Wszystkie metalodendrymery wykazywały znacznie wyższą toksyczność wobec komórek nowotworowych w porównaniu z komórkami prawidłowymi. Cytotoksyczność dendrymerów była zależna od ich stężenia i generacji. Przy czym, w przypadku dendrymerów z ligandami chlorkowymi, dendrymer generacji pierwszej był bardziej cytotoksyczny w porównaniu z dendrymerami generacji zerowej i drugiej. Dodatkowo wykazano, że nanocząstki z ligandem azotanowym były bardziej cytotoksyczne niż z ligandem chlorkowym. Wyniki badań przedstawione w tej pracy potwierdziły, że obecność miedzi znacząco wpływa na cytotoksyczne właściwości dendrymerów w stosunku do komórek nowotworowych.

Celem drugiego etapu badań, na podstawie którego powstała praca, **Sanz del Olmo, N.; Holota, M.; Michlewska, S.; Gómez, R.; Ortega, P.; Ionov, M.; de la Mata, F.J.; Bryszewska, M. Copper (II) Metallodendrimers Combined with Pro-Apoptotic siRNAs as a Promising Strategy Against Breast Cancer Cells. Pharmaceutics 2020, 12, 727,** było określenie zdolności dendrymerów CCD do tworzenia kompleksów z proapoptotycznymi siRNA, a także ocena zdolności utworzonych siRNA/dendrymer kompleksów (dendrypleksów) do internalizacji komórek nowotworowych oraz określenie ich cytotoksyczności względem komórek raka piersi linii MCF-7. Charakterystykę kompleksów siRNA/dendrymer określono z wykorzystaniem techniki DLS i pomiaru potencjału zeta, a także przeanalizowano morfologię powstałych kompleksów za pomocą transmisyjnej mikroskopii elektronowej. W przeciwieństwie do dendrymerów pierwszej i drugiej generacji nanocząstki generacji zerowej nie tworzyły kompleksów z siRNA. Kompleksy utworzone z dendrymerami pierwszej generacji były większe od kompleksów tworzonych z dendrymerami generacji drugiej, wykazywały też większą wartość potencjału zeta. Pomiary DLS i potencjału zeta umożliwiły wyznaczenie optymalnego stosunku molowego siRNA/dendrymer 1:30 do zastosowania w badaniach *in vitro*. Dane uzyskane technikami polaryzacji fluorescencji, dichroizmu kołowego i agarozowej elektroforezy żelowej potwierdziły uzyskane wyniki. Dodatkowo za pomocą elektroforezy żelowej wykazano, że dendrymery generacji pierwszej i drugiej nie tylko tworzą kompleksy z siRNA, ale dodatkowo chronią go przed degradacją w obecności nukleaz. W kolejnym etapie badań określono zdolność utworzonych dendrypleksów do internalizacji komórek nowotworowych. Za pomocą cytometrii przepływowej i mikroskopii konfokalnej wykazano, że dendrymery drugiej generacji wydajniej wprowadzają kwas nukleinowy do komórek w porównaniu do dendrymerów pierwszej generacji, a procent zinternalizowanych komórek jest większy po 3 niż po 24 godzinach. Ostatnim etapem badań było określenie cytotoksyczności utworzonych kompleksów w stosunku do komórek nowotworu piersi MCF-7. Po 72 godzinach inkubacji w obecności dendrypleksów żywotność komórek linii MCF-7 znacząco spadła w porównaniu do samych dendrymerów. Uzyskane wyniki mogą sugerować, że badane metalodendrymery mogą być potencjalnymi wektorami do dostarczania siRNA do komórek.

Wyniki ostatniego etapu badań zostały zaprezentowane w publikacji: **Hołota, M.; Michlewska, S.; Garcia-Gallego, S.; del Olmo, N.S.; Ortega, P.; Bryszewska, M.; de la Mata, F.J.; Ionov, M. Combination of Copper Metallodendrimers with Conventional Antitumor Drugs to Combat Cancer in In Vitro Models. Int. J. Mol. Sci. 2023, 24, 4076**. Ta cześć pracy obejmowała analizę zdolności dendrymerów do kompleksowana z lekami przeciwnowotworowymi, takimi jak: doksorubicyna, metotreksat i 5-flurouracyl. Dodatkowo została określona cytotoksyczność powstałych kompleksów w stosunku do komórek nowotworowych (MCF-7 i HepG2). Uzyskane wyniki wskazują, że dendrymery oddziałują z wymienionymi lekami, tworząc stabilne kompleksy dendrymer/lek. Kompleksy

przygotowane z udziałem dendrymerów zerowej generacji miały najmniejszą średnicę hydrodynamiczną i wykazywały najniższy potencjał zeta.

W celu wykrycia ewentualnego synergistycznego efektu leków skoniugowanych z dendrymerami przeprowadzono badania określające właściwości cytotoksyczne nieskompleksowanych dendrymerów, leków i ich kompleksów w odpowiednich stężeniach z wykorzystaniem nowotworowych linii komórkowych, raka wątroby HepG2 oraz raka piersi MCF7. Dodatkowo przeprowadzono analizę zmian poziomu reaktywnych form tlenu i zmian mitochondrialnego potencjału błonowego wywołanych działaniem badanych nanosystemów. W wybranych stężeniach kompleksy dendrymer/lek wykazywały znacznie wyższą cytotoksyczność w stosunku do obu typów komórek zarówno w porównaniu z samymi lekami, jak i dendrymerami. Badane nanokompleksy powodowały też zwiększoną produkcję reaktywnych form tlenu (RFT) oraz wyraźniejszą depolaryzację błony mitochondrialnej, co prawdopodobnie wskazuje na rozpoczęcie procesu apoptozy. Dla potwierdzenia wywołanej przez nanokompleksy aktywacji programowanej śmierci komórkowej zastosowano metodę podwójnego barwienia komórek (aneksyna V-FITC/jodek propidyny). Za pomocą techniki cytometrii przepływowej stwierdzono zwiększenie puli komórek wczesno i późno apoptotycznych oraz nekrotycznych inkubowanych z badanymi związkami. Wyniki uzyskane metodą cytometrii przepływowej zostały dodatkowo potwierdzone metodą mikroskopii konfokalnej.

Podsumowując, stwierdzamy, że zastosowanie dendrymerów CCD i leków przeciwnowotworowych (DOX, MTX lub 5-Fu) połączonych w jednym nanokompleksie przynosi pożądane efekty cytotoksyczne wobec komórek nowotworowych przy niższych stężeniach komponentów, co może być rozważane w dalszych badaniach i rozwoju perspektywicznych nanonośników.

WNIOSKI

1. Karbokrzemowe dendrymery z atomami miedzi są bardziej cytotoksyczne wobec komórek nowotworowych niż komórek prawidłowych.
2. Badane metalodendrymery tworzą kompleksy z proapoptotycznymi siRNA i lekami przeciwnowotworowymi.
3. Dendrymery CCD skutecznie wprowadzają lecznicze siRNA do komórek, chroniąc je przed degradacją w obecności nukleaz.
4. Utworzone kompleksy Dendrymer/Lek-(DOX, MTX lub 5-Fu) w zastosowanych stężeniach są bardziej cytotoksyczne wobec komórek nowotworowych niż nieskompleksowane komponenty.

STRESZCZENIE W JĘZYKU POLSKIM

Nowotwory są grupą chorób, które powodują ogromne problemy na skalę globalną. Stanowią one drugą najczęstszą przyczynę zgonów na świecie, a liczba nowych przypadków stale rośnie. Współcześnie stosowane terapie mają wiele ograniczeń, a ponadto wiążą się z efektami ubocznymi, negatywnie wpływającymi na jakość życia pacjentów. W związku z tym stale poszukuje się nowych strategii w terapii przeciwnowotworowej, które byłyby pozbawione tych wad i poprawiłyby efektywność stosowanego leczenia. Obecnie medycyna pokłada duże nadzieje w wykorzystaniu nanosystemów opartych na nanocząstkach jako nośnikach terapeutycznych molekuł.

W niniejszej pracy doktorskiej oceniono możliwość wykorzystania karbokrzemowych dendrymerów z atomami miedzi (CCD) z ligandami azotanowymi i chlorkowymi jako nośników proapoptotycznego siRNA oraz leków w terapii przeciwnowotworowej.

Przeprowadzona charakterystyka biofizyczna dendrymerów potwierdziła, że mają one dodatni ładunek i oddziałują z HSA oraz błonami lipidowymi komórek. Wyniki badań wykazały również, że badane dendrymery są bardziej toksyczne wobec komórek nowotworowych niż prawidłowych.

W pracy wykorzystano szereg metod biofizycznych mających na celu sprawdzenie czy badane metalodendrymery tworzą kompleksy z przeciwnowotworowym siRNA. W tym celu zastosowano nastepujace techniki: agarozową elektroforezę żelową, pomiary potencjału zeta i średnicy hydrodynamicznej, oraz spektropolarymetrię dichroizmu kołowego. Wykazano, że w przeciwieństwie do dendrymerów generacji zerowej, dendrymery generacji pierwszej i drugiej tworzą kompleksy z siRNA. Wykryto protekcyjne działanie tych dendrymerów przed degradacją kwasu nukleinowego w obecności RNaz. Badania *in vitro* potwierdziły, że dendrymery CCD mogą wprowadzać lecznicze siRNA do komórek nowotworowych linii MCF-7. Zaobserwowano, że po 72 godzinach inkubacji komórek nowotworowych z kompleksami CCD_n/siRNA następuje znaczny spadek ich żywotności.

Następna część pracy obejmowała ocenę zdolności badanych dendrymerów do tworzenia kompleksów z trzema komercyjnie dostępnymi lekami przeciwnowotworowymi: doksorubicyną (DOX), metotreksatem (MTX) i 5-flurouracylem (5-Fu). Pomiary potencjału zeta i średnicy hydrodynamicznej potwierdziły hipotezę, iż dendrymery CCD tworzą kompleksy z wybranymi lekami. Badania *in vitro* wykazały synergistyczny efekt działania dendrymerów skoniugowanych z lekami (DOX, MTX i 5-FU). Powstałe kompleksy wykazywały większą cytotoksyczność wobec komórek nowotworowych niż wolne dendrymery lub leki, powodując wzmożoną produkcję RFT i depolaryzację błony mitochondrialnej. Za pomocą techniki cytometrii przepływowej z zastosowaniem podwójnego barwienia (aneksyną V-FITC - jodek propidyny) wykazano, że obecność dendrymerów powoduje wzrost odsetka komórek apoptotycznych i nekrotycznych w zawiesinie komórek linii MCF7 w zależności od zastosowanego dendrymeru.

Podsumowując wyniki przeprowadzonych badań, można wnioskować, że karbokrzemowe dendrymery z atomami miedzi wykazują właściwości przeciwnowotworowe i mogą być rozważane jako potencjalne nośniki leków i kwasów nukleinowych do komórek nowotworowych.

STRESZCZENIE W JĘZYKU ANGIELSKIM

Cancers are a group of diseases that pose significant global challenges. They are the second leading cause of death worldwide, and the number of new cases continuously rising. Currently used conventional therapies have many limitations and are associated with side effects that negatively impact the quality of patients' life. Consequently, there is a constant search for new strategies in anticancer therapy that would be devoid of these drawbacks and improve the effectiveness of treatment. Currently, oncology holds huge hopes for utilizing nanosystems based on nanoparticles, as therapeutic carriers.

This PhD thesis describes the potential of carbosilane copper dendrimers (CCD) with nitrate and chloride ligands as carriers of proapoptotic siRNA and anticancer drugs to tumour cells. Performed biophysical characterization of studied dendrimers shows that they are positively charged and are able to interact with proteins and cell membranes. Obtained results indicate that CCD dendrimers are more toxic towards cancer cells than to normal cells.

In this work, a number of biophysical methods were applied to verify that CCD dendrimers are able to form the complexes with anti-tumor siRNA. The gel electrophoresis, DLS, zeta potential, fluorescence polarisation, circular dichroism, flow cytometry, transmission microscopy, and confocal microscopy techniques were used for this purpose. It was shown that, unlike "0" generation, dendrimers of the generations 1^{st} and 2^{nd} were able to form the complexes with siRNA. The protective effect of the dendrimers against siRNA degradation at the presence of RNases was additionally demonstrated. *In vitro* studies indicate that CCD dendrimers were able to transfect siRNAs into MCF-7 cells. The 72 hours of incubation of MCF-7 cells with siRNA/dendrimer complex decreased the cells viability significantly.

Next part of work was aimed to analyse the ability of CCD dendrimers to complex anticancer drugs such as doxorubicin (DOX), methotrexate (MTX), and 5-fluorouracil (5-Fu). The results of zeta potential and DLS measurements have shown that cooper dendrimers can form a stable complexes with mentioned drugs. *In vitro* studies demonstrated a synergistic effect between dendrimers and drugs (DOX, MTX, and 5-FU). Formed complexes were significantly more cytotoxic against cancer cells than uncomplexed drugs or dendrimers. Dendrimer/drug complexes caused increase in ROS production and mitochondrial membrane depolarization. The flow cytometry assay with the use of double staining

(annexin V-FITC - propidium iodide) showed an increase in the percentage of both apoptotic and necrotic cells depending on the dendrimer used.

Summarizing, we conclude that carbosilane copper dendrimers show significant anticancer effect, and are able to complex and deliver the drugs and siRNA to cancer cells, which allow us to consider them as prospective drug carriers.

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Article

In Vitro Anticancer Properties of Copper Metallodendrimers

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Abstract: Newly synthesized carbosilane copper dendrimers (CCD) with chloride and nitrate surface groups seem to be good candidates to be used as gene and drug carriers in anti-cancer therapy, due to their properties such as size and surface charge. Copper attached to the nanoparticles is an important element of many biological processes and recently their anti-cancer properties have been widely examined. Zeta size and potential, transmission electron microscopy (TEM), circular dichroism (CD), analysis of haemolytic activity, and fluorescence anisotropy techniques were used to characterize copper dendrimers. Additionally, their cytotoxic properties toward normal (PBMC) and cancer (1301; HL-60) cells were examined. All tested dendrimers were more cytotoxic against cancer cells in comparison with normal cells.

Keywords: copper metallodendrimers; anticancer therapeutic agent; nanocarrier; structure; cytotoxicity

1. Introduction

Copper is an important element involved in many essential biological processes. Copper compounds are known as anti-oxidants and they have anti-bacterial and anti-fungal activity [1]. This metal is a crucial trace element necessary for the action of several enzymes and proteins, such as cytochrome oxidase, superoxide dismutase, ascorbate oxidase, and tyrosinase. Copper takes part in energy metabolism, respiration, and DNA synthesis. Biological molecules with copper are involved in oxidation-reduction reactions, reacting directly with molecular oxygen to produce free radicals. It is known that an excess or deficiency of copper causes Wilson and Menkes diseases, respectively. Therefore, to avoid the toxic effects of copper, the mechanism of its level regulation is required. Due to the fact that elevated levels of copper were observed in many types of human tumours, the attention of researchers is focused on the activity of this metal as a therapeutic anti-cancer agent [2–4].

Copper compounds, similar to other anti-cancer metals such as gold, silver, and ruthenium, are known as inducers of apoptosis, especially in cancer cells [4–7]. Additionally, copper complexes with phenanthroline derivatives with various alkyl chains were shown to have not only anti-tumour activity, but also anti-metastatic and anti-angiogenic activity [8]. Therefore, copper can be considered an alternative to other metal-based drugs, particularly those with platinum compounds, which have many side effects, such as neurotoxicity, ototoxicity, emetogenesis, nephrotoxicity, fatigue, petechial, alopecia, diarrhoea, anaemia [1,9,10]. Currently, copper gluconate complex co-administered with disulfiram is subject to clinical research in therapy for refractory solid malignancies [8,11]. However, the main problem in the use of copper in anti-cancer therapy is its poor water-solubility, which can significantly reduce the bioavailability of copper-based drugs [12].

Nowadays, oncology uses the achievements of nanotechnology [13,14]. Nanotechnology is a field of research dealing with the synthesis of particles with sizes not exceeding 100 nm [15,16], such as quantum dots, carbon nanotubes, and dendrimers [17,18]. Dendrimers were synthesized for the first time in the 1970s and now are quite popular in the field of drug delivery [12,19]. These nanoparticles have unique properties, such as specific structure and a high degree of monodispersity [20–22]. Most of them show thermal and chemical stability and a hydrophobic character, which may contribute to their interaction with biological membranes [13]. Additionally, the presence of functional groups determines their specific properties, such as size and surface charge [12,13,23]. Moreover, attaching metal molecules to the dendrimer surface can increase their water-solubility and enhance bioavailability [13].

In the present study, two groups of copper carbosilane metallodendrimers were tested as candidates for use in anticancer therapy.

2. Materials and Methods

2.1. Dendrimers

Two families of copper carbosilane metallodendrimers, with chloride and nitrate ligands, were used in the current study (Figure 1, Table 1).

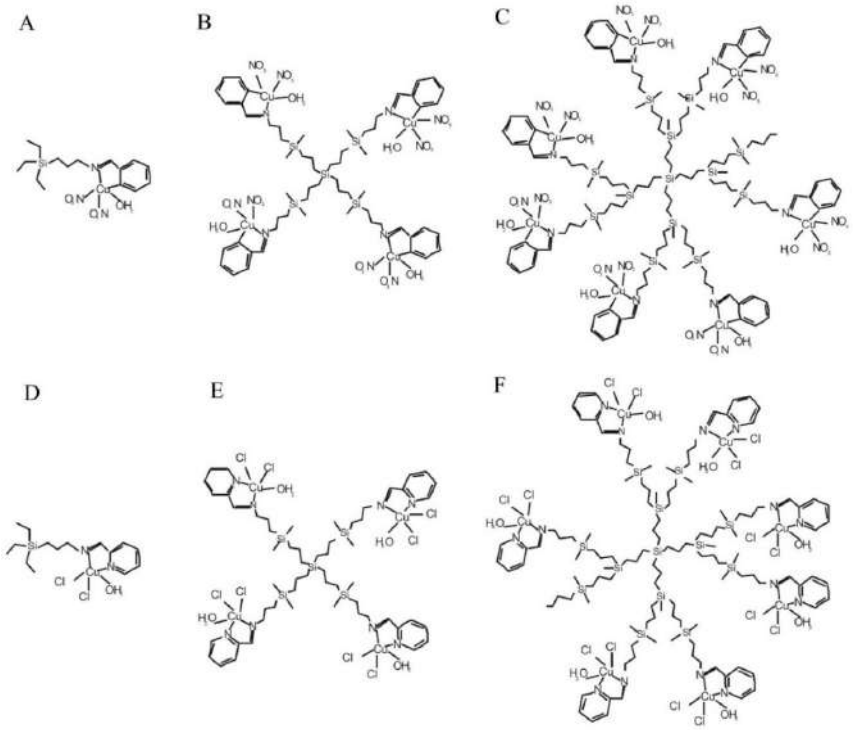

Figure 1. Structure of copper metallodendrimers with the nitrate (A) – CCD-NO-0, (B) – CCD-NO-1, (C) – CCD-NO-2 and chloride (D) – CCD-Cl-0, (E) – CCD-Cl-1, (F) – CCD-Cl-2- surface groups.

Table 1. Characterisation of copper metallodendrimers with chloride and nitrate surface groups.

	CCD-NO-0	**CCD-NO-1**	**CCD-NO-2**	**CCD-Cl-0**	**CCD-Cl-1**	**CCD-Cl-2**
Generation	0	1	2	0	1	2
Surface groups number	1	4	8	1	4	8
Molecular weight [g/Mol]	468.04	1840.10	3992.90	414.93	1627.68	3696.01
Solubility	MeOH/DMF/DMSO			DMF/DMSO/$CHCl_3$/CH_2Cl_2		

2.2. Zeta Potential Technique

Zeta potential was measured using a Photon Correlation spectrometer Zetasizer Nano ZS, Malvern Instruments (UK). Helmholtz–Smoluchowski's equation was used to calculate the data; seven measurements of five cycles of each sample were made.

2.3. Measurement of the Hydrodynamic Diameter of the Particles

The hydrodynamic diameter of the particles was measured using a Malvern Zetasizer Nano ZS spectrometer (UK). The dynamic light scattering technique was applied. Wavelength was set at 633 nm, a detection angle of 90°, and the refraction factor was 1.33. The measurements were conducted in distilled water. For each sample seven measurements in five cycles were made. The data were analyzed using Malvern software.

2.4. Transmission Electron Microscopy (TEM)

TEM was used to evaluate the structure, shape, and size of the copper metallodendrimers. Ten microliters of dendrimers at concentration 20 μmol/L were placed on 200-mesh copper grids with carbon surface. The samples were stained using uranyl acetate solution for 20 min, then washed with deionized water and dried at room temperature. The JEOL-1010 (JEOL, Akishima, Japan) transmission electron microscope was applied.

2.5. Circular Dichroism

Circular dichroism (CD) was assessed with the J-815 CD spectrometer (Jasco, Japan). The human serum albumin (HSA) concentration was 0.25 μmol/L. Complexes of dendrimer/HSA were prepared in a 10 mmol/L Na-phosphate buffer, pH 7.4, at molar ratios ranging from 0.5 to 10. The measurements were made from 195 to 260 nm in a Helma quartz cell with a thickness of 0.5 cm. The scan parameters were as follows: 50 nm/min scan speed, 0.5 nm step resolution, 4 s response time, 1.0 nm bandwidth, with the slit set to auto. The mean ellipticity was calculated using software provided by Jasco.

2.6. Haemotoxicity

Blood from healthy donors from Central Blood Bank, Lodz was used. Erythrocytes were isolated by centrifugation and washed three times with PBS 10 mM, pH 7.4. After isolation, the dendrimers in rising concentrations from 0.1 to 100 μmol/L were added to the erythrocytes with 14% hematocrit. Then the samples were incubated at 37 °C for 24 h. The absorbance was measured at 540 nm using a Jasco V-650 spectrophotometer. The percentage of haemolysis was calculated using the following formula:

$$H(\%) = (A\ 540\ nm/A_{water}\ 540\ nm) \times 100\%.$$

2.7. Erythrocyte Membrane Isolation

To estimate the changes of membrane fluidity caused by dendrimers, the erythrocyte membranes were isolated by centrifugation (15 min, 15,000× *g*, 4 °C) and washed several times with 30 mmol/L Na-phosphate buffer, PH 7.4, diluted with water (1:1). The protein concentration was determined by the Lowry method. Final protein concentration was 0.5 mg/mL.

2.8. Fluorescence Anisotropy

The fluorescence anisotropy of two fluorescent probes, DPH (1,6-diphenyl-1,3,5-hexatriene) and TMA-DPH (1-[4-(trimethyl-ammonium) phenyl]-6-phenyl-1,3,5-hexatriene), intercalating in erythrocyte membranes, was measured after the addition of increasing concentrations of dendrimers using PerkinElmer LS-50B spectrofluorometer (Perkin-Elmer, Waltham, MA, USA). The excitation wavelengths were 348 nm and 358 nm and the emission wavelengths were 426 nm and 428 for DPH for TMA-DPH, respectively. The slit width of the excitation monochromator was 6 nm and that of the emission monochromator was 8 nm.

The fluorescence anisotropy values were calculated from Jablonski's equation:

$$r = (I_{VV} - GI_{VH})/(I_{VV} + GI_{VH}),$$

where r = fluorescence anisotropy, I_{VV} and I_{VH} = the vertical and horizontal fluorescence intensities, respectively, to the vertical polarization of the excitation light beam used. $G = I_{VH}/I_{VV}$ (grating correction factor) corrects the polarization effects of the monochromator. The measurements were performed with Perkin Elmer software.

2.9. Cell Lines

To assay the dendrimers' cytotoxicity, two cancer cell lines of leukaemia (HL-60 and 1301, ATCC cell lines, Manassas, Virginia, USA) and a normal cell line PMBC (peripheral blood mononuclear cells) (isolated from blood of healthy donors from Central Blood Bank, Lodz) were applied. PMBC cells were obtained from blood samples with Histopaque 1077 gradient (1500 rpm, 15 min, 24 °C) in a RPMI-1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USAwith 10% heat-inactivated fetal bovine serum (FBS, HyClone, GE Healthcare Life Sciences, Chicago, Illinois, USA) contained 1% of antibiotic. The cells were grown in plastic tissue culture flasks (Falcon, GE Healthcare Life Sciences, Chicago, Illinois, USA) at a temperature of 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

2.10. Cytotoxicity

To study the cytotoxicity of dendrimers at concentrations of 1–50 μmol/L, the Alamar Blue test was applied. The cells were seeded on a black 96-well plate at a density of 10,000 per well. After 24 h incubation, the absorbance/fluorescence of the samples was measured at 528 and 590 nm. Viability was estimated from the following formula:

$$\% \text{ viability} = (A/A_c) \times 100\%.$$

2.11. Statistical Analysis

For the statistical analysis, the results were collected out of a minimum of three independent experiments and presented as mean ± standard deviation (SD). The Kruskal–Wallis non-parametric test was applied. Significance was accepted at * $p < 0.05$.

3. Results

3.1. Particle Size and Zeta Potential Analysis

Measurements of the dendrimers' zeta potential provided information of their surface charges. All studied dendrimers were positively charged, and the charge values depended on dendrimer generation. The highest zeta potential was shown for the dendrimers of 2nd generation. Additionally, the zeta potential was higher for dendrimers possessing the nitrate groups than for those with chloride groups, the respective values were in the range of 14.79 ± 1.92–39.3.78 ± 3.78 mV and 10.45 ± 1.25–37.48 ± 3.09 mV (Table 2).

Table 2. Zeta potential and zeta size of copper metallodendrimers with nitrate and chloride surface groups.

Dendrimer	Zeta Potential, [mV]	Zeta Size, [nm]
CCD-NO-0	14.79 ± 1.92	135.28 ± 9.27
CCD-NO-1	25.90 ± 2.32	51.59 ± 6.74
CCD-NO-2	39.23 ± 3.78	63.12 ± 5.28
CCD-Cl-0	10.45 ± 1.25	152.13 ± 7.52
CCD-Cl-1	19.68 ± 1.78	59.53 ± 8.92
CCD-Cl-2	37.48 ± 3.09	74.27 ± 7.26

Means ± SD.

An analysis of the hydrodynamic diameter of dendrimers indicated that nanoparticles with chloride groups were bigger than those with nitrate groups. The respective values were in the range of 59.53 ± 8.92 to 152.13 ± 7.52 nm and 51.59 ± 6.74 to 135.28 ± 9.27 nm (Table 2). It has been shown that, in both cases, the size of dendrimers of generation 0 was higher than dendrimers of the 1st and 2nd generations. This effect can be explained by possible nanoparticle aggregation. The highest polydispersity index (PDI) values were registered for the dendrimers of generation 0 in both groups. An increase of the dendrimer generation led to a decrease of the PDI value. For dendrimers containing chlorides, PDI values were higher than for dendrimers with nitrate groups (Table 3).

Table 3. Polydispersity index (PDI) of copper metallodendrimers with nitrate and chloride surface groups.

Dendrimer	PDI
CCD-NO-0	0.537 ± 0.146
CCD-NO-1	0.370 ± 0.087
CCD-NO-2	0.229 ± 0.022
CCD-Cl-0	0.542 ± 0.122
CCD-Cl-1	0.423 ± 0.068
CCD-Cl-2	0.356 ± 0.062

Means ± SD.

3.2. Transmission Electron Microscopy (TEM)

The morphological structure of the dendrimers was analyzed using transmission electron microscopy. Opposite to the results of the Zeta technique, the smallest nanoparticles, with a size of 5–50 nm, were observed for the samples with dendrimers of generation 0 (CCD-NO-0) (Figure 2). The 1st generation dendrimer (CCD-NO-1) was visible both as a single nanoparticle of about 5–10 nm and as a bigger aggregated form. Dendrimers of the 2nd generation (CCD-NO-2) formed aggregated structures with the size of about 500 nm. In contrast, all dendrimers with chloride groups were seen as aggregated systems. Dendrimers of generation 0 (CCD-Cl-0) were visible as small, clumped structures. Dendrimers of the 1st (CCD-Cl-1) and 2nd generations (CCD-Cl-2) formed aggregate structures with a size of about 50–150 nm and 450–500 nm, respectively (Figure 2).

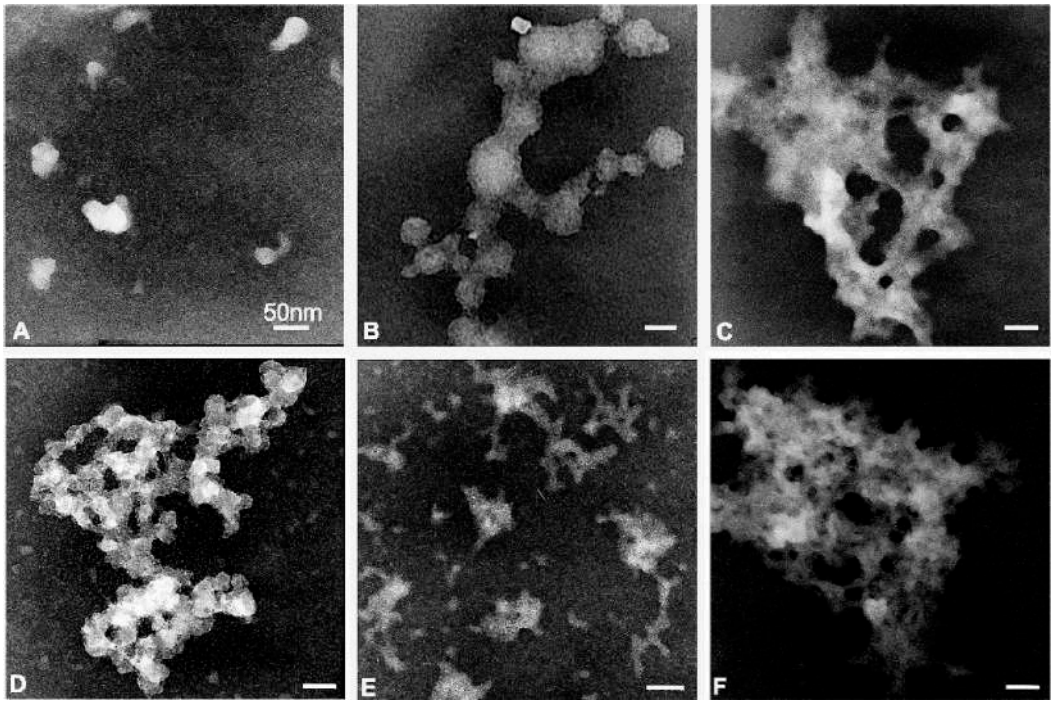

Figure 2. Ultrastructure of copper metallodendrimers visualized by transmission electron microscopy (TEM): (**A**) CCD-NO-0, (**B**) CCD-NO-1, (**C**) CCD-NO-2, (**D**) CCD-Cl-0, (**E**) CCD-Cl-1, (F) CCD-Cl-2. Dendrimers were dissolved in Na-phosphate buffer 10 mmol/L, pH 7.4. Bar = 50 nm.

3.3. Circular Dichroism

To analyze the ability of dendrimers to affect the proteins' secondary structure we applied the circular dichroism technique. The graphics in Figure 3 indicate that the addition of increasing amounts of dendrimers into a protein solution practically did not change the typical alpha helix shape of the HSA CD spectra. Figure 4 shows the changes in mean residue ellipticity of HSA, at λ = 210 nm in the presence of CCD. The highest increase of HSA spectra ellipticity was caused by the presence of the dendrimers of the 2nd generation, for both groups. The smallest ellipticity changes were caused by the 1st generation of dendrimers with nitrate end groups and by the 0 generation of the dendrimers with chloride groups.

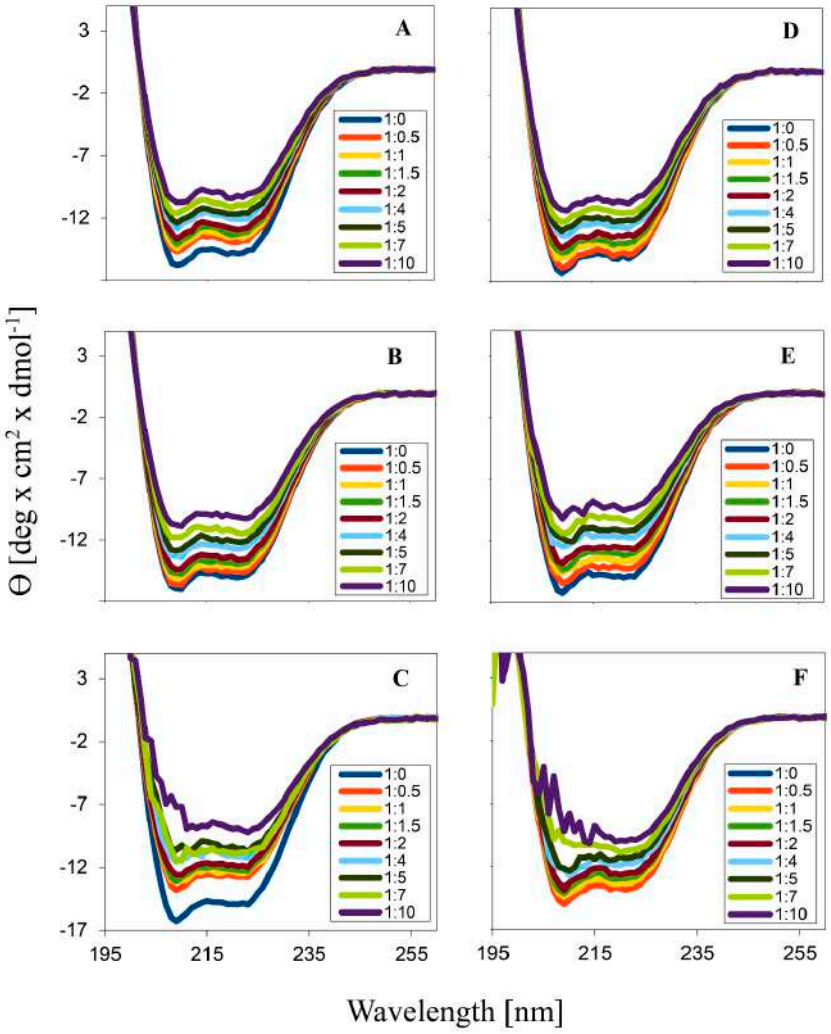

Figure 3. The CD spectra of human serum albumin (HSA) in the presence of copper metallodendrimers: (**A**) CCD-NO-0, (**B**) CCD-NO-1, (**C**) CCD-NO-2, (**D**) CCD-Cl-0, (**E**) CCD-Cl-1, (**F**) CCD-Cl-2. HSA concentration 0.25 μmol/L, wavelength 195–260 nm, scan speed 50 nm/min, bandwidth 1.0 nm, Na-phosphate buffer 10 mmol/L, pH 7.4.

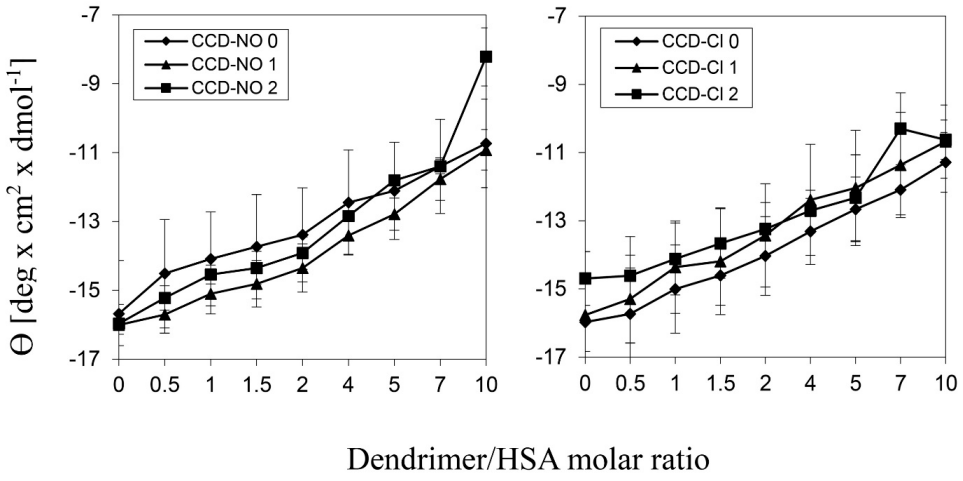

Figure 4. Changes in mean residue ellipticity of HSA, at λ = 210 nm in the presence of metallodendrimers. Results are mean ± standard deviation (SD), n = 3. HSA concentration 0.25 μmol/L, wavelength 195–260 nm, scan speed 50 nm/min, bandwidth 1.0 nm, Na-phosphate buffer 10 mmol/L, pH 7.4.

3.4. Erythrocyte Membrane Fluidity

To estimate the way CCD dendrimers interact with biological membranes, the fluorescence anisotropy technique using of DPH and TMA-DPH fluorescent probes was applied. By this method, it is possible to analyze which part of the lipid membrane can be influenced by the dendrimers. The fluorescence anisotropy of the DPH probe reflects the fluidity state of the hydrophobic region of the bilayer, whereas changes in the TMA-DPH probe anisotropy show the fluidity changes in the region of the membrane surface. The results indicate that all tested dendrimers increased the fluorescence anisotropy of both probes. In the case of the dendrimers with nitrate groups, the highest anisotropy values of the DPH probe were registered after the addition of the 1st and 2nd generation dendrimers, and the lowest by the generation 0, while in the case of the TMA-DPH probe the highest increase was caused by the dendrimer of generation 0 and lowest of generation 1 (Figure 5, left panels).

Dendrimers with chloride groups caused the highest increase in DPH probe anisotropy in the case of the CCD-Cl-1 dendrimer, while for the other two the parameter was just slightly changed. TMA-DPH fluorescence anisotropy was the highest in the presence of CCD-Cl-0 and the smallest for CCD-Cl-2 dendrimers (Figure 5, right panels).

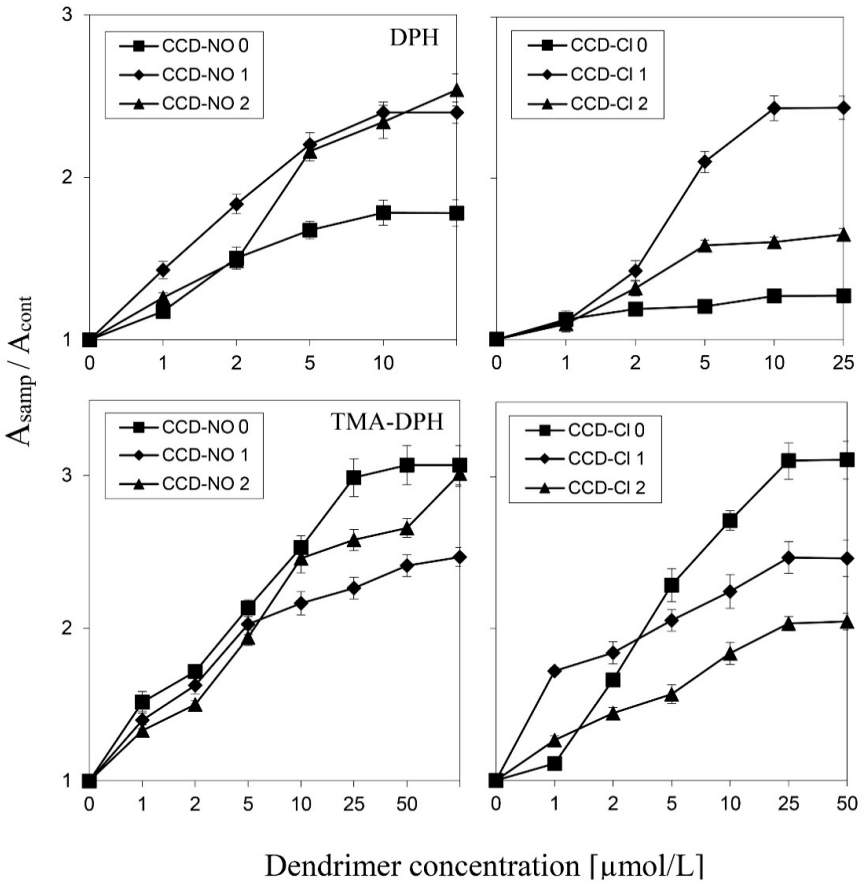

Figure 5. Changes in fluorescence anisotropy of DPH (top panels) and TMA-DPH (bottom panels) of erythrocyte membranes incubated with copper metallodendrimers at rising concentrations from 1 to 70 µmol/L. PBS buffer, pH 7.4, 37 °C. CCD-Cl: left panels, CCD-NO: right panels. The values are the mean ± SD, $n = 3$.

3.5. Hemotoxicity

The hemotoxicity test was used to study the interaction of dendrimers with the erythrocyte membrane. Destruction of the membrane triggers the release of proteins, including haemoglobin. Figure 6 presents the results of erythrocyte haemolysis caused by CCD dendrimers after 24 h incubation.

The intensity of membrane destruction depended on the dendrimer kind, generation, and applied concentration. Results show that at lower concentrations dendrimers with chloride groups were more hemotoxic than those with nitrate groups. However, along with the increase of concentration the opposite result was observed, where the effect of CCD-NO dendrimers was higher.

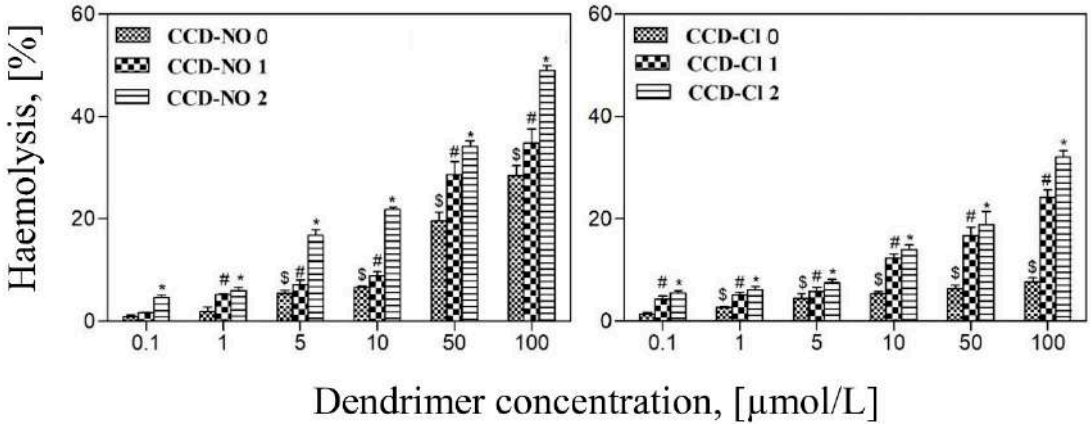

Figure 6. Erythrocyte haemolysis induced by copper metallodendrimers after 24 h of incubation. The concentration range 0.1–100 µmol/L. 2% haematocrit in PBS buffer, pH 7.4, 22 °C. Results are mean ± SD, $n = 6$.

3.6. Cytotoxicity

The influence of the CCD dendrimers on normal PBMC and cancer 1301 and HL-60 cells was evaluated (Figure 7). The performed cytotoxicity tests showed that the tested compounds affected the PBMC viability less than cancer cells. In contrast, they caused concentration- and generation-dependent decreases in the viability of both cancer cell lines. All dendrimers were more cytotoxic to 1301 than to HL-60 cell line. The concentration up to 1 µmol/L did not decrease the viability of the cancer cell lines. CCD-NO-1 and CCD-NO-2 dendrimers at a concentration of 5 µmol/L decreased 1301 cell viability up to 59.2% and 58.0% compared to the control, respectively. The increase in dendrimer concentration resulted in a drop of the 1301 cell viability to 14.2% and 14.6% compared to the control, respectively. Similarly, the 2nd generation dendrimer (CCD-NO-2) at concentrations 5–50 µmol/L caused a decrease in the HL-60 cell viability up to 48.4%–8.7% more than the control. The 1st generation dendrimer (CCD-NO-1) from 0.1 to 5 µmol/L did not significantly affect the HL-60 cell viability. An increase in its concentration up to 50 µmol/L decreased cell viability to 8.4% of the control. The dendrimer of generation 0 (CCD-N-0) caused a statistically significant decrease in both 1301 and HL-60 cell viability to 14.8% and 22.4% compared to the control, respectively, however only at a concentration of 50 µmol/L.

Dendrimers with chloride groups (CCD-Cl-1), at a concentration of 5 µmol/L, significantly decreased the viability of 1301 cells to 53.7% compared to the control, while the effect of generation 0 (CCD-Cl-0) and generation 2 (CCD-Cl-2) at same concentration was smaller—cell viability decreased up to 82.9% and 87.5%, respectively. At a concentration of 10 µmol/L, the dendrimers of generation 0 and 2 decreased the viability of 1301 cells up to 62.5% and 28.7%, respectively. The treatment of 1301 cells with all chloride dendrimers, CCD-Cl-0, CCD-Cl-1, and CCD-Cl-2, at a concentration of 50 µmol/L decreased their viability up to 12.6%, 13.3%, and 15.4%, respectively. The viability of HL-60 cells treated with 5 µmol/L of CCD-Cl-2 decreased up to 37.6% compared to the control, then with increasing dendrimer concentrations up to 50 µmol/L, the cells' viability did not change significantly. CCD-Cl-0 generation 0 and CCD-Cl-1 generation 1 at concentrations of up to 50 µmol/L decreased cell viability to 25% and 7.6%, respectively. The IC_{50} values of each dendrimer for all studied cell lines considered in this study are summarized in Table 4.

Table 4. IC_{50} values of copper metallodendrimers in normal peripheral blood mononuclear cell (PBMC) and cancer: 1301, HL-60 cell lines after 24 h incubation. The values are the mean ± SD of $n \geq 6$.

Dendrimer	PBMC	1301	HL60
CCD-NO-0	62.64 ± 0.2	27.84 ± 1.2	29.05 ± 0.6
CCD-NO-1	56.55 ± 0.4	5.03 ± 0.2	24.32 ± 1.7
CCD-NO-2	35.02 ± 0.2	4.85 ± 2.4	4.01 ± 0.4
CCD-Cl-0	62.23 ± 0.3	12.44 ± 0.7	5.94 ± 0.4
CCD-Cl-1	31.57 ± 7.3	4.58 ± 2.4	5.57 ± 0.2
CCD-Cl-2	50.95 ± 1.1	7.12 ± 0.3	2.37 ± 0.3

Means ± SD.

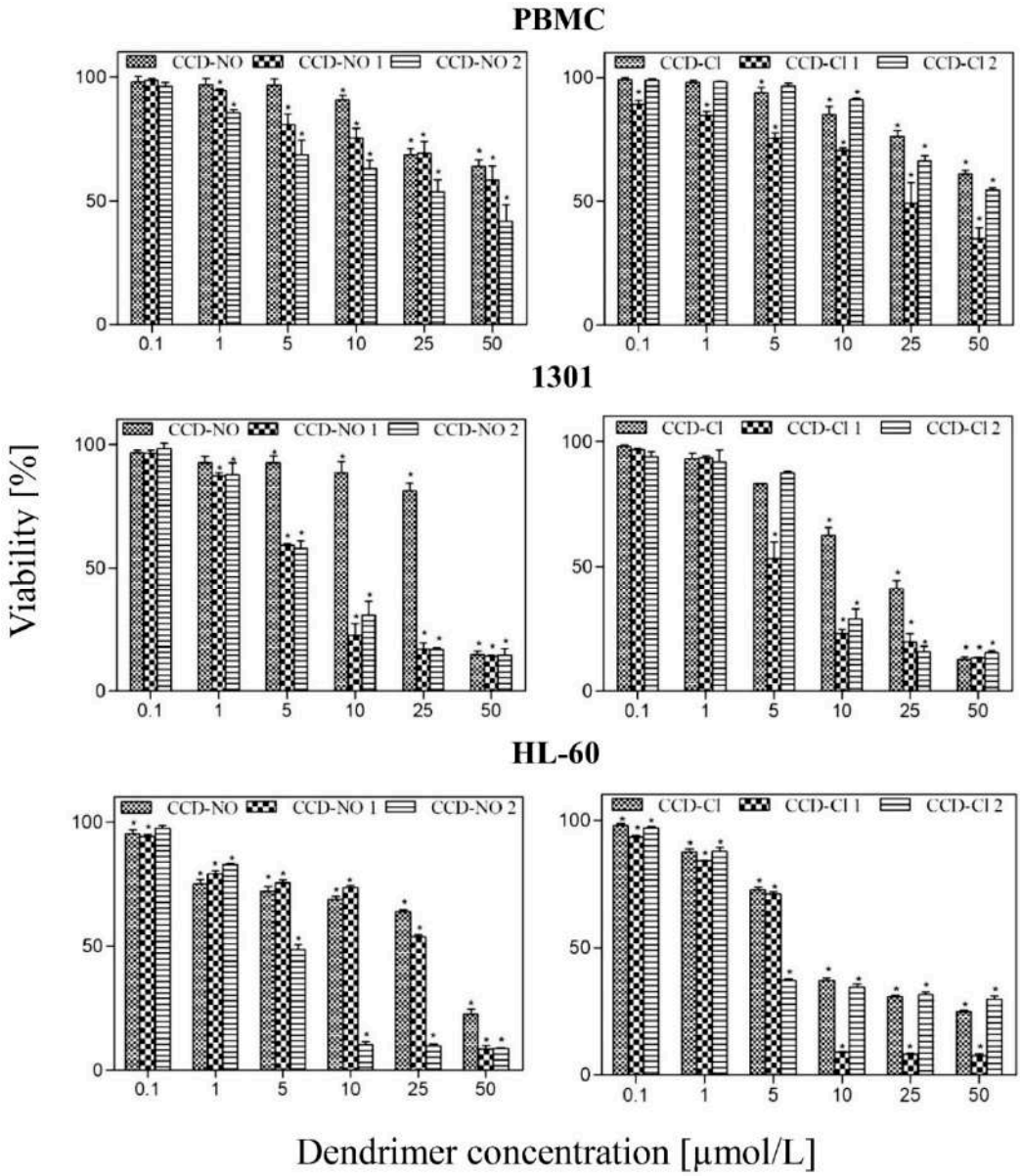

Figure 7. Effect of copper metallodendrimers on the viability of normal PBMC and cancer: 1301, HL-60 cell lines after 24 h incubation. The concentration range was 0.1–50 µmol/L. The values are the mean ± SD of $n \geq 6$.

4. Discussion

Copper is one of the anti-cancer metals and due to low toxicity seems to be an interesting alternative in cancer therapy. In this paper we investigated its biophysical properties and cytotoxicity to normal and cancer cells of carbosilane dendrimers containing copper molecules in their structure (Figure 1).

Measurements of the size of CCD dendrimers using the dynamic light scattering technique showed that dendrimers of generation 0 (CCD-NO-0) and (CCD-Cl-0) were the largest in their respective groups, while in other generations this parameter depended on generation. This effect can indicate a trend to aggregate the dendrimers of generation 0. A similar tendency was described for the carbosilane ruthenium dendrimers (CRD) of generation 0. This tendency can be due to electrostatic interactions between single molecules of dendrimers. On the contrary, TEM analysis showed that dendrimers of generation 0 were seen as single 5–50 nm nanoparticles (Figure 2.). This discrepancy between sizes of nanoparticles is probably due to the different methods applied. The zeta size measurements were conducted in a solution while TEM was conducted in a dry state [13,24].

The measurements of the zeta potential in a solution can provide information about dendrimer surface charges. The tested nanoparticles were positively charged. Similar results were described earlier in the experiments with CRD dendrimers [13]. It is known that the positive charge makes interaction with negatively charged biological membranes easier [13,25–27]. Due to this positive charge, cationic dendrimers were shown to be cytotoxic for normal Hippo-18 cells [28].

Analysis of the fluorescence anisotropy changes of two fluorescent labels, DPH and TMA-DPH, that are located at different membrane depths, confirmed that all tested dendrimers interacted with both regions of membranes and changed membrane fluidity. The observed increase in the fluorescence anisotropy indicated the rising membrane stiffness [29], which in turn reflected the ability of the tested dendrimers to interact with bilayers [30,31]. Phosphorothioate dendrimers of the 5th generation with peripheral hydroxyl groups [32] and viologen-phosphorus dendrimers were also shown to enhance the stiffness of biological membranes, although the intensity of this process was lower [33].

Next, to check the effect of dendrimers on the cell membrane, the degree of haemolysis was determined. Damage to the membrane caused by interaction with ligands results in the outflow of proteins, in particular haemoglobin [13]. Carbosilane copper dendrimers caused haemolysis after 24 h of incubation, the intensity of haemolytic effect depended on their generation and concentration. At lower concentrations, the dendrimers with the chloride ligands were more efficient than those with nitrate ones. However, with rising concentrations the opposite effect was observed. The lowest efficiency of the 0 generation dendrimers may have resulted from the smaller number of active groups compared to the higher generation dendrimers [13,34–37]. A similar effect was demonstrated in the experiments with a carbosilane dendrimer terminated with ruthenium [13], phosphoric dendrimers [38], and PAMAM dendrimers [35]. In contrast, after 24 h incubation, the haemolytic activity of the dendrimers containing titanium was not observed [39]. The surface charge of dendrimers is therefore an important haemotoxicity agent [38]. Cationic dendrimers can interact strongly with negatively charged membranes and can be more toxic compared to neutral or anionic dendrimers [20,34,40].

Circular dichroism spectroscopy can be used to check the interaction between dendrimers and albumin and their effect on the secondary structure of this protein [41]. CCDs caused minor changes to the albumin secondary structure. The addition of the increasing dendrimer concentrations to the HSA solution did not change the characteristic alpha-helix spectrum, while an increase in ellipticity was observed. This is advantageous because a large change in the original protein structure in the complexes would probably result in a loss of the biological activity of the protein, causing a limitation in the potential medical use [42].

The impact of copper-terminated carbosilane dendrimers on the viability of PBMC normal cells and 1301, HL60 cancer ones was evaluated and the dendrimers' cytotoxicity was checked. It is worth noting that all dendrimers were significantly more toxic to both cancer cell lines than to PBMCs.

The cytotoxic effect depended on the kind, generation, and concentration of the dendrimers. Among all tested dendrimers the dendrimer of the 1st generation with chloride groups (CCD-Cl-1) was the most toxic to HL-60. The same dendrimer was equally toxic to the human prostate cancer cell line PC3 [1]. However, Brahmi et al. [43] showed that the toxicity of phosphorus dendrimers with copper increased with its generation. Similarly, a generation-depended impact of ruthenium-terminated carbosilane dendrimers (CRD) on HL-60 cells was demonstrated [13]. The obtained findings are in good agreement with the previous studies [44–46], which suggests the importance of polymeric and hybrid nanoparticles as efficient carriers in drug delivery.

5. Conclusions

All studied CCD dendrimers strongly interact with cell membranes. They show significantly higher toxicity to tumour cells compared to normal cells. The cytotoxicity of dendrimers is concentration- and generation-dependent. Dendrimers with a nitrate ligand are more toxic than chloride dendrimers. On the basis of the obtained results, it can be proposed that the studied dendrimers are an alternative to other non-viral carriers to be used in classic anti-cancer therapy.

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Copper (II) Metallodendrimers Combined with Pro-Apoptotic siRNAs as a Promising Strategy Against Breast Cancer Cells

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Abstract: Cancer treatment with small interfering RNA (siRNA) is one of the most promising new strategies; however, transfection systems that increase its bioavailability and ensure its delivery to the target cell are necessary. Transfection systems may be just vehicular or could contain fragments with anticancer activity that achieves a synergistic effect with siRNA. Cationic carbosilane dendrimers have proved to be powerful tools as non-viral vectors for siRNA in cancer treatment, and their activity might be potentiated by the inclusion of metallic complexes in its dendritic structure. We have herein explored the interaction between Schiff-base carbosilane copper (II) metallodendrimers, and pro-apoptotic siRNAs. The nanocomplexes formed by metallodendrimers and different siRNA have been examined for their zeta potential and size, and by transmission electron microscopy, fluorescence polarisation, circular dichroism, and electrophoresis. The internalisation of dendriplexes has been estimated by flow cytometry and confocal microscopy in a human breast cancer cell line (MCF-7), following the ability of these metallodendrimers to deliver the siRNA into the cell. Finally, in vitro cell viability experiments have indicated effective interactions between Cu (II) dendrimers and pro-apoptotic siRNAs: Mcl-1 and Bcl-2 in breast cancer cells. Combination of the first-generation derivatives with chloride counterions and with siRNA increases the anticancer activity of the dendriplex constructs and makes them a promising non-viral vector.

Keywords: Pro-apoptotic siRNA; copper; dendrimers; delivery vectors; in vitro; anticancer activity

1. Introduction

New therapeutic approaches for cancer treatment are constantly being researched, especially where the side-effects of current therapies can be minimised. Within the new lines of research, suppressing the expression of specific genes is of particular importance in processes of cell development and differentiation in cancer and defence against viruses. RNA interference currently seems to be a

growing technique in molecular biology due to its high potential in terms of therapeutic value [1]. In this sense, siRNA is the subject of clinical research, with encouraging results in the treatment of viral infections, neurodegenerative diseases, and cancer [2,3]. siRNA is formed by 2-stranded RNA molecules of approximately 20 nucleotides in length, whose strands complement each other perfectly. The mechanism of action of siRNA begins when the 'antisense' strand assembles into the RNA-induced silencing complex (RISC), which is used to identify the complementary messenger RNA. RISC then catalyses the cutting of the mRNA into halves, which are degraded by cellular mechanisms, thereby blocking the expression of the gene [4,5].

However, RNAi-based therapies are not without technical challenges, and the efficacy of their activity is conditioned due to their rapid degradation by nucleases, as well as to their negatively charged molecules being unable to easily penetrate the lipid cell membranes without a carrier to assist them. Many studies now focus on the use of a wide variety of nanoparticles as carriers for nucleic acids, such as inorganic or polymeric nanoparticles, liposomes, and dendrimers [6–9]. Furthermore, the nanocarriers have the potential to improve the pharmacokinetics and biodistribution of siRNA significantly and can be chemically modified to anchor another bioactive molecule into its structure to implement its therapeutic action for which the siRNA has been designed. For example, mesoporous silica nanoparticles that incorporate folic acid promoting endocytosis [10] or amphiphilic dendrimer with RGDK peptide sequences [11] have been described as efficient vectors for transfection of various siRNAs. Dendrimers are monodisperse materials that have interesting properties that can be used in different biomedical fields and have proved useful transfecting agent [12]. The possibility of incorporating different therapeutic fragments in a controlled way to dendritic scaffold opens the door to use heterofunctional systems with different modes of action. In the case of cancer, combination of siRNA with anticancer organometallic compounds supported on dendritic systems is a new and powerful therapeutic approach to potentiate their activity [13].

Organometallic complexes of platinum, ruthenium, silver, and copper, among others, have proven to be excellent anticancer agents; many of them have been introduced in different nanoparticles or dendritic systems to increase their bioavailability and improve their therapeutic action [14–16]. Some of them, such as cationic ruthenium carbosilane dendrimers [13] or multifunctional selenium nanoparticles [17] used as siRNA carriers, have a synergistic effect through their combined action. In the search for other alternatives among different transition metals, copper is emerging as a good candidate for the development of drugs for the treatment of cancer. This is a consequence due to its rich redox chemistry propitiated by its two states of oxidation, giving rise to the generation of reactive oxygen species (ROS) and excellent properties as an anti-cancer agent, along with high biocompatibility [18,19]. The mechanism by which the different copper (II) complexes exert their anti-cancer activity is still unknown. Furthermore, copper compounds are able to induce apoptosis in cancer cells [15]. Our research group has now shown how the inclusion of copper (II) complex into the carbosilane dendritic skeleton potentiated its anticancer activity in different cancer cell lines (PC-3, HL-60, HeLa), with promising results from both in vitro and in vivo experiments [15,20]. Copper (II) carbosilane dendrimers have shown an extraordinary interaction with different model cell membranes, such as lecithin liposomes and CTAB micelles [21].

The objective of our work has been to study the capacity of copper (II) carbosilane dendrimers as transfecting agents of pro-apoptotic siRNA, and to assess any cooperative effect between both fragments showing anticancer activity. For this purpose, we selected two different anticancer siRNAs—Mcl-1 (Myeloid cell leukaemia-1) and Bcl-2 (B-cell lymphoma 2)—which play a crucial role in the regulatory genes of apoptosis [22,23]. Herein, we describe the biophysical characterisation of complexes formed between copper (II) carbosilane dendrimers (G_n-[Cu]) and pro-apoptotic siRNA, their ability to internalise the siRNA into cancer cells, and the cytotoxicity in human breast cancer cell line (MCF-7) in which Mcl-1 and Bcl-2 are overexpressed [22,24].

2. Materials and Methods

2.1. General Considerations

Copper(II) metallodendrimers: 2 groups have been investigated: Schiff-base copper (II) carbosilane metallodendrimers comprising chloride G_n-$[CuCl_2]_m$ (n = 0, m = 1; n = 1, m = 4; n = 2, m = 8), and nitrate G_n-$[Cu(ONO_2)_2]_m$ (n = 0, m = 1; n = 1, m = 4; n = 2, m = 8) as counterions previously reported [18] (Figure 1).

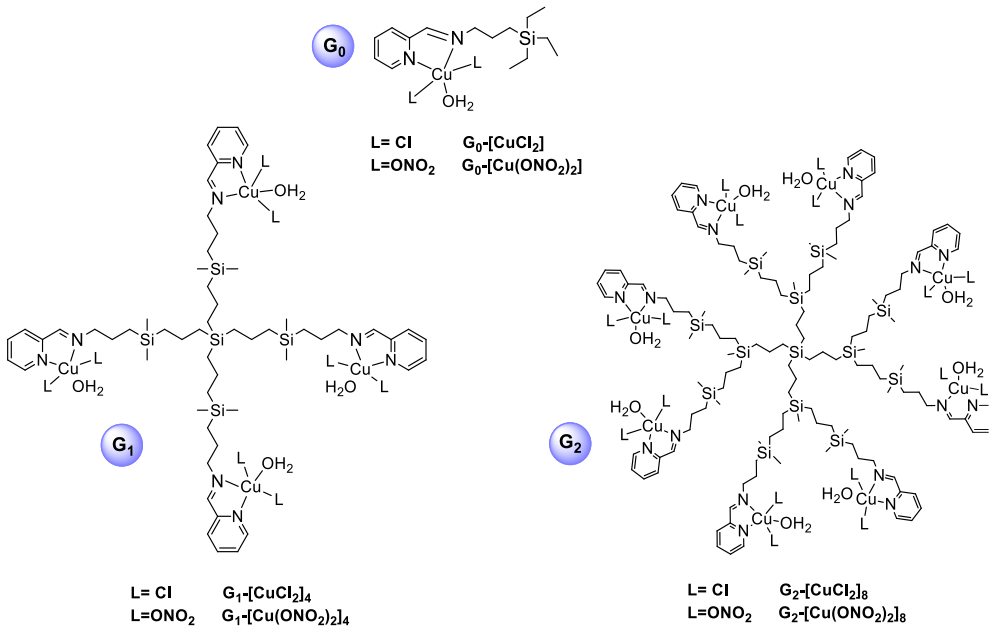

Figure 1. Chemical representation and nomenclature to the Schiff-base copper (II) metallodendrimers of zero (G_0), first (G_1) and second (G_2) generation.

siRNA structures: 2 different anticancer siRNAs—Mcl-1 (Myeloid cell leukaemia-1) and Bcl-2 (B-cell lymphoma 2)-were synthesised (Darmacon, Inc., Lafayette, CO, USA). The experiments were carried out with the fluorescein-labelled siRNAs for electrophoresis, fluorescence measurements, and internalisation techniques. Sense and antisense sequences are included in Table 1.

Table 1. Sense and antisense sequences of two pro-apoptotic siRNAs (Mcl-1 and Bcl-2).

Strand	Mcl-1	Bcl-2
Sense	5′-GGACUUUUAUACCUGUUAUtt 3′	5′-G CUG CAC CUG ACG CCC UUCtt 3′
Antisense	5′-AUAACAGGUAUAAAAGUCCtg 3′	5′-GAA GGG CGU CAG GUG CAG Ctt 3′

2.2. Biophysical Evaluation of the Dendriplexes

2.2.1. Gel Electrophoresis

[siRNA] = 2 µM, G_n-[Cu]/siRNA complexes were prepared at pH 7.4 in 10 mM phosphate-buffered saline. G_n-[Cu]/siRNA molar ratios ranged from 1:1 to 1:100. Electrophoretic conditions: 3% agarose gel with GelRed stain was run in Tris-acetate-EDTA (TAE) buffer for 45 min at 90 V/35 mA and detected with UV Protective assays. G_n-[Cu]/siRNA was incubated with 3 µg/mL RNase at 37 °C for 30 min. The samples were incubated on ice for 10 min before 0.082 mg/mL heparin was added.

2.2.2. Transmission Electron Microscopy (TEM)

10 μL of dendriplex solutions (molar ratio [G_n-[Cu]]/[siRNA] = 30) were coated on 200-mesh copper grids with carbon surface after 15 min incubation. The samples were stained with uranyl acetate solution for 20 min. The grids were washed with deionised water and dried at room temperature before examination in a JEOL-1010 (JEOL, Tokyo, Japan) transmission electron microscope.

2.2.3. Zeta Potential Measurements

Zeta potential values were measured using a Laser Doppler Velocimetry technique by Zetasizer Nano ZS-90, Malvern Instruments (UK) and calculated using the Helmholtz-Smoluchowski equation. Conditions: [siRNA] = 0.3 μM, [G_n-[Cu]]/[siRNA] molar ratios ranged from 0.5 to 50 with water as solvent. Analysis of data was carried out using Malvern software and given as mean ± standard deviation (SD) obtained by 7 measurements in 5 cycles at room temperature for each sample.

2.2.4. Hydrodynamic Diameter of the Dendriplexes

Conditions: [siRNA] = 0.3 μM, [G_n-[Cu]]/[siRNA] molar ratios ranged from 0.5 to 50 with distilled water as solvent. The Malvern Zetasizer was used to determine the hydrodynamic diameter of the dendriplexes by dynamic light scattering in the same manner as in 2.2.3.

2.2.5. Fluorescence Polarisation Measurements

Fluorescence polarisation of labelled siRNA was measured with a PerkinElmer LS-50B spectrofluorimeter (Perkin-Elmer, Waltham, MA, USA). The results are shown as the ratio between sample and control values (r/r_0). Conditions of measure: [siRNA] = 0.35 μM, complex formed at 37 °C, pH 7.4, in 10 mM Na-phosphate buffer at molar ratios [G_n-[Cu]]/[siRNA] ranging from 0.5 to 50. Excitation wavelength of 485 nm was with an excitation-slit width set at 2.5 nm, and emission wavelength 516 nm with an emission-slit set of 3 nm. Data collected are mean ± standard deviation (SD) of a minimum of 3 independent experiments.

2.2.6. Circular Dichroism

Circular dichroism (CD) measurements involved a Hellma quartz cells with a thickness of 0.5 cm in a J-815 CD spectrometer (Jasco, Tokyo, Japan), with software provided by Jasco being used to calculate the mean ellipticity values. Complex formation: on to 1 μM siRNA, different amounts of G_n-[Cu] dendrimers were added in a 10 mM Na-phosphate buffer, pH 7.4, in a range of molar ratios [G_n-[Cu]]/[siRNA] from 1 to 31. Wavelength was set from 235 to 300 nm, and the parameter assays were: 0.5 nm step resolution, 1.0 nm bandwidth, 4 s response time, and 50 nm/min scan speed. The slit was set on auto.

2.3. Evaluation of Anticancer In Vitro

2.3.1. Cell Cultures

MCF-7, purchased from ATCC cell lines (Manassas, VA, USA), were grown in plastic tissue culture flasks (Falcon, GE Healthcare Life Sciences, Chicago, IL, USA) at 37 °C in a humidified air atmosphere with 5% CO_2. DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) was supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone, GE Healthcare Life Sciences, Chicago, IL, USA) and 1% antibiotic (penicillin/streptomycin).

2.3.2. Cytotoxicity

MTT (MTT, 3-(4,5dimethyl 2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) was measured. Cells were seeded on a 96-well plate at 1×10^4 cells per well. After 72 h incubation with G_n-[Cu] dendrimers and G_n-[Cu]/siRNA complexes, MTT (0.5 mg/mL of MTT in PBS) was added for 3 h to

allow formazan crystals to form, which were dissolved in DMSO. Optical density directly proportional to the surviving cells was measured at 580 nm (background correction at 720 nm) using a multiwell plate reader (BioTek PowerWave HT, BioTek Instruments, Inc. Winooski, VT, USA). Results were calculated from the following equation:

$$\% \text{ viability} = (A/Ac) \times 100$$

where Ac, is the absorbance of the control cells (non-treated) and A is the absorbance of the sample. The results are given as mean ± standard deviation (SD) from 3 independent experiments.

2.3.3. Statistical Analysis

The results were collected from a minimum of 3 independent experiments and given as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and Tukey's test was applied. Significance was accepted at $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***).

3. Results and Discussion

Two families of copper (II) metallodendrimers, (G_n-$[CuCl_2]_m$ (n = 0, m = 1; n = 1, m = 4; n = 2, m = 8) and G_n-$[Cu(ONO_2)_2]_m$ (n = 0, m = 1; n = 1, m = 4; n = 2, m = 8) (Figure 1), were selected to assess the influence of dendrimer generation and the metal counter-ion (chloride and nitrate) on their ability to form complexes with siRNA and be delivered to cancer cells. These compounds had positive charges in zeta potential measurements, corroborating their ionic nature, with the positive charge on the metal complex and the negative charge on the counterion (chloride and nitrate) [15]. The siRNAs chosen to carry out the study were Mcl-1 and Bcl-2, which are overexpressed in MCF-7cells.

3.1. Evaluation of the Interaction Between Ru(II) Metallodendrimers and siRNA

Electrophoresis Assays

Gel electrophoresis is commonly applied to characterise nanocomplex (also called dendriplex) formation and calculate [G_n-[Cu]]/[siRNA] molar ratios. To visualise the dendriplex G_n-[Cu]/siRNA formation, it is necessary to use a fluorescein-labelled Mcl-1 siRNA. Negatively charged siRNA can migrate freely in the gel, whereas the addition of positively charged Cu (II) metallodendrimers to the siRNA solution significantly delays siRNA migration, confirming the ability of copper metallodendrimers to complex with siRNA. To calculate the amount of dendrimers needed to complex a total of 2 μM siRNA, different molar ratios of G_n-[Cu]/siRNA were used, ranging from 1 to 100. The electrophoregram shows that dendrimers of the first and second generation interacts with siRNA by forming positively charged complexes, whereas the dendrimers of zero generation do not retain the siRNA migration in the gel, probably due to their small size (Figure 2). It was also possible to observe different patterns of migration depending on the metal counterion (chloride and nitrate) in the dendrimer. Complexes formed with chloride counterion dendrimers (G_n-$[CuCl_2]_m$) need molar ratios ranging between 1:25–1:50 to saturate the G_n-[Cu]/siRNA, whereas nitrate complexes (G_n-$[Cu(ONO_2)_2]_m$) reach saturation at molar ratios ranging between 1:15–1:25 (Figure 2). This effect could be explained by the higher positive density of the charge of nitrate compounds, as the lability of the bond between the nitrate and the metal centre is higher than in chloride derivatives.

Once the capacity of Cu (II) metallodendrimers to form dendriplexes had been proven, one of the essential issues related to the efficient delivery of siRNA is its protection against digestion by nucleases. Based on the results obtained in a previous electrophoresis gel, we selected a molar ratio of G_n-[Cu]/siRNA = 30 as the most suitable to assess protection against RNase-TEM assay, in vitro internalisation, and cell viability experiments.

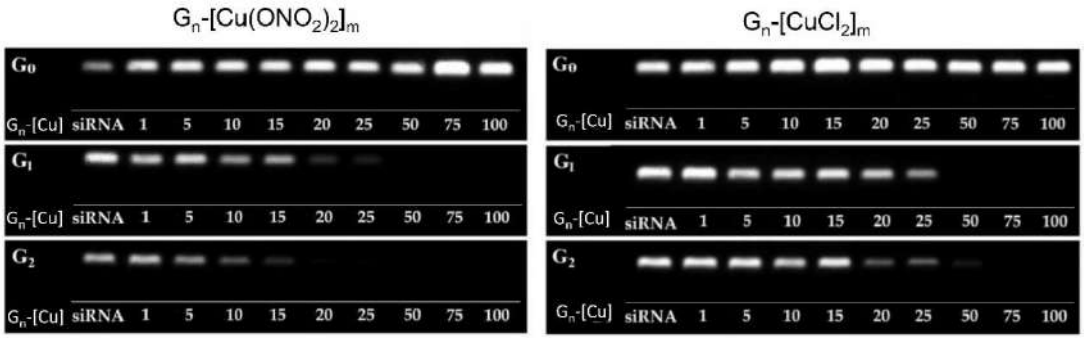

Figure 2. Formation of G_n-[Cu]/siRNA complexes at different molar ratios. G_n-[Cu]: dendrimer, siRNA naked [siRNA] = 2 μM, 1–100 dendriplexes migration at different molar ratios. Complexes prepared as described in the text were analysed by electrophoresis on 3% agarose gel with siRNA migration being identified by fluorescein-labelled siRNA.

As a control, naked siRNA incubated with RNAse showed no fluorescence signal in the gel that would indicate its degradation. Treatment of the G_n-[Cu]/siRNA complexes with heparin after incubation with RNAse displaces the dendrimers from the complex, making it possible to observe the signal of undamaged siRNA in the agarose gel, thereby indicating the protective properties of all copper (II) metallodendrimers of both first and second generation (Figure 3).

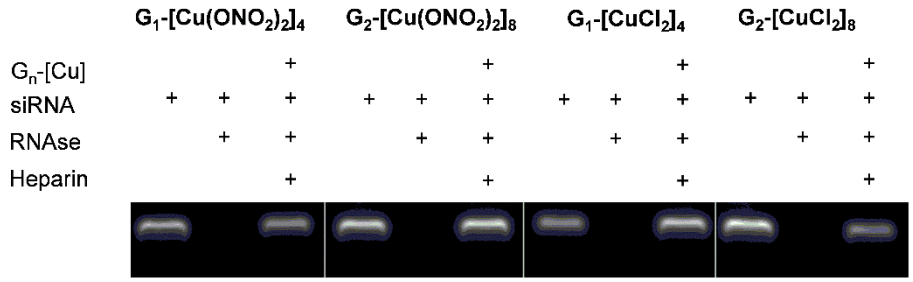

Figure 3. Protective effect of G_n-[Cu(X)$_2$]$_m$ (X = Cl or ONO$_2$) to siRNA against degradation by RNase. [siRNA]= 2 μM, [RNase A] =3.0 μg/mL for 30 min at 37 °C, [heparin] = 0.082 mg/mL.

3.2. Biophysical Characterisation of Dendriplexes

The dendrimers flexibility, as well as the nanoconjugates size, are determining factors in the transfection capacity; in general, higher dendritic flexibility and a smaller size are related to a more powerful transfection ability.

3.2.1. Zeta Potential, the Hydrodynamic Diameter of Dendriplexes, and TEM Assays

Zeta potential measurements determine the surface charges of complexes formed between the siRNA (negative zeta potential values) and dendritic systems. Addition of increasing amounts of first and second generation of cationic Cu (II) metallodendrimers induced a change towards positive values. In general, the lesser amount of the second-generation dendrimer compared to the first generation was needed to compensate the ionic charge, due to the high positive density of 8 positive charges against 4. Furthermore, as in electrophoresis assays, one could note a different behaviour depending on the counterion. Again, nitrate derivate dendrimers (G_n-[Cu(ONO$_2$)$_2$]$_m$) saturated the complex more quickly, with less concentration than chloride derivates (G_n-[CuCl$_2$]$_m$), due to the nature of the metal-ligand bond. In contrast, the dendriplexes formed with the monometallic compound, regardless of the metal counter-ion present in the structure and the concentration used, did not increase the zeta potential values, indicating a lacking complexes formation (Figure 4).

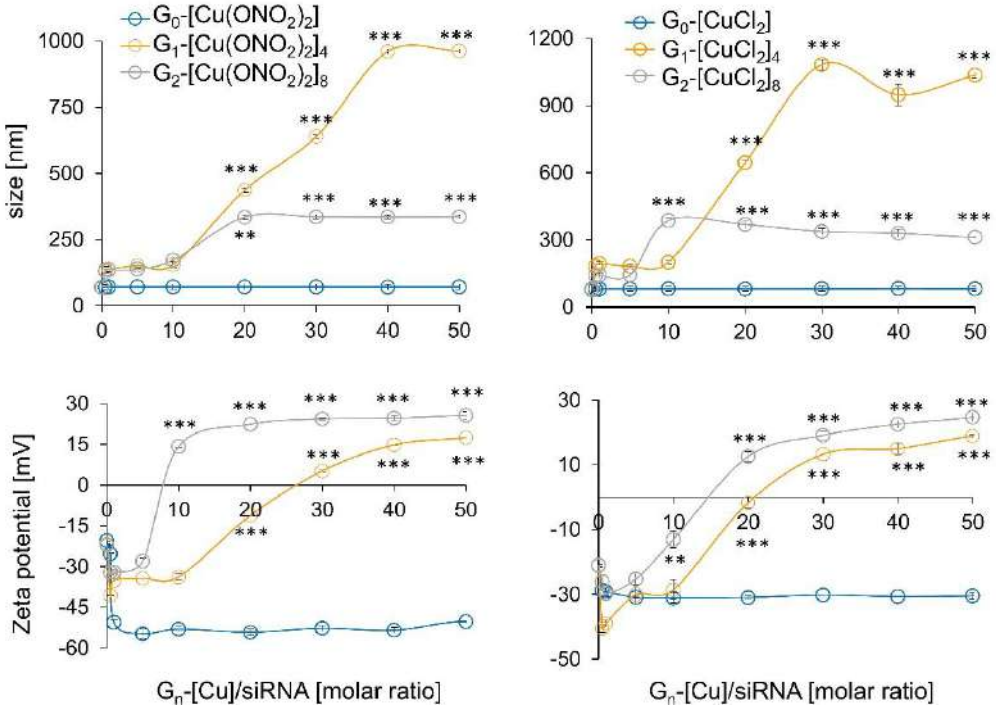

Figure 4. Zeta potential and zeta average size of G_n-[Cu]/siRNA complexes at different molar ratios in water as a solvent. [siRNA] = 0.3 μM. Results were represented as mean ± standard deviation (n = 3). Statistically significant differences compared to the control cells $p \leq 0.01$ (**); $p \leq 0.001$ (***).

The size and morphological structure of dendriplexes can be determined by using dynamic light scattering (DLS) to find their hydrodynamic diameter in solution and TEM for morphological structure, shape, and size. The DLS results show that those formed with dendrimers of the first generation were more prominent than those composed of the second generation, with sizes ranging from 980–1110 nm and 290–340 nm, respectively (Figure 4), and for the monometallic derivative, the size parameters were unchanged in respect to naked siRNA. These findings indicate the existence of aggregation between different dendriplexes confirmed by TEM, where, in all dendriplexes, visible aggregates with globular electron-dense structures inside were present (Figure 5). TEM images indicate that conjugates formed by G_1-$[CuCl_2]_4$ are smaller in size (~50 nm), whereas other dendriplexes, G_1-$[Cu(ONO_2)_2]_4$, G_2-$[Cu(ONO_2)_2]_8$ and G_2-$[CuCl_2]_8$, were of greater dimensions (~500 nm) (Figure 5). This phenomenon has been previously observed for other carbosilane systems of a cationic nature [25–27] or other dendritic skeletons as PAMAM [28,29] or phosphorus dendrimers [30]. The size and charge of the nanoparticle can be responsible for their interaction with immune cells [31]. Moreover, the larger nanoparticles (130 and 70 nm) were strongly affected by the drag force leading to the removal from endothelial cell surfaces [32]. Khopr et al. (2018) suggests that the anionic PISA nanoparticles (40 nm) seem to be good candidates for anti-cancer drug delivery while the bigger nanoparticles can be applied for the creation of vaccines and could be used as immunomodulators for B cells [32].

The difference in size found by DLS and TEM for the same dendritic structure can be explained by differences in sample preparation; while DLS is a technique carried out in solution, TEM looks at dry samples [4,33]. In all the cases analysed, there was also an effect on the hydrodynamic diameter of the complexes formed by siRNA and delivery systems, which was concentration-dependent, being higher with an increasing molar ratio.

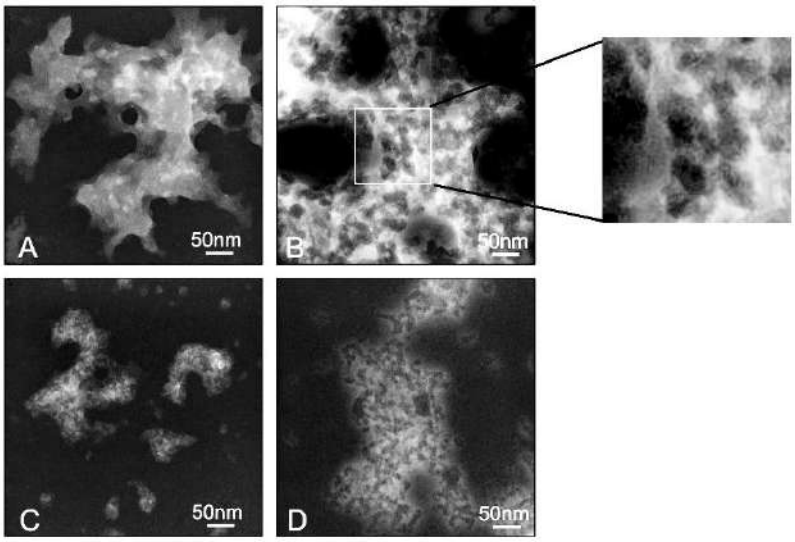

Figure 5. Ultrastructure of G_n-[Cu]/siRNA complexes visualised by TEM: (**A**) G_1-$[Cu(ONO_2)_2]_4$, (**B**) G_2-$[Cu(ONO_2)_2]_8$ (**C**) G_1-$[CuCl_2]_4$, (**D**) G_2-$[CuCl_2]_8$.

3.2.2. Fluorescence Polarisation and Circular Dichroism

The stability of dendriplexes was corroborated by fluorescence polarisation measurements of fluorescein-labelled Mcl-1. Changes in fluorescence polarisation of a probe attached to the siRNA structure can reflect an interaction between dendrimers and nucleic acids. Fluorescence polarisation decreased with first and second-generation dendrimers, whereas for monometallic ones, this was not observed (Figure 6). Plateau phase was reached at a molar ratio [G_n-[Cu]]/[siRNA] = 20 for the dendrimers of both nitrate and chloride counterions. Increasing concentration of dendrimers of zero generation did not significantly change the level of fluorescence polarisation.

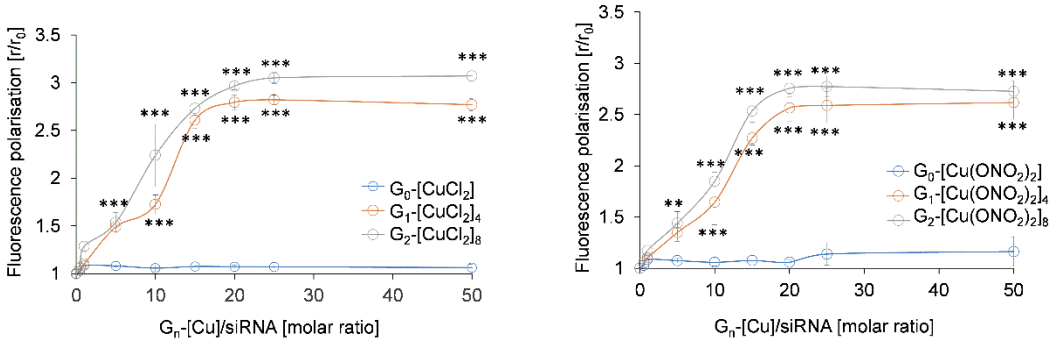

Figure 6. Changes in fluorescence polarisation of fluorescein-labelled Mcl-1 in the presence of increasing concentrations of different G_n-[Cu] metallodendrimers. [siRNA] = 0.35 μM in Na-phosphate buffer 10 mM (pH 7.4). Results are represented as mean ± standard deviation (n = 3). Statistically significant differences compared to the control cells $p \leq 0.01$ (**); $p \leq 0.001$ (***).

The influence of nanoconjugates on the secondary structure of siRNA was measured by circular dichroism [13,30]. The typical siRNAs CD spectra contains two characteristic peaks at λ = 210 and 260 nm. [7] However, we focused on the analysis of the ellipticity changes in the region of λ = 240–280 nm. A decrease in ellipticity (θ) in the range of a wavelength from 235 to 300 nm, accompanied by a reduction in the peak at 258 nm, was detected with increasing G_n-[Cu]/siRNA molar ratios (Figure 7). CD spectra of siRNA in the presence of dendrimers reflected an alteration in the secondary structure of siRNA due to the binding of dendrimer molecules. The second-generation dendrimers showed the most intensive changes, whereas the monometallic derivatives do not significantly affect the structure of

siRNA. Analysis of the changes in θ/θ_0 parameter at 258 nm showed that at this wavelength, ellipticity values reached a plateau at a molar ratio G_n-[Cu]/siRNA of 10 for G_1-$[Cu(ONO_2)_2]_4$, G_2-$[Cu(ONO_2)_2]_8$, G_1-$[CuCl_2]_4$1 and 8 for G_2-$[CuCl_2]_8$.

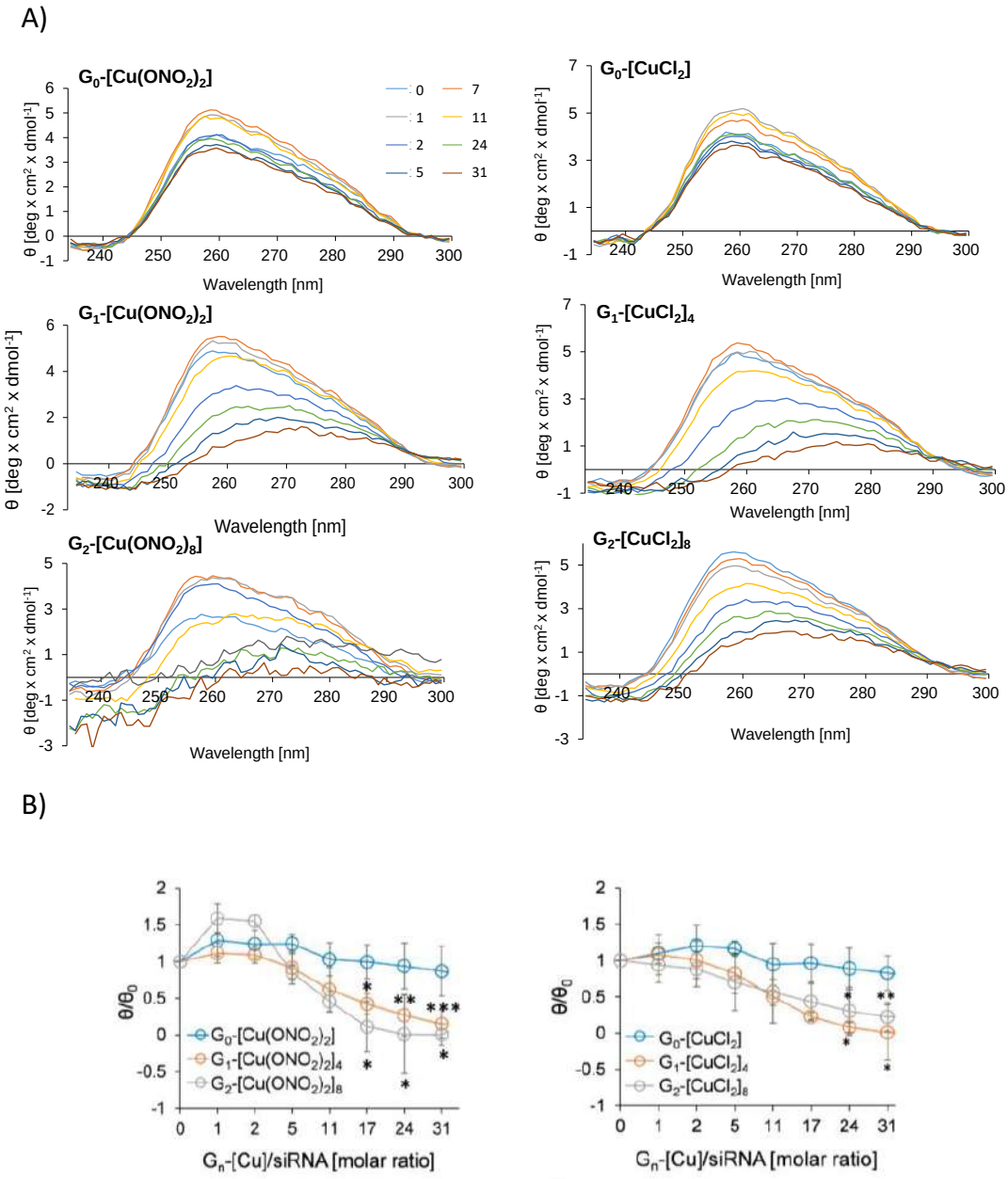

Figure 7. (**A**) CD spectra of siRNA at the presence of G_n-[Cu] metallodendrimers in increasing molar ratios. G_n-[Cu]/siRNA (**B**) Changes in the mean residue ellipticity (θ) of siRNA at λ = 258 nm in the presence of dendrimers. [siRNA] = 1 μM. Results are represented as mean ± standard deviation (SD), n = 3. Statistically significant differences compared to the control cells $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***).

3.3. Biological Evaluation of CCD-siRNA Complexes: Cellular Uptake and Anticancer Activity

Cells treated with pro-apoptotic siRNA silences the expression of anti-apoptotic genes, which may result in the induction of apoptosis and, consequently, a decrease in cancer cell viability [4,34]. Cancer cells use different mechanisms to evade apoptosis and favour tumour progression. Family members of Bcl-2 proteins control mitochondrial-dependent apoptosis. High levels in these anti-apoptotic proteins,

including Bcl-2 and Bcl-xL, prevent apoptosis and therefore impede cancer therapy. The influence of dendriplexes on MCF-7 involved the use of 2 different types of siRNAs, which were Mcl-1 and Bcl-2, leading to its overexpression. On the one hand, the involvement of Mcl-1 in breast cancer has been widely confirmed by the high basal levels of the mRNA found in the cancer compared to other subtypes. Moreover, it has become a target for breast cancer treatment and a crucial indicator in prognosis [22]. This human protein is encoded by the Mcl-1 gene that induces myeloid leukemia cell differentiation.

On the other hand, the Bcl-2 gene is overexpressed in 50–70% of breast cancer patients, giving rise to resistance to conventional treatments, making it a promising target. Silencing of this gene by siRNA in orthotopic xenograft models has shown that siRNA against Bcl-2 given intravenously significantly suppresses growth of MCF-7 cells. Moreover, this strategy significantly increases the efficacy when combined with doxorubicin [35].

3.3.1. Cellular Uptake

Confocal microscopy shows the ability of dendritic systems to transport siRNA into the cytoplasm, whereas naked siRNA does not cross the membrane without a transfection agent (Figure 8A). The results of confocal microscopy and flow cytometry techniques showed that the internalisation of the G_n-[Cu]/siRNA complexes is more favourable after 3 h of incubation than after 24 h and is dependent on dendrimer generation and the nature of the counterion. The percentage of cell internalisation (≈50%) is raised after 3 h incubation with second-generation Cu(II) metallodendrimer with a nitrate counter-ions, G_2-$[Cu(ONO_2)_2]_8$, (Figure 8, bottom panel). In addition, the microphotographs show that Mcl-1 siRNA complexed with G_n-$[Cu(ONO_2)_2]_m$ dendrimers was more visible than siRNA complexed with G_n-$[CuCl_2]_m$ dendrimers. Whereas G_n-$[Cu(ONO_2)_2]_m$ dendriplexes appeared inside cells as green dots, the complexes created with G_n-$[CuCl_2]_m$ were mainly near the cell membranes, rarely in the cytoplasm (Figure 8, top panels). Although transfection will depend on the type of cell to be transfected, the applied dendrimer generation, and the time of incubation, we can say that copper (II) metallodendrimers, in general, have a greater transfection efficiency than the Ru(II) analogues in the HL60 a cell line (Ru(II)-second generation (≈30% efficiency) [12], and close to cationic carbosilane dendrimers with 16 positive charges on the surface [5]. The extension of incubation time up to 24 h can cause a gradual siRNA release from the complex, and, as a result, the siRNA interference phenomenon may be initiated [29].

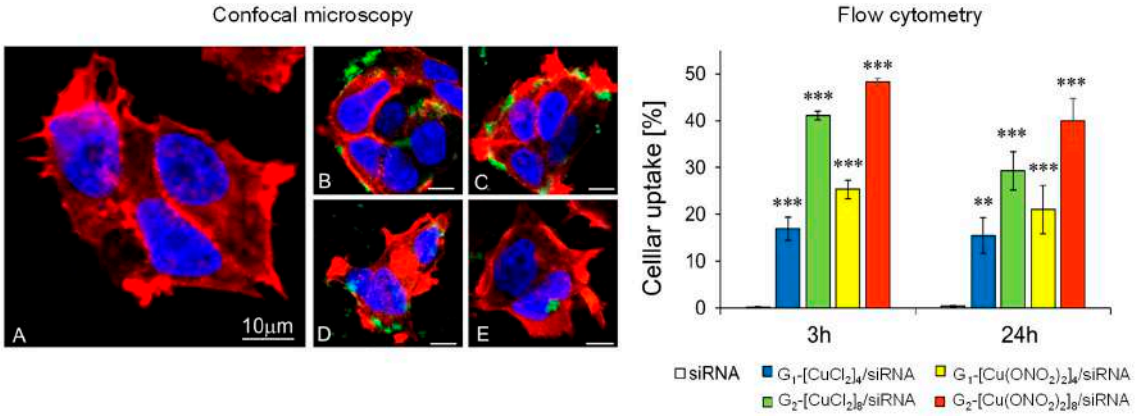

Figure 8. Confocal microscopy: (**A**) naked siRNA, (**B**) G_1-$[Cu(ONO_2)_2]_4$/siRNA (**C**), G_2-$[Cu(ONO_2)_2]_8$/siRNA (**D**) G_1-$[CuCl_2]_4$/siRNA and (**E**) G_1-$[CuCl_2]_4$/siRNA and flow cytometry data of uptake by MCF-7 cells. General conditions: incubation time: 3 h and 24 h; Bar = 10 µM; [siRNA] = 100 nM; molar ratio [G_n-[Cu]/[siRNA]] = 30. The results are mean ± standard deviation (SD), n = 3. Statistically significant differences compared to the control cells $p \leq 0.01$ (**); $p \leq 0.001$ (***).

3.3.2. Anticancer Activity

A possible cooperativity between Cu(II) metallodendrimers, which are active in a wide panel of cancer cell lines, and pro-apoptotic Mcl-1 or Bcl-2 siRNA, have been studied by MMT assay in MCF-7 cells after 72 h treatment. Previously, we reported the IC_{50} values for Cu(II)-systems in MCF-7 [21] and PBMC [15] cell line. The G_n-[Cu] concentration chosen to carry out the viability assays here was a subtoxic concentration of 3 μM. Figure 8 shows the treatment of MFC-7 cells with dendrimer alone and dendriplex (molar ratio [G_n-[Cu]/siRNA] = 30; G_n-[Cu] = 3 μM and for siRNA = 100 μM) expressed as percentage viability. The results indicate that G_n-[Cu]/siRNA complexes were more toxic to MCF-7 cells compared with uncomplexed dendrimers. As an example, MCF-7 treated with 3 μM G_1-$[CuCl_2]_4$ had a viability of up to 95%, whereas dendriplexes G_1-$[CuCl_2]_4$/Mcl-1 and G_1-$[CuCl_2]_4$/Bcl-2 reduced viability to 32% and 43%, respectively (Figure 9). With second-generation systems, a reduction of ~90% in viability was seen when MFC-7 cells were treated with dendriplexes. This result shows a cooperative effect between fragments with anticancer activity and a different mode of action. In addition, regarding dendrimer size and nature of counterion, this finding shows that the dendritic generation affects the cytotoxicity of the dendriplexes, i.e., the first-generation dendrimers show the most promise due to the lack of toxicity when the dendrimers are administered alone. However, the presence of the different counterion (chloride or nitrate) did not appear to significantly affect the anticancer activity. Results previously obtained for other cationic carbosilane dendrimers of the second and third generations with 12 and 24 functional groups, respectively, in HeLa and HL-60 cell lines, showed that the combination of these dendrimers with si-Mcl-1, si-Bcl-2 and si-Bcl-xL, required a siRNA concentration of 250 nM and a molar ratio of [G_n-[Cu]]/[siRNA] = 10 to have promising activity, despite the greater number of functional groups compared to copper metallodendrimers.

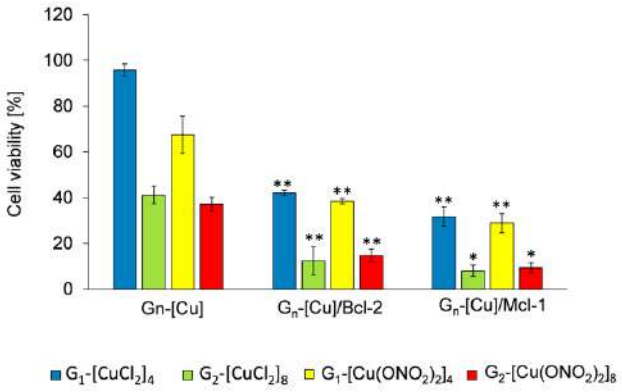

Figure 9. Viability of MCF-7 cells after 72 h of incubation with G_n-[Cu] and their complexes with pro-apoptotic siRNAs (Mcl-1; Bcl-2) at molar ratio [G_n-[Cu]]/[siRNA] = 30. Conditions: [G_n-[Cu]] = 3 μM, [siRNAs] = 100 nM. Results are mean ± standard deviation (SD), n = 3. Statistically significant differences compared to the control cells $p \leq 0.05$ (*); $p \leq 0.01$ (**).

4. Conclusions

We have demonstrated the ability of cationic Cu (II) carbosilane dendrimers to transfect the pro-apoptotic siRNAs Mcl-1 and Bcl-2 and protect it against nuclease degradation. All the biophysical procedures carried out in this work indicate that monometallic derivatives under the applied conditions do not complex with siRNA, probably as a result of their small size, which does permit the creation of stable conjugates. In contrast, first- and second-generation copper (II) metallodendrimers not only formed dendriplexes with pro-apoptotic siRNA, but also protected them against degradation by nucleases. Moreover, Cu (II) metallodendrimers act as pure vehicles as well as enhancing the anticancer action of siRNA. To be highlighted is the fact that the first-generation dendrimer G_1-$[CuCl_2]_4$ at 3 μM does not have a cytotoxic activity (viability 90%), but when combined with siRNAs, it can decrease

viability by 35–40%, depending on the pro-apoptotic siRNA used. These findings show that copper (II) dendrimers can be powerful agents in cancer therapies as siRNA carriers or anticancer agents.

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Combination of Copper Metallodendrimers with Conventional Antitumor Drugs to Combat Cancer in In Vitro Models

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Abstract: Copper carbosilane metallodendrimers containing chloride ligands and nitrate ligands were mixed with commercially available conventional anticancer drugs, doxorubicin, methotrexate and 5-fluorouracil, for a possible therapeutic system. To verify the hypothesis that copper metallodendrimers can form conjugates with anticancer drugs, their complexes were biophysically characterized using zeta potential and zeta size methods. Next, to confirm the existence of a synergetic effect of dendrimers and drugs, in vitro studies were performed. The combination therapy has been applied in two cancer cell lines: MCF-7 (human breast cancer cell line) and HepG2 (human liver carcinoma cell line). The doxorubicin (DOX), methotrexate (MTX) and 5-fluorouracil (5-FU) were more effective against cancer cells when conjugated with copper metallodendrimers. Such combination significantly decreased cancer cell viability when compared to noncomplexed drugs or dendrimers. The incubation of cells with drug/dendrimer complexes resulted in the increase of the reactive oxygen species (ROS) levels and the depolarization of mitochondrial membranes. Copper ions present in the dendrimer structures enhanced the anticancer properties of the whole nanosystem and improved drug effects, inducing both the apoptosis and necrosis of MCF-7 (human breast cancer cell line) and HepG2 (human liver carcinoma cell line) cancer cells.

Keywords: dendrimers; copper; anticancer drug; drug delivery; apoptosis; necrosis

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1. Introduction

Cancer is a serious global disease and is the second most common reason of death in the world [1,2]. The most commonly used methods of treatment for cancer are chemotherapy, radiation therapy and surgical excision of affected tissues [3]. However, commonly practiced therapies are not always effective, and many side effects connected with the treatment of cancers can negatively affect the quality of the patient's life [2].

Currently, many studies are focusing on the development of anticancer drug carriers to improve their bioavailability and increase selectivity, thereby reducing side effects [4]. Using nanoparticle-based delivery systems can result in the increase of drug concentration in tumor tissue and a reduction of toxicity in healthy tissues. Presently, non-viral drug delivery involves the use of various types of nanoparticles, including polymeric nanoparticles, micelles, liposomes, nanotubes or dendrimers [5].

Dendrimers, due to their unique properties such as specific branching structures, monodispersity, thermal/chemical stability and hydrophobic/hydrophilic natures, have

promise. Cationic dendrimers could be good candidates for use in chemotherapeutical transport systems [6].

Doxorubicin (DOX), methotrexate (MTX) and 5-fluorouracil (5-FU) are drugs that are commonly used in cancer chemotherapy [7–9]. Doxorubicin, by intercalating into DNA (deoxyribonucleic acid), inhibits the replication and transcription of cancer cells [10,11]. Although doxorubicin is one of the most effective anticancer drugs, its use is limited due to side effects such as cardiotoxicity, which can cause cardiomyopathies with fatal consequences [4]. The mechanism of methotrexate is similar and causes replication and transcription arrest, blocking cells developing in the G1 phase of the cell cycle [12]. Again, even though methotrexate is one of the most effective chemotherapeutics, it has dangerous side effects. The consequence of methotrexate application is myelosuppression, hepatotoxicity and pulmonary fibrosis [13]. 5-fluorouracil causes the damage of DNA and RNA (ribonucleic acid) of cancer cells [14] and is also cytotoxic against normal cells and tissues and can cause life-threatening cardiotoxicity [15].

Drugs used in classical chemotherapy do not distribute to specific parts of the body; therefore, they affect all cells, not just cancer cells. Consequently, there is a limitation of the possible doses for cancer treatment [5]. Additionally, many types of cancer cells are drug-resistant due to quick removal of the therapeutic material from the cytoplasm [16]. Delayed release of anticancer drugs from the complex with nanomaterials would be a desired effect.

A combined therapy of drugs to reduce side effects provides good results since, in combination, lower doses of the drugs are necessary to be effective. Moreover, the use of controlled release systems such as nanomaterials to increase the bioavailability of antimetastatic drugs seems to be promising.

Dendrimers, well-defined macromolecular structures, have proven to be good transfection vehicles and have been studied in combination therapy. Dendrimers can also undergo continuous modifications directed to increase their efficiency in cancer therapy. According to the literature, hydrophilic nanoparticle surface chemistry has been characterized as less toxic and has exhibited better biocompatibility than the cationic surface of nanoparticles for medical applications [17]. However, Shcharbin's team displayed that only high generation of dendrimers with unmodified surface in high doses has some toxicity in vivo. The modifications of nanoparticle surface decrease their toxicity and determine the desired location of multifunctional dendrimer-based conjugates [18]. Additionally, the effects depend on the changing surface properties that influence the uptake of nanoparticles and their conjugates with drugs [17,19].

These modifications include the introduction of metal atoms that exhibit anticancer properties, for example, platinum, gold, silver, ruthenium or copper [6,20], into the dendrimer structure to improve their biomedical applications. In particular, carbosilane copper metallodendrimers bearing iminopyridine moieties have demonstrated outstanding anticancer activity in both solid tumors and myeloid cell lines [6,21–24].

It is known that copper is one of the most important micronutrients that takes part in biological oxidation-reduction (redox) reactions employed by critical enzymes, including cytochrome c oxidase (COX), NADH dehydrogenase-2 (ND2) or Cu/Zn–superoxide dismutase (SOD1). Additionally, it possesses redox activity that leads to the production of reactive oxygen species (ROS), which is connected with homeostatic regulation of copper within the body [25]. Invalid copper levels have been repeatedly reported about in cancer tissues [26–28].

Subtle changes, such as the metal counterion or the presence of ligands on the iminopyridine ring, can modulate antitumor activity, probably through different ROS-production pathways. This exemplifies the importance of adequately designing the metallodendrimer.

Several examples of the use of metallodendrimers in combination therapy are, for example, the combination of a third-generation copper (II) metallophosphate dendrimer in combination with DOX, cisplatin, paclitaxel and MG132, which showed stronger inhibition of HL60 cell proliferation compared to drugs and dendrimer use alone. The combination of

ruthenium(II) carbosilane metallodendrimers showed synergism when used in combination therapy with MTX, DOX or 5-Fu against leukemia [16].

In the present study, copper carbosilane metallodendrimers were chosen for their conjugation with the previously mentioned anticancer drugs. Some preclinical and clinical studies show the positive effects of copper in cancer therapy [25] due to copper limiting many aspects such as growth, angiogenesis and metastasis of cancer progression [29]. Additionally, copper increases the level of reactive oxygen species (ROS) in cancer cells, the consequence of ROS generation being damage to DNA [23]. It is presumed that the ability to produce ROS underlies the copper toxic effects toward cancer cells that is not observed in normal cells. This could result in higher sensitivity of cancer cells for changes in ROS levels than normal cells [25].

This study presents the first results from experiments analyzing the effects of copper carbosilane metallodendrimers containing chloride Gn-[NCPh(*o*-N)$CuCl_2 \cdot H_2O]_m$ (n = 0, m = 1 CCD-Cl-0; n = 1, m = 4 CCD-Cl-1; n = 2, m = 8 CCD-Cl-2) and nitrate ligands Gn-[NCPh(*o*-N)$Cu(ONO_2)_2 \cdot H_2O$]m (n = 0, m = 1 CCD-NO-0; n = 1, m = 4 CCD-NO-1; n = 2, m = 8 CCD-NO-2) conjugated with conventional anticancer drugs against cancer cells. It was shown that studied dendrimers are more toxic towards cancer than normal cells and were able to improve the anticancer effect of traditional drugs.

2. Results

2.1. Zeta Potential and Size

It is known that the surface charge, size and binding parameters of nanovectors are significant when evaluating them for drug carriers. Based on the previously published results of carbosilane metallodendrimer interaction with drugs [16], it was hypothesized that copper metallodendrimers can form complexes with DOX, MTX and 5-Fu. To prove this hypothesis, the size and surface charge of naked drugs were checked and then complexed with dendrimers. To determine the mentioned parameters, the zeta technique was applied. Obtained results indicate that both zeta size and zeta potential values were increased when dendrimers were added to the drug solution in all tested formulations. The addition of the CCD-NO of first and second generations (CCD-NO-1 and CCD-NO-2) to the drug suspension increased the zeta potential of the formed nanosystem up to 50 mV, while the presence of CCD-NO of generation "0" did not exceed 40 mV, giving the lowest values of size and zeta potential of all of them (see Figure 1).

The highest zeta potential was registered for the formulation DOX/CCD-NO-1 (Figure 1A). Similar results were obtained for the complexes formed by CCD-Cl dendrimers with drugs. While CCD-NO-1,2 and CCD-Cl-1,2 dendrimers were added to the MTX solution, the zeta potential values were near 40 mV. The dendrimers of generation "0" again showed the lowest values not exceeding 20 mV. CCD-Cl dendrimers exhibit a less cationic character with more lipophilic properties than CCD-NO, due to the stronger Cu-Cl bond. This behavior will impact the stability of the complexes formed with each of the drugs, which intrinsically exhibit different properties. For example, doxorubicin and methotrexate show pending ionizable groups and nicely interact with CCD-NO with no relevant differences between G1 and G2. However, the increase in lipophilicity from CCD-Cl-1 to CCD-Cl-2 is counterproductive in the interaction with DOX and MTX (but slightly favorable with 5-FU).

The size of nanocomplexes formed with CCD-NO and CCD-Cl dendrimers of generations one and two with DOX, 5-FU and MTX were near 450 nm, 800 nm and 600 nm, respectively. The compounds of generation "0" formed smaller complexes with the size being about 300 nm for DOX, 600 nm for 5-FU and 400 nm for MTX, as expected (Figure 1B).

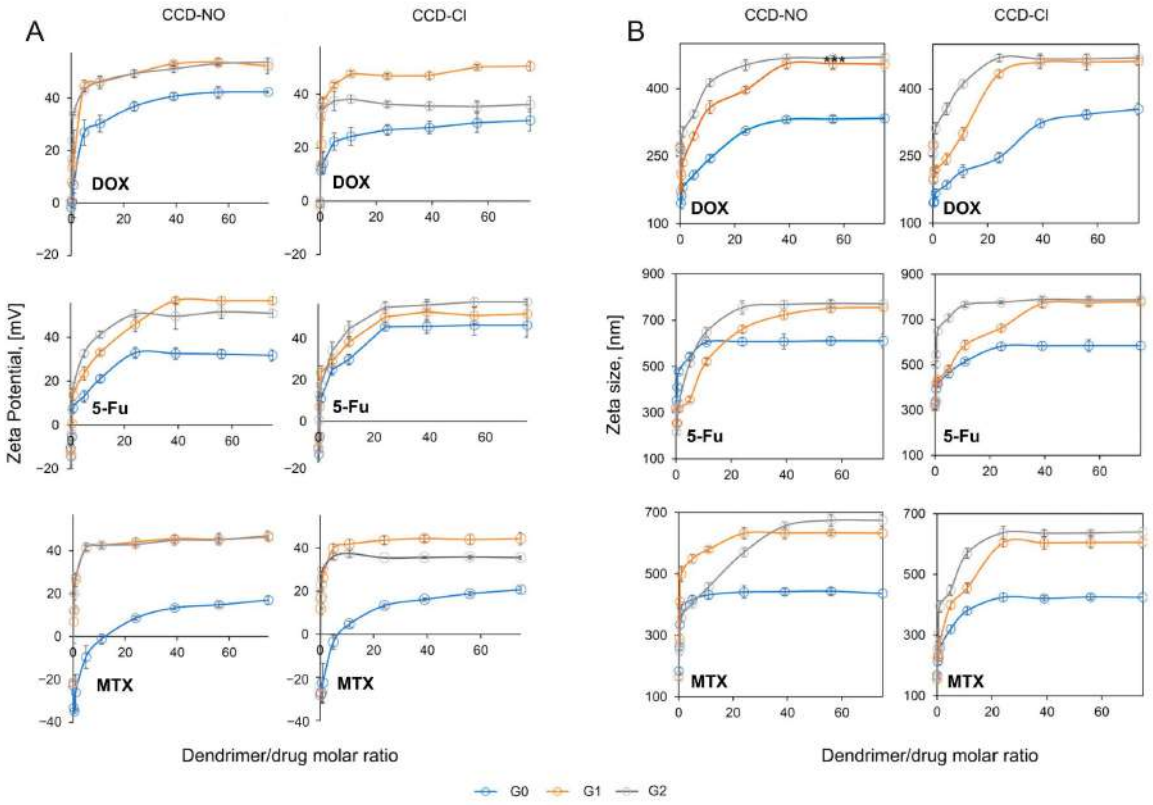

Figure 1. Zeta potential (**A**) and zeta average size (**B**) of DOX—top panels; 5-FU—middle panels; and MTX—bottom panels, at the presence of increasing concentrations of copper dendrimers. Drugs concentration 10 µmol/L. The measurements were performed using sodium phosphate buffer 10 mmol/L, pH 7.4. Results are mean ± standard deviation (SD), n = 3.

2.2. Cytotoxicity

Since the main aim of this study was to analyze the synergetic effect of complexes together with traditional anticancer drugs with copper metallodendrimers and additionally show the suitability of using CCDs as drug carriers, the experiments on cell viability with the presence of both components were performed in two different lines: MCF-7 (Figure 2) and HepG2 cells (Figure 3).

The MCF7 and HepG2 cells were incubated for 72 h with drugs, dendrimers or drug/dendrimer complexes. The amount of drugs established on the base of drug effective concentrations (80% of viability for considered cells) was as follows: DOX 0.1 µmol/L, 5-FU 1µmol/L and MTX 0.02 nmol/L.

The results presented in Figures 2 and 3 show significant synergistic effect of copper metallodendrimers complexed with drugs. All tested concentrations of the dendritic systems and individual drugs were shown to be of low cytotoxicity in both cell lines, MCF7 (Figure 3) and HepG2 (Figure 3), while their combination (dendrimer/drug) in different concentrations significantly reduced cell viability. The synergistic effect is more pronounced in the combination with doxorubicin and 5-fluorouracil, where it is possible to observe reductions of 40 to 80% with respect to the control, as the concentration of the dendritic system in the formulation is increased.

It is worth highlighting that among CCD-NO dendrimers, antitumor activity increases with the dendrimer generation, G2 being the better carrier. However, among CCD-Cl dendrimers, it is the first-generation metallodendrimer which is the most efficient. This behavior highlights the impact of the metal counterion, with chloride generating a more hydrophobic environment which improves the interaction with the drugs, even at lower generations.

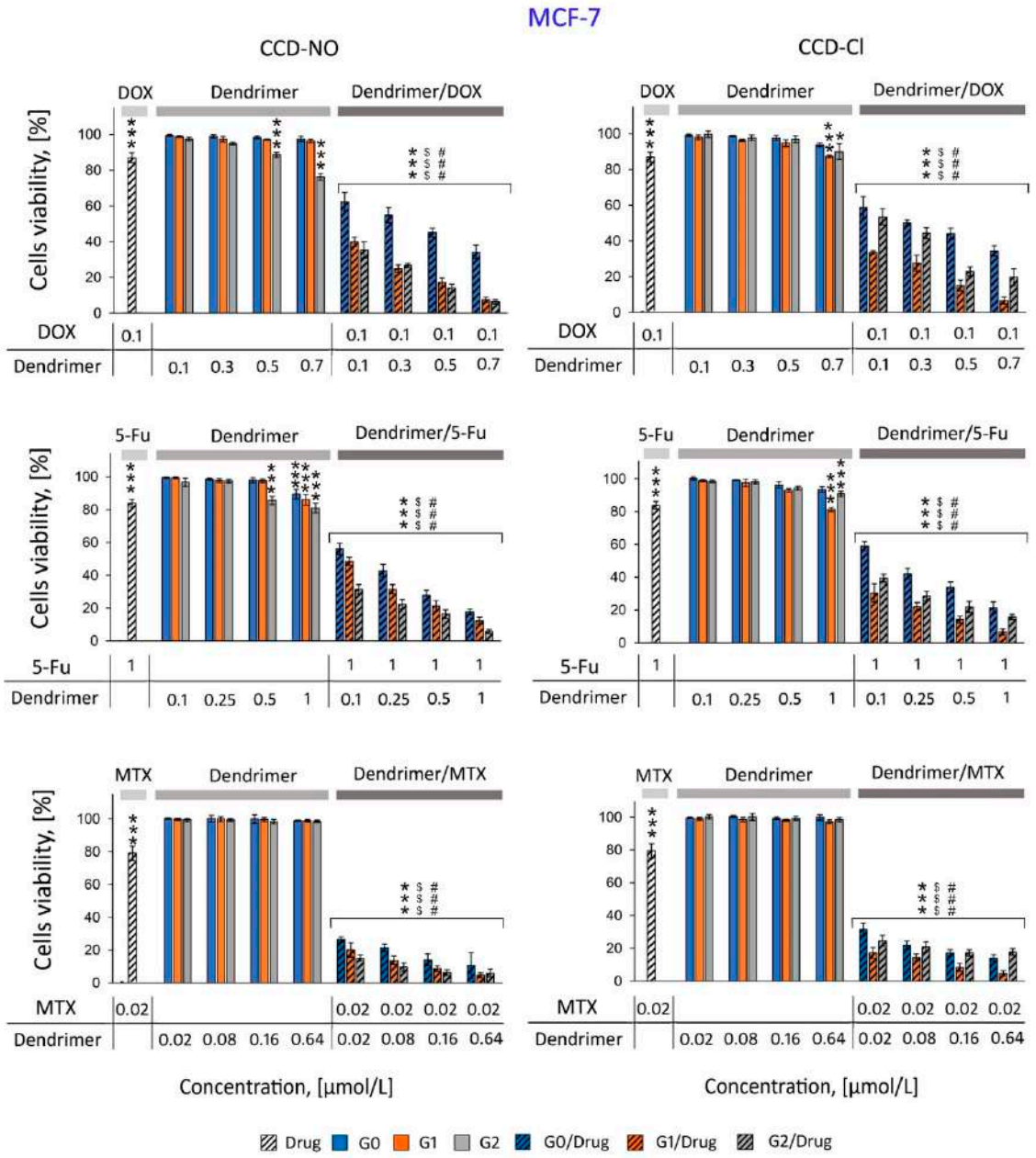

Figure 2. Cytotoxicity profiles of anticancer drugs, copper dendrimers and their (dendrimer/drug) complexes towards MCF7 cells. MTT assay, incubation time 72 h in phosphate saline buffer 10 mmol/L, pH 7.4. Results are means ± SD, from a min. 3 independent experiments. Statistically significant differences vs. control: * p <0.05, *** p < 0.001, vs. free drug $$$ p < 0.001, vs. free dendrimer ### p < 0.001.

The complexes formed by all drugs with CCD-NO and CCD-Cl dendrimers had similar effects; however, the cytotoxicity of CCD-NO formulations was slightly higher, maybe due to the major solubility of dendrimers in aqueous medium when the copper ligand is nitrate. The most cytotoxic CCD-NO-1/MTX formulation in the highest applied concentration reduced the number of living cells by 95.25% vs. the control. The other tendency was observed for 5-FU/dendrimer nanocomplexes. While CCD-NO complexes showed generation-dependent cytotoxic activity, among the CCD-Cl formulations the most effective was the system formed with the first generation of dendrimers. CCD-NO-2/5-FU reduced the number of living cells by 94.37% and 91.92% for MCF7 and HEPG2 cells, respectively, and the CCD-Cl-1/5-FU decreased the viability of cells by 93.38% for MCF7

and 92.02% for HEPG2 cells. Similarly, while CCD-NO/DOX complexes decreased the viability of both cell lines in a generation-dependent manner, among the CCD-Cl/DOX systems the most cytotoxic was CCD-Cl-1/DOX. The number of living cells decreased by about 90% for MCF7 and by about 70% for HEPG2 cells.

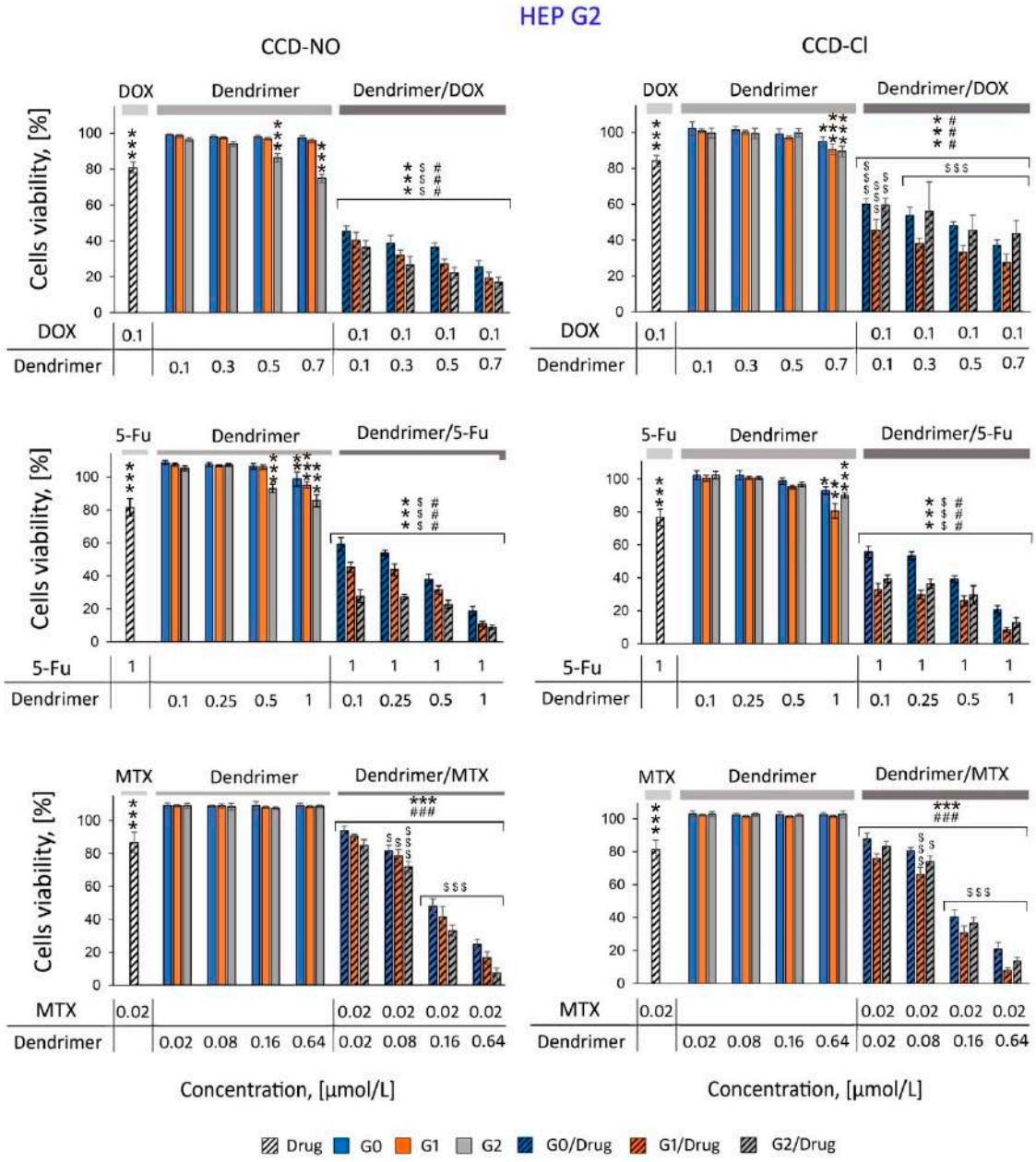

Figure 3. Cytotoxicity profiles of anticancer drugs, copper dendrimers and their (dendrimer/drug) complexes towards HEP G2 cells. MTT assay, incubation time 72 h in phosphate saline buffer 10 mmol/L, pH 7.4. Results are means ± SD, from a min. 3 independent experiments. Statistically significant differences vs. control: * p <0.05, ** p < 0.01, *** p < 0.001, vs. free drug $ p <0.05, $$$ p < 0.001, vs. free dendrimer ### p < 0.001.

2.3. Generation of Intracellular Reactive Oxygen Species (ROS)

Carbosilane metallodendrimers have cytotoxic responses towards cancer cells [6,30–32] that are consistent with our present studies on cell viability described above. To understand the toxicity mechanisms of the dendrimers and their drug complexes better, several additional analyses were performed. One of the studies carried out was the evaluation of an in vitro generation of intracellular reactive oxygen species (ROS) where MCF 7 and HEP G2

cancer cells are exposed to copper metallodendrimers and combination dendrimer/drugs. An increased production of intracellular ROS level due to in vitro exposure of MCF 7 and HEP G2 cancer cells to copper dendrimers and analyzed dendrimer/drug complexes was evaluated. The levels of ROS were detected at 0.5, 3, 24 and 48 h incubation of cells with studied samples. Neither of the used dendrimer concentrations significantly increased the level of ROS in both tested cell lines during applied incubation time (Figure 4).

The figure of ROS level changes involved by naked drugs is present in the Supplementary File (Figure S1A). When dendrimers were complexed with drugs and added to the cell suspension, the data showed that although ROS production initially increased in an approximately linear fashion at the beginning, at 24 h of incubation there is maximum ROS production, with decreases after 48 h of incubation. Interestingly, the application of higher doses only slightly influenced the ROS level. The results are similar in both MCF7 and HepG2 cells, the highest increase in the generation of ROS being observed after 24 h of incubation, and in selected cases a massive increase in the ROS level has been observed: 160% vs. control (100%).

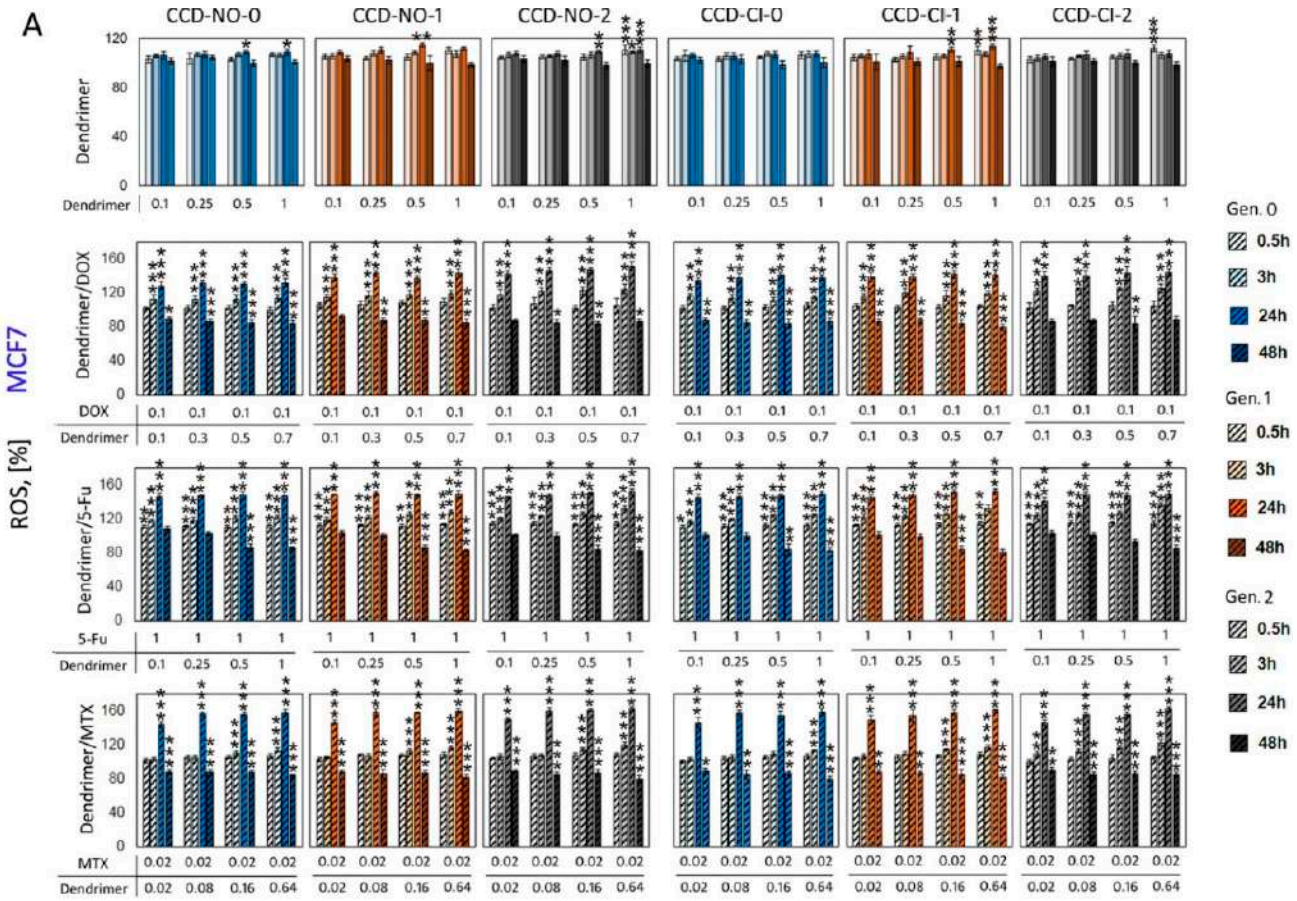

Figure 4. *Cont.*

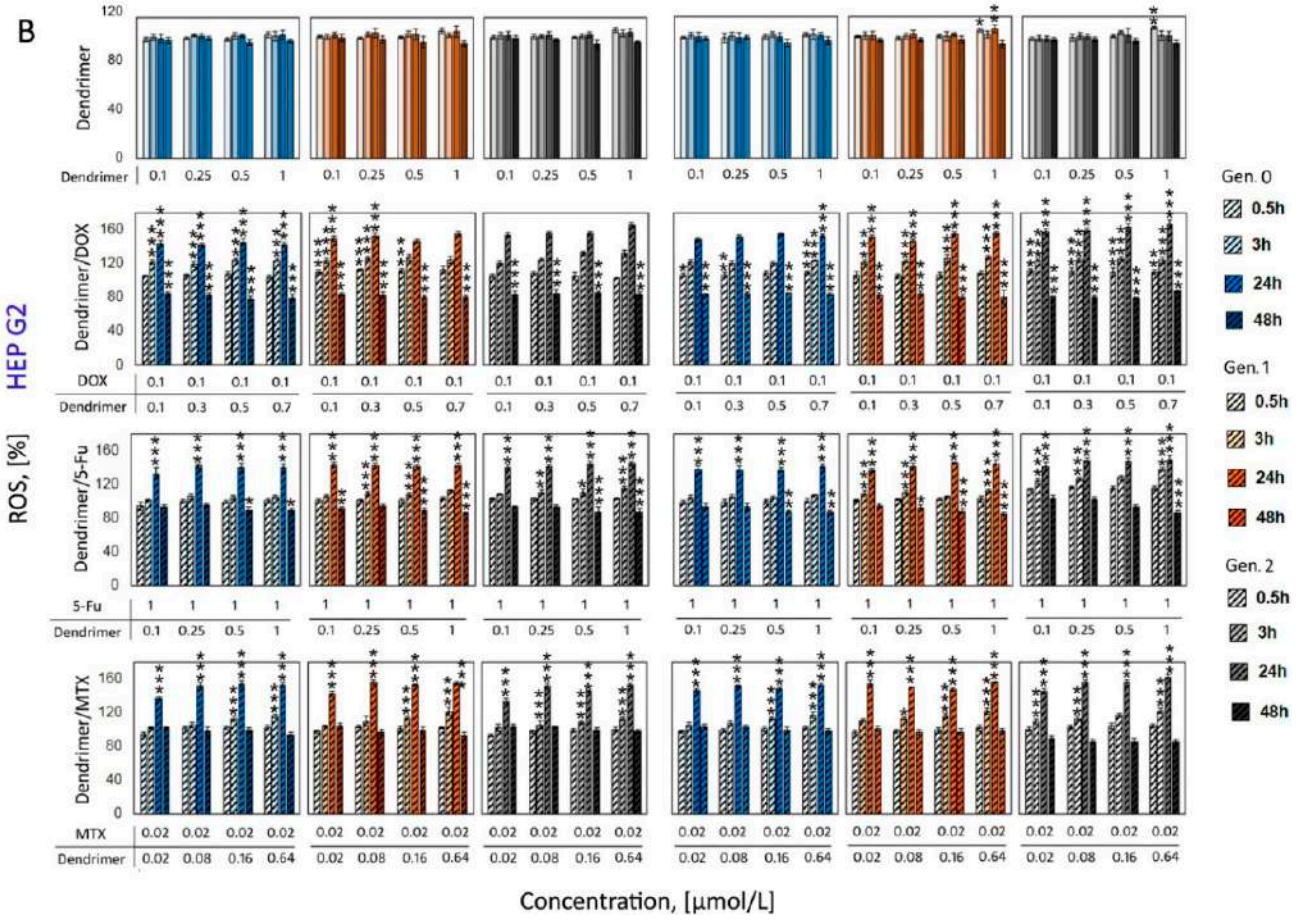

Figure 4. Time-dependent ROS production in MCF7 (**A**) or HEP G2 (**B**) cells induced by copper dendrimers or their complexes with anticancer drugs DOX, 5-Fu and MTX. Fluorescent probe H_2DCFDA 2 µmol/L, incubation time 0.5 h, 3 h, 24 h, 48 h, phosphate-buffered saline 10 mmol/L, pH 7.4. Results are means ± SD, from a min. 3 independent experiments. Statistically significant differences vs. control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Control (untreated cells) is 100% ROS production.

2.4. Mitochondrial Membrane Potential

After the analysis of the reactive oxygen species formation, alterations in mitochondrial membrane potential were studied. Carbosilane copper metallodendrimers have been reported to selectively alter the mitochondrial membrane potential in tumor cells [24]. At 10 µmol/L, the metallodendrimers produced a pro-oxidative effect at 24–48 h in U937 cells, which collapsed at 48 h and continued decreasing until 72 h. This effect was different among nitrate and chloride dendrimers and also depended on the generation. In PBMC, a similar effect was observed, but from 48 h a recovery was observed. In this study, a JC-1 fluorescent probe was used to determine the mitochondrial membrane potential changes after 24 and 48 h incubation of cells with copper dendrimers, drugs or dendrimer/drug complexes. Results show that changes in mitochondrial membrane potential were not significant in cells treated with noncomplexed dendrimers at low concentrations (0.02–0.64 µmol/L) (Figure 5) or naked drugs (Figure S1B), regardless of the concentrations tested or the applied incubation time.

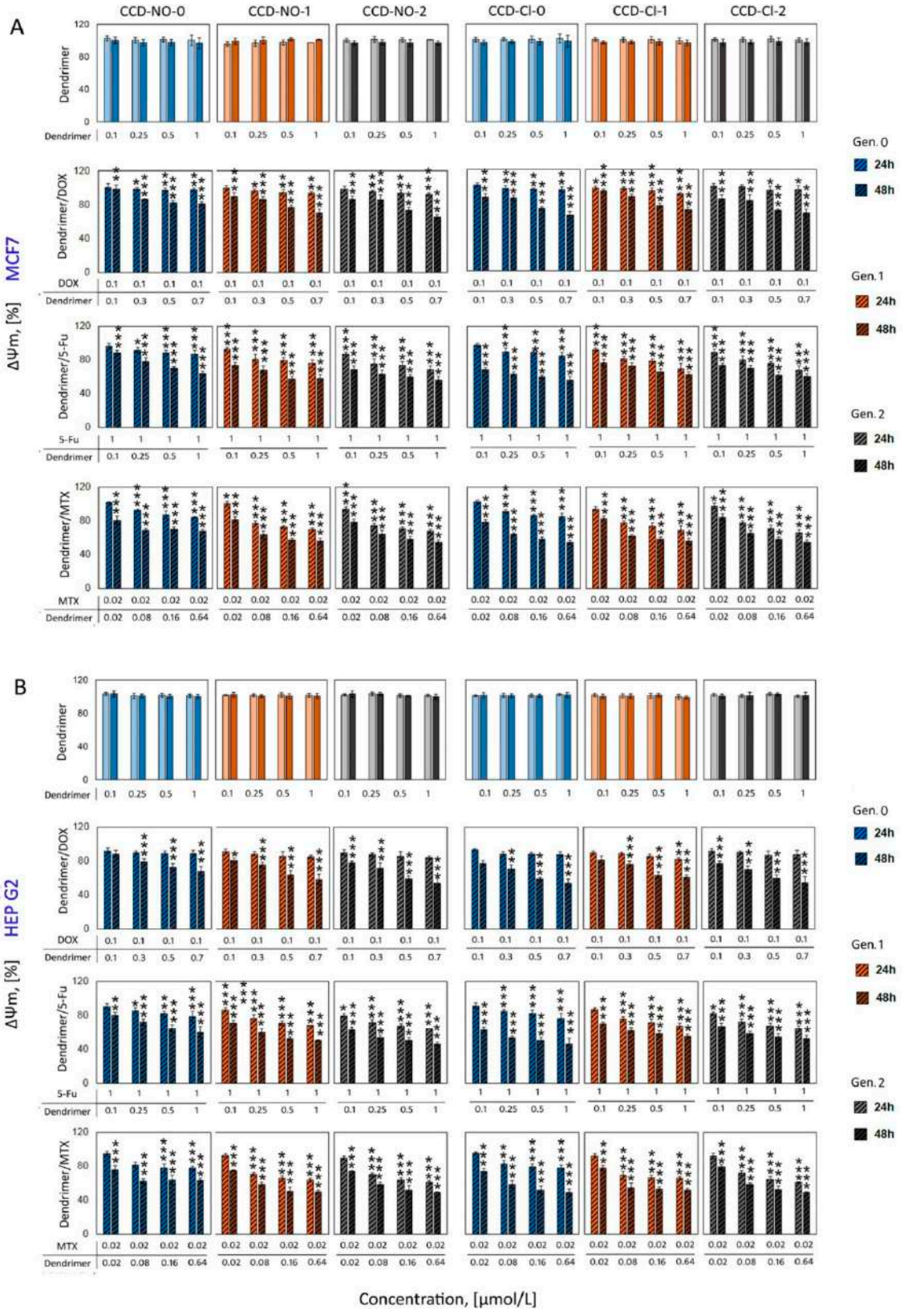

Figure 5. Changes in the mitochondrial membrane potential ($\Delta\Psi_m$) in MCF7 (**A**) and HEP G2 (**B**) cells after exposure to copper dendrimers or their complexes with anticancer drugs DOX, 5-Fu and MTX, measured by JC-1 fluorescent probe in PBS10 mmol/L, pH 7.4. Results are means ± SD, from a min. 3 independent experiments. Statistically significant differences vs. control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The 24 h incubation of cells with dendrimers/drug complexes just slightly decreased the mitochondrial potential of cells of both used cell lines. After 48 h of incubation, this effect was more pronounced for all formulations studied (Figure 5), showing that this effect depends on the dendrimer concentration but not on the dendrimer generation. The biggest drop in Ψ_m was observed for second-generation dendrimer CCD-2/MTX formulation, independent of the ligand nature (NO or Cl). The mitochondrial potential decreased by 40% for MCF7 and 35% for HEPG2 cells vs. control untreated cells. The results showed that the highest concentrations of complexes generated by the second generation of both groups of dendrimers with MTX and 5-FU caused a decrease in the mitochondrial potential of 50% for both cell lines. A similar effect was observed for DOX nanocomplexes. The values of mitochondrial potential decreased 40% and 30% vs. control for MCF7 cells and HEPG2, respectively.

2.5. Confocal Microscopy Imaging and Flow Cytometry Analysis of Cell Cycle and Apoptosis

The results obtained previously in cell viability, mitochondrial function and ROS level have been connected with the induction of apoptosis and necrosis involved by the studied formulations in MCF7 and HEP G2 cells. Obtained confocal images show visible morphological changes in cells incubated with drugs and dendrimer/drug complexes. Since naked drugs in applied concentrations were not toxic, they were not considered in imaging experiments. Results show that 48 h incubation of cells with dendrimer/drug complexes led to a decrease in the fraction of healthy cells in both cell lines, while the early apoptotic and necrotic cell fractions were significantly increased compared to the cells treated by noncomplexed drugs (Figure 6). These results have good correlation with cytotoxicity studies.

To further identify the apoptotic and necrotic fractions formed in MCF7 and HEP G2 cells in the presence of nanocomplexes, the flow cytometry analysis of double-stained annexin V/PI was applied. The 24 h incubation of cells for all studied formulations did not cause significant changes in the analyzed cell fractions (Figure S2). Increasing the incubation time to 48 h shows that the highest percentage of the healthy cell fraction was accounted for after their treatment with noncomplexed drugs (Figure 7). This trend was observed for both studied cell lines. Results indicate that similar low changes in cell fractions were visible when cells were incubated with nanocomplexes formed with compounds of generation "0" (CCD-Cl-0 and CCD-NO-0). However, there was a significant decrease in the percentage of the healthy cell fraction when complexes containing the dendrimers of generations one and two were applied. In that case, the percentage of late apoptotic and necrotic cell fractions increased significantly. The highest percentage increase, >60% of a late apoptotic fraction, was registered for the CCD-Cl-1/5-FU nanocomplex incubated with HEP G2 cells, whereas the biggest amount of necrotic and dead cells (>90%) was caused by the presence of CCD-Cl-2/DOX and CCD-Cl-2/5-FU nanocomplexes (Figure 7).

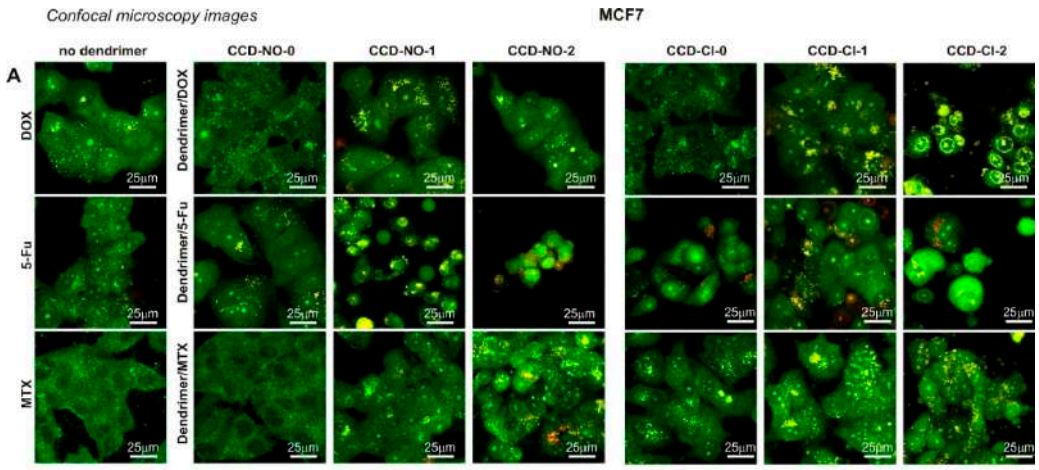

Figure 6. *Cont.*

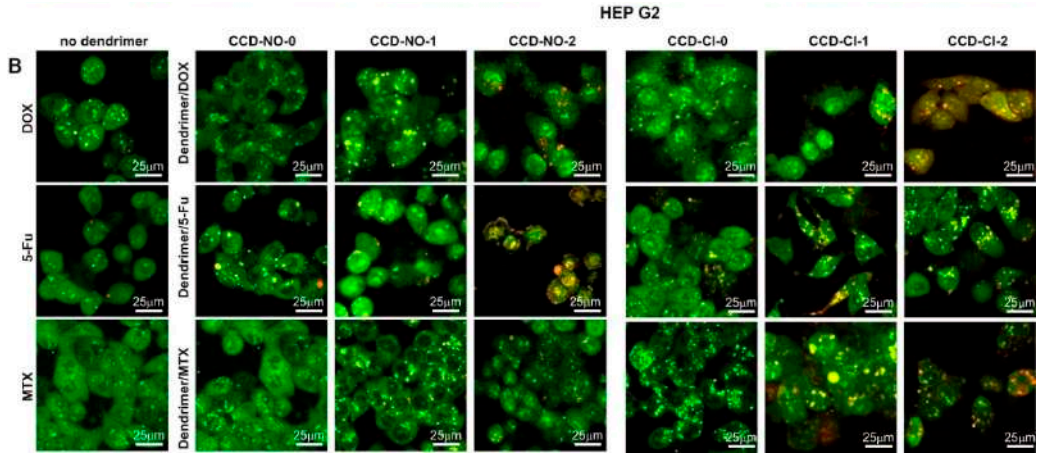

Figure 6. Images of MCF7 (**A**) and HEP G2 (**B**) cells after 48 h exposure to dendrimers, anticancer drugs or dendrimer drug complexes. Incubation time 48 h. The concentrations of drugs were as follows: DOX, 0.1 μmol/L (DOX/dendrimer molar ratio 1:7); 5-FU, 1 μmol/L (5-FU/dendrimer molar ratio 1:1); MTX, 2 nmol/L (MTX/dendrimer molar ratio: 1:32). Cells were stained with orange acridine (OA) and ethidium bromide (EB) and visualized by confocal microscopy. Living cells: morphologically normal (green nucleus); early apoptotic cells: condensed or fragmented chromatin (green nucleus); late apoptotic cells: fragmented and condensed (red chromatin); necrotic: red morphologically normal cells. Scale bar = 25 μm.

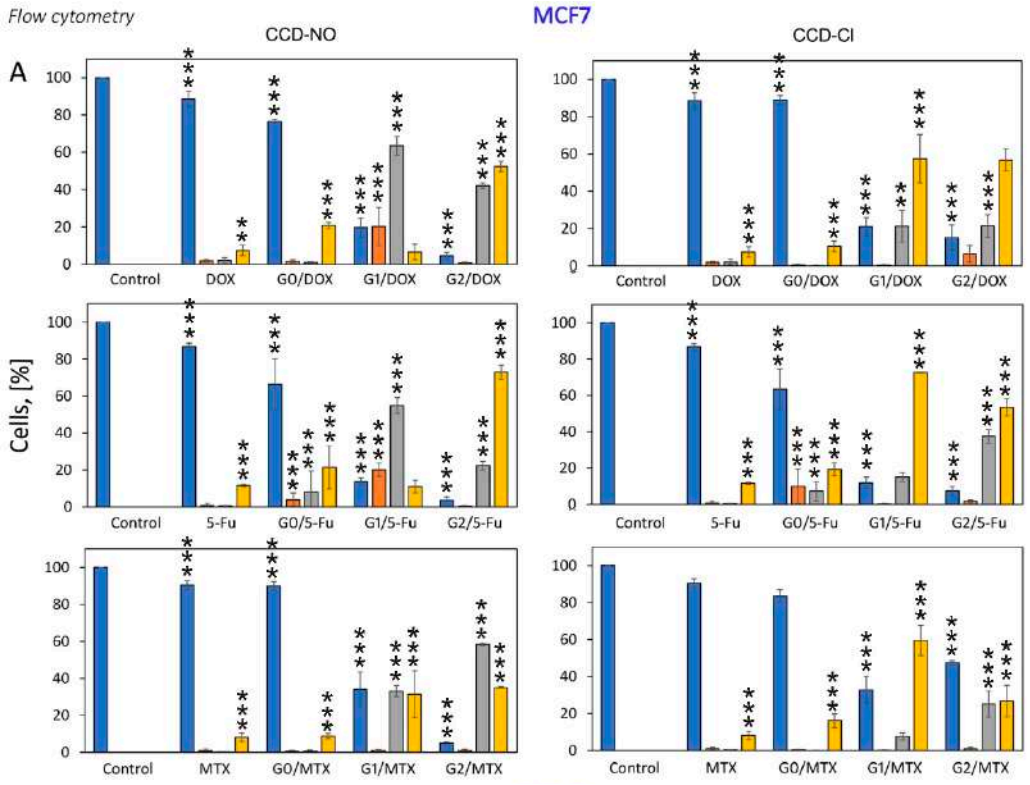

Figure 7. *Cont.*

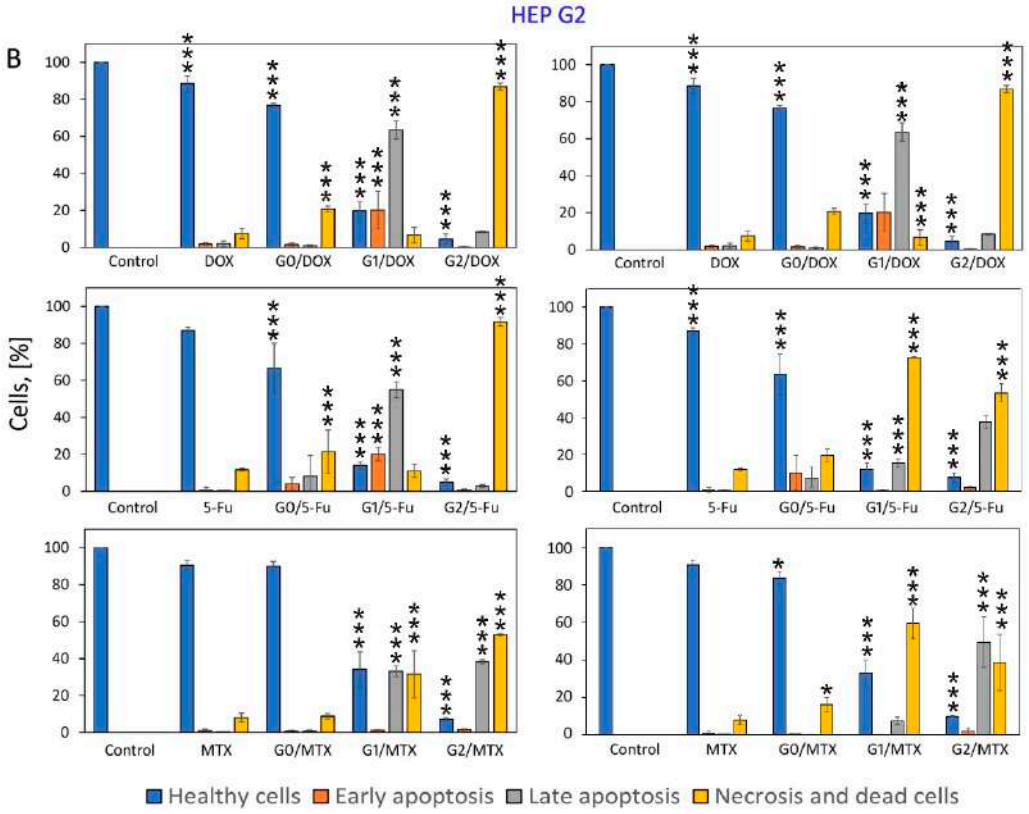

Figure 7. The percentages of cells in different phases and apoptosis profile evaluated by flow cytometry and measured using annexin V/propidium iodide staining in PBS 10 mmol/L, pH 7.4. MCF7 (**A**) and HEP G2 (**B**) cells interacted with dendrimers, anticancer drugs or dendrimer drug complexes. Incubation time 48 h. The concentrations of drugs were as follows: DOX, 0.1 µmol/L (DOX/dendrimer molar ratio 1:7); 5-FU, 1 µmol/L (5-FU/dendrimer molar ratio 1:1); MTX, 2 nmol/L (MTX/dendrimer molar ratio: 1:32). Results are means ± SD, from a min. 3 independent experiments. Statistically significant differences vs. control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Discussion

Cancer treatment is still struggling with many difficulties, one being the side effects of classical chemotherapy. Therefore, numerous studies are focused on new therapeutic approaches such as combinatory therapy or the use of drug carriers that would increase the bioavailability, biocompatibility and stability of conventional drugs and ensure their selective delivery to cancer cells. Due to unique characteristics, dendrimers seem to be good candidates for therapeutic agents or as platforms for delivering chemotherapeutic agents directly to neoplastic cells [6,33,34]. Here, we have evaluated the effect of the combination of carbosilane metallodendrimers with peripheral copper atoms, which have shown anticancer activity in several tumor lines together with conventional drugs such as DOX, MTX and 5-FU that are currently used in clinics. We focused on the evaluation and biophysical characteristics of nanosystems formed by copper (II) carbosilane dendrimers and anticancer drugs. We have also carried out several studies to elucidate the mechanism of cell death when cells are treated with the conjugates.

As expected, the measurements of the zeta potential and size of dendrimer/drug complexes showed increased values with rising dendrimer concentrations in all tested formulations, and the zeta potential and size were lower when complexes were formed with compounds of G0 regardless of metal coordination spheres containing either chloride or nitrate (CCD-NO and CCD-Cl). However, the dendriplexes containing 5-FU showed a sharp drop in their hydrodynamic diameter. The increased surface charge of formed complexes could improve their interaction with cell membranes facilitating the delivery of complexes into cells [34–36]. The same tendency was observed previously in a study of the

interaction of carbosilane ruthenium dendrimers (CRD) of the first and second generations with MTX, DOX and 5-FU [16].

As previously mentioned, a therapeutic approach in the treatment of cancer is the co-administration of drugs to obtain a synergistic effect in therapeutic activity using lower doses of both drugs, implying a reduction of side effects. To determine the existence of a synergistic effect between the copper dendrimers and the drugs selected for this work, MCF7 and HepG2 cell lines were chosen.

The anticancer activity exhibited by copper (II) metallodendrimers against leukemic 1301, HL-60 and U937 cancer cells had a slight effect on the viability of normal PBMC cells [6,24]. Therefore, our first step was to measure the subtoxic concentrations of drugs and dendrimers against MCF7 and HepG2 in order to determine the concentration of drugs that reduce the cell viability to approximately 80%.

All three generations of compounds were shown to be non-toxic in both cell lines at concentrations <1 μM with different nanoconjugates prepared by keeping a constant drug concentration and increasing the dendrimer concentration to safe concentration levels.

The cytotoxicity studies presented in this work show that 72 h of incubation of MCF7 and HepG2 cells with dendrimer/drug complexes significantly reduced the number of living cells compared to the effect of noncomplexed drugs or dendrimers.

The cytotoxicity of G0/drugs complexes were lower than the effect of formulations prepared with a higher generation of dendrimers (G1/drugs and G2/drugs), probably due to the interaction strength between different drugs and dendritic systems. Interactions are weaker with smaller systems such as zero-generation compounds, with the reduced ability of these dendrimers to cross the membrane barrier and transfect the cells.

On the other hand, the smaller number of metal centers in the zero-generation system compared to the first- and second-generation dendrimers makes them less cytotoxic. Again, these results are in agreement with similar effects observed previously for ruthenium dendrimers complexed with DOX, 5-FU and MTX against HL-60 and 1301 cells [16].

The analysis of the different data obtained indicated that in general and for both cell lines, it is possible to observe that combinations with first-generation metallodendrimers with chloride (CCD-Cl-1) or nitrate ligands (CCD-NO-1) exhibit matching activities. However, a slight increase in activity is observed for second-generation formulations with CCD-NO-2 and a significant decrease for CCD-Cl-2, despite having a higher number of metal centers on the surface. This is in agreement with previous EPR studies carried out for this type of system showing that the dendritic generation and metal center counterion have a clear influence in the interaction with tumor cells and subsequently the effectivity and selectivity of the therapy [24]. In the case of MCF7 cells, the combination of dendritic structures and drugs showed the greatest synergistic effect with methotrexate by decreasing viability around 95.25% vs. control. Similar to previous results, cytotoxicity increases with increasing concentration of the dendritic system in the nanoconjugates. It has been previously demonstrated that the PAMAM dendrimer conjugated with MTX induced a synergistic effect towards MES-SA endometrial cancer cells [8]. Moreover, a third-generation copper (II) metallophosphate dendrimer in combination with DOX, cisplatin, paclitaxel and MG132 showed stronger inhibition of HL60 cell proliferation compared to drugs and dendrimer used alone. However, no synergistic properties were found for the combinations of this dendrimer with camptothecin [37].

It is well known that cytotoxicity effects can be tightly connected with intracellular reactive oxygen species generation [38]. Considering the important role of ROS production for cell viability and the possibility of using this parameter as an indicator of apoptosis regulation [39], we studied the changes of intracellular ROS level with the presence of dendrimer/drug complexes. Despite one of the mechanisms associated with the antiproliferative effects of copper metallodendrimers being ROS production, our current studies showed that at subtoxic concentrations, copper metallodendrimers did not generate an increase of ROS. However, when they were combined with the different drugs tested it was possible to observe the significantly increased level of ROS compared to noncomplexed

drugs or noncomplexed dendrimers. The highest increase of ROS levels reached 160% vs. control, in both MCF7 and HepG2 cells, and was observed after 24 h of incubation in selected cases. Following incubation (48 h), this parameter dropped below control values.

Increased and uncontrolled generation of ROS can lead to a situation where the ion channels in the mitochondrial membrane are opened [40]. The open channels result in a collapse of the mitochondrial potential and additional increased production of ROS. The consequence of these changes occurring in the mitochondria can be cell death [41–43]. Therefore, we analyzed the changes of mitochondrial potential ($\Delta\Psi_m$) in the presence of the studied nanocomplexes and compared these results with the data obtained for ROS level. While the incubation of cells with naked drugs or dendrimers did not influence this parameter at the tested concentrations, the presence of drug/dendrimer nanosystems significantly depolarized the mitochondrial membrane. These results have good correlation with those obtained for ROS experiments, indicating that this misbalance in the redox status was present in the alteration of mitochondrial membrane potential, again with higher activity of complexes containing dendrimers of generations one and two.

It is known that mitochondrial depolarization is characteristic of the early stages of apoptosis [41,42,44]. Thus, the percentage of apoptotic and necrotic cells in the cell suspension was determined after treatment with dendrimer/drug complexes. According to results obtained by flow cytometry (AV/PI double staining) and confocal microscopy (OA/EB fluorescent staining), the studied nanosystems induced apoptosis and necrosis in both applied cell lines. During 48 h incubation of cells with the dendrimer/drug nanosystems, the highest degree of apoptosis was observed for complexes formed with dendrimers of the first and second generations. For MCF-7 cells, it was mainly early apoptosis, but late apoptosis prevailed in HEP G2 cells. Higher dendrimer generations and concentrations led to the increase of percentage of necrotic and dead cells. In contrast, complexes containing dendrimers of generation "0" were practically not apoptotic in effect, with a high percentage of healthy cells. The results obtained by flow cytometry correlate with data from confocal microscopy. Obtained microimages show that treatment of the cells with dendrimer/drug complexes led to an increase of the number of apoptotic and necrotic MCF7 and HEP G2 cells. Similar results were shown by amphiphilic dendritic nanomicelle. The 12 h incubation of MDA-MB-231 cells with nanocomplex contained dendritic micelles, and DOX or 5-FU initiated the apoptosis and necrosis in cell suspensions [45]. Confocal microscopy images show that the morphology of cells treated by nanocomplexes was influenced strongly when compared to cells incubated with noncomplexed drugs.

4. Materials and Methods

4.1. Metallodendrimers

Two families of copper carbosilane metallodendrimers containing chloride ligands were used: Gn-[NCPh(*o*-N)$CuCl_2 \cdot H_2O]_m$ (n = 0, m = 1 CCD-Cl-0; n = 1, m = 4 CCD-Cl-1; n = 2, m = 8 CCD-Cl-2) and nitrate ligands Gn-[NCPh(*o*-N)$Cu(ONO_2)_2 \cdot H_2O$]m (n = 0, m = 1 CCD-NO-0; n = 1, m = 4 CCD-NO-1; n = 2, m = 8 CCD-NO-2) (structures are shown in Figure 8). The compound synthesis procedure was carefully described in [46]. The basic parameters of dendrimers are given in Table 1.

Table 1. Characterization of copper metallodendrimers with chloride and nitrate ligands.

Compound	Generation/Number of Surface Groups	Molecular Weight (g/mol)
CCD-Cl-0	0/1	414.93
CCD-Cl-1	1/4	1627.68
CCD-Cl-2	2/8	3696.01
CCD-NO-0	0/1	468.04
CCD-NO-1	1/4	1840.10
CCD-NO-2	2/8	3992.90

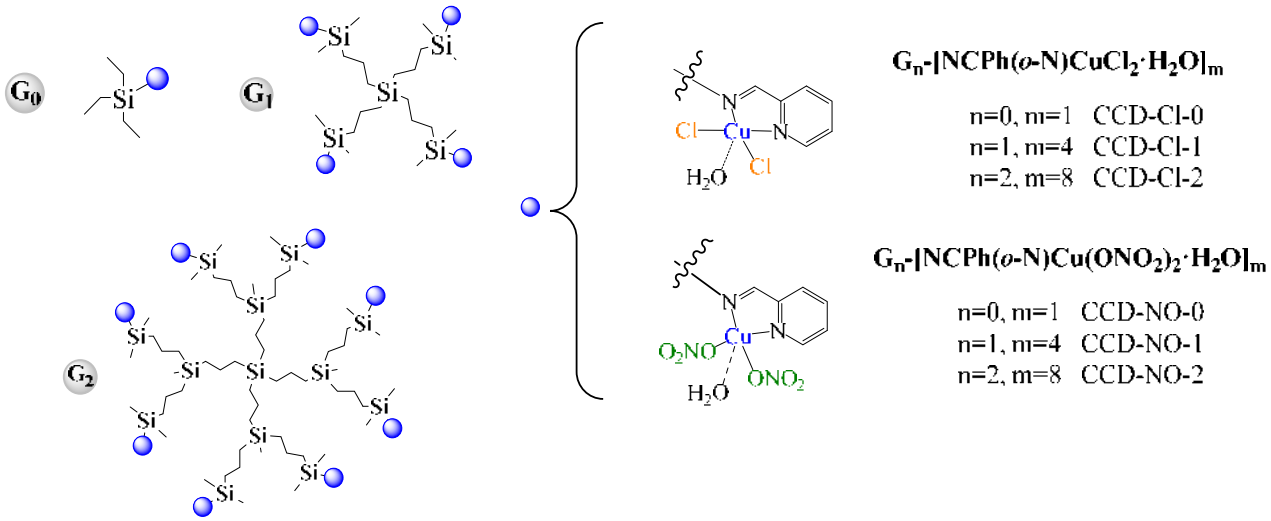

Figure 8. Dendritic structures of Gn-[NCPh(*o*-N)$CuCl_2$·H_2O]m (CCD-Cl) and Gn-[NCPh(o-N)Cu$(ONO_2)_2$·H_2O]m (CCD-NO) systems.

4.2. Drugs

Three commercially available anticancer drugs, doxorubicin (DOX), methotrexate (MTX) and 5-flurouracil (5-FU), were used in the current study for their complexes with copper dendrimers. The drugs were purchased from Sigma-Aldrich Sp. Z.O.O., Poznan, Poland.

4.3. Zeta Size

The hydrodynamic diameter of the CCD/drug complexes was estimated by dynamic light scattering technique using the Malvern Zetasizer Nano ZS-90 spectrometer, (Malvern Instruments, Malvern, UK). Complexes were prepared in distilled water. Drug concentration was always 10 µmol/L, and the concentration of dendrimers varied from 1:0.25 to 1:75 of dendrimer/drug molar ratio. For each sample, 15 measurements in 5 cycles were taken at room temperature. The experiment was repeated 3 times for each compound. The data were analyzed by Malvern software and presented as a mean ± standard deviation (SD).

4.4. Zeta Potential

Zeta potential values of formed complexes were evaluated by the Laser Doppler Velocimetry technique using the Zetasizer Nano ZS-90, Malvern Instruments UK. The Helmholtz–Smoluchowski equation was applied to calculate zeta potential. Complexes were prepared in distilled water. Drug concentration was always 10 µmol/L, and the concentration of dendrimers varied from 1:0.25 to 1:75 of dendrimer/drug molar ratio. For each sample, 15 measurements in 5 cycles were made at room temperature. The experiment was repeated 3 times for each compound. The data were analyzed by Malvern software and presented as a mean ± standard deviation (SD).

4.5. Cells

To determine the effect of studied dendrimer/drug complexes on the cell viability level of reactive oxygen species, mitochondrial membrane potential and the percentage of apoptotic and necrotic cells, 2 cancer cell lines, MCF-7 (human breast cancer cell line) and HepG2 (human liver carcinoma cell line), were used. Cells were purchased from ATCC Company, Manassas, VA, USA. The cells were grown in plastic tissue culture flasks (Falcon, GE Healthcare Life Sciences, Chicago, IL, USA) at the temperature of 37 °C in a humidified atmosphere containing 5%, CO_2 and 95% air. DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) with 10% heat-inactivated fetal bovine serum (FBS, HyClone, GE

Healthcare Life Sciences, Chicago, IL, USA) containing 1% antibiotic for cell culture was used.

4.6. Cytotoxicity

To evaluate the cytotoxicity effects of studied complexes, the MTT (Sigma, USA) assay was applied. Cells were seeded on a 96-well plastic plate at 1×10^4 cells per well and incubated with formed dendriplexes for 72 h. Next, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added. To dissolve the formazan crystals, the DMSO was added after 3 h of incubation. Absorbance of samples was measured at 580 nm and 720 nm. The cells' viability was calculated using following equation:

$$\% \text{ viability} = A/A_c \times 100$$

where A is the absorbance measured for sample; A_c is the absorbance of the control cells. The results are presented as mean ± standard deviation (SD) from 3 independent experiments.

4.7. ROS Level

The H_2DCFDA (Thermo Fisher Scientific, Waltham, MA, USA) fluorescent probe was used to measure the changes of the reactive oxygen species level in the cells treated by dendrimer/drug complexes. H_2DCFDA is not able to emit fluorescence until the acetate groups are removed by esterases inside of the cell. During the oxidation process, highly fluorescent DCF (dichlorofluorescein) formed from H_2DCF. The changes in fluorescence intensity of DCF reflect the alterations in the intracellular level of ROS. Cells were seeded on a 96-well black plate, at 1.5×10^4 cells per well. Cells were incubated with dendriplexes for 0.5, 3, 24 and 48 h, the medium was removed, and cells were washed with PBS. Next, the H_2DCFDA probe at final concentration 2 µmol/L was added in each well. Cells were incubated for 15 min at 37 °C and washed with PBS. After these procedures, the fluorescence was measured at the excitation and emission wavelength of 485 nm and 530 nm, respectively. The results are presented as mean ± standard deviation (SD) from 3 independent experiments.

4.8. Mitochondrial Membrane Potential

The fluorescent probe JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine changes in mitochondrial membrane potential (Ψ_m) for cells treated with dendrimer/drug complexes. JC-1 accumulates in mitochondria and exhibits red fluorescence with an excitation and emission wavelength of 530 and 590 nm, respectively. When the mitochondrial membrane is depolarized, JC-1 exhibits green fluorescence with an excitation and emission wavelength of 485 and 540 nm, respectively. An increased ratio in the red-to-green fluorescence can indicate the increase of Ψ_m. As in the previous technique, the cells were seeded on a 96-well black plate at 1.5×10^4 cells per well. After 24 and 48 h of incubation of cells with dendriplexes, the medium was removed, and cells were carefully washed with PBS. After that, cells were incubated for 20 min at 37 °C, in the presence of JC-1 at the final concentration of 5 µmol/L. Cells were then washed with PBS. Measurements were made at an excitation and emission wavelength of 530 nm and 590 nm, respectively. The results are presented as mean ± standard deviation (SD) from 3 independent experiments.

4.9. Annexin V/Propidium Iodide Double-Staining Assay

Flow cytometry technique using annexin V-FITC Apoptosis Detection Kit was applied to determine the percentage of apoptotic and necrotic cells after their treatment with dendrimer/drug complexes. The final concentrations of drugs were as follows: DOX, 0.1 µmol/L (DOX/dendrimer molar ratio 1:7); 5-FU, 1 µmol/L (5-FU/dendrimer molar ratio 1:1); MTX, 2 nmol/L (MTX/dendrimer molar ratio: 1:32). Cells were incubated with drugs or dendrimer/drug complexes for 24 and 48 h and washed twice in cold PBS. After

washing, cells were suspended with a binding buffer (HEPES/NaOH, pH 7.4). The cell suspension was adjusted for a concentration of 10^6 cells/mL. An amount of 100 µL of the suspension was then placed in a measuring tube, and 5 µL of annexin V-FITC + 10 µL of propidium iodide (PI) was added and mixed.

The following controls samples were additionally prepared: (1) negative control (unlabeled cells), (2) PI positive control (cold ethanol was used to induce necrosis), (3) annexin V positive control (hydrogen peroxide was used to induce apoptosis). Each sample was analyzed by flow cytometry (LSRII, Becton Dickinson, NJ, USA). The results are presented as mean ± standard deviation (SD) from 3 independent experiments.

4.10. Orange Acridine/Ethidium Bromide Fluorescent Staining

The ability of dendrimer/drug complexes to induce the apoptosis and necrosis in MCF7 and HEP G2 cells was evaluated by confocal microscopy using double fluorescent dye staining of orange acridine (OA) and ethidium bromide (EB). Both of these fluorescent dyes can stain cell nuclei after intercalation with DNA. While OA can be easily uptaken by cells and stains the nucleus green, EB is able to stain the nucleus of damaged cells only in red. Confocal analysis allows the indication of the fractions of the cells that are early and late apoptotic, necrotic or healthy. The cells were treated with dendriplexes for 24 h and stained with dual fluorescent staining solution (2 µL) containing 100 µg/mL AO and 100 µg/mL EB for 2 min and covered with a coverslip. Then, cells were washed with PBS and visualized using a Leica TCS SP8 confocal microscope (Wetzlar, Germany) with the objective 63×/1.40 (HC PL APO CS2, Leica Microsystems, Wetzlar, Germany).

The cells with normal morphology and a green nucleus were recognized as living cells, with green nucleus and condensed or fragmented chromatin as early apoptotic cells, with condensed or fragmented red chromatin as late apoptotic cells and with red nucleus as necrotic cells. Leica Application Suite X (LAS X, Leica Microsystems, Germany) software was used for the export of images.

4.11. Statistical Analysis

The results come from a minimum of 3 independent experiments. To assess the significance of differences, one-way analysis of variance (ANOVA) and Bonferroni test were applied. Significance was accepted at $p \leq 0.05$ or less.

5. Conclusions

In summary, conjugation of copper carbosilane metallodendrimers with doxorubicin, 5-florouracyl or methotrexate at subtoxic concentrations appears to be a promising strategy that could be considered for anticancer drug delivery, due to the synergistic effect of the used components. In vitro experiments show that copper dendrimers significantly increase the anticancer effect of the applied drugs and probably can serve as drug transporters. The synergistic effect of dendrimer/drug complexes has been confirmed by several biophysical assays. Rapid increase of the ROS level and depolarization of mitochondrial membranes caused by the action of nanocomplexes indicated their ability to induce apoptosis in MCF7 and HEP G2 cancer cells. Observed cytotoxicity associated with the onset of cell apoptosis and necrosis depended on the generation of dendrimers. In vivo experiments are planned to determine the ability of the studied systems for cancer therapy.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms24044076/s1.

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Supporting information

Combination of copper metallodendrimers with conventional antitumor drugs to combat cancer in *in vitro* models

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The copper metallodendrimers with chloride and nitrate ligands were synthesized accordingly with previously published protocol (*Sanz Del Olmo, N., Maroto-Díaz, M., Gómez, R., Ortega, P., Cangiotti, M., Ottaviani, M. F., & de la Mata, F. J. (2017). Carbosilane metallodendrimers based on copper (II) complexes: Synthesis, EPR characterization and anticancer activity. Journal of inorganic biochemistry, 177, 211–218. https://doi.org/10.1016/j.jinorgbio.2017.09.023*). Its purity was evaluated by elemental analysis, nuclear magnetic resonance and mass spectrometry techniques.

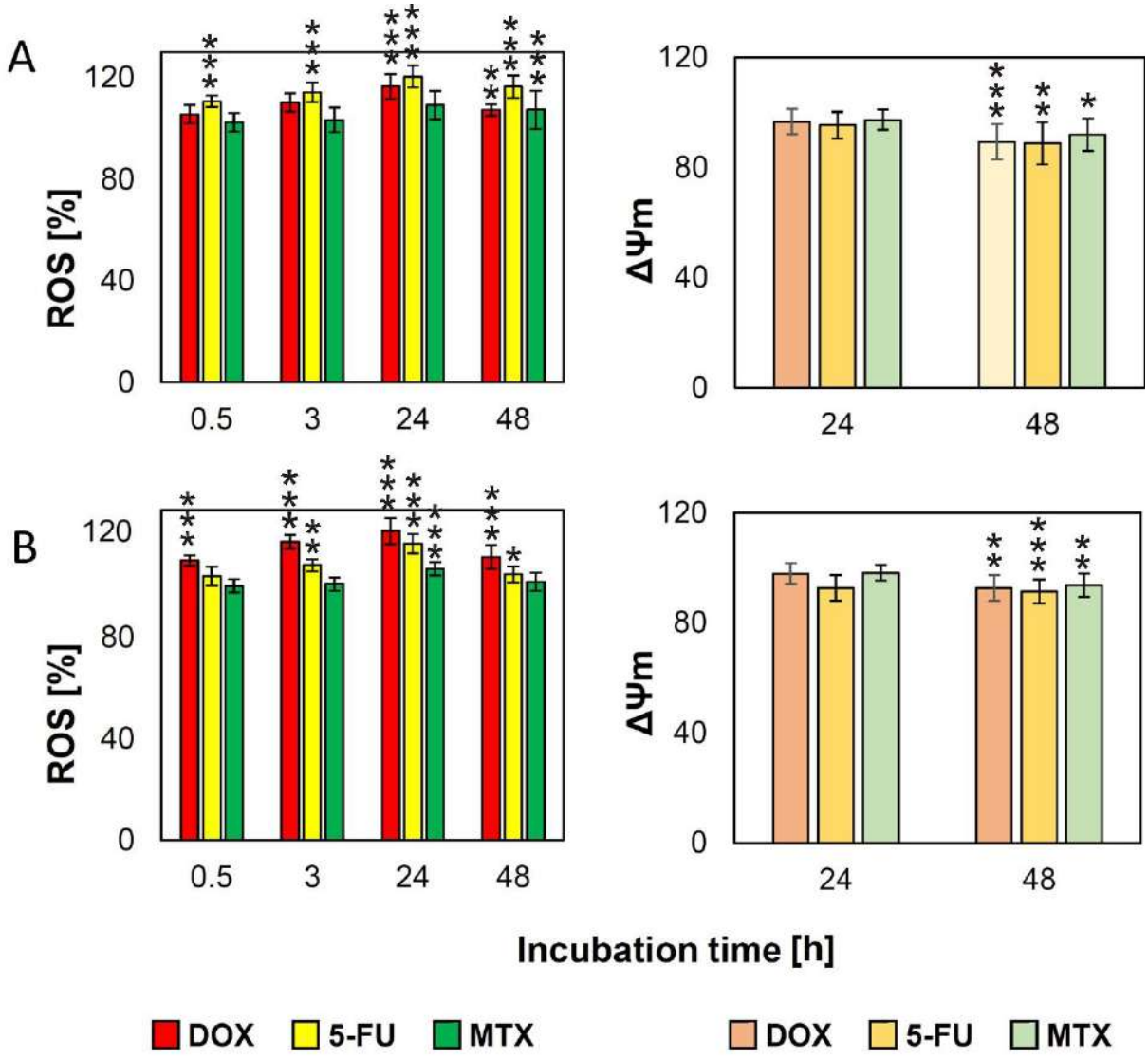

Figure S1. Time-dependent ROS production and changes in mitochondrial membrane potential (Ψm**)** in MCF7 – (A) or HEP G2 – (B) cells involved by anticancer drugs DOX, 5-Fu and MTX. ROS - fluorescent probe H_2DCFDA; ΔΨm - fluorescent probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), PBS 10 mmol/L, pH-7.4. Results are means ± SD, from a min. of 3 independent experiments. Statistically significant differences vs. control *p <0.05, **p < 0.01, ***p < 0.001.

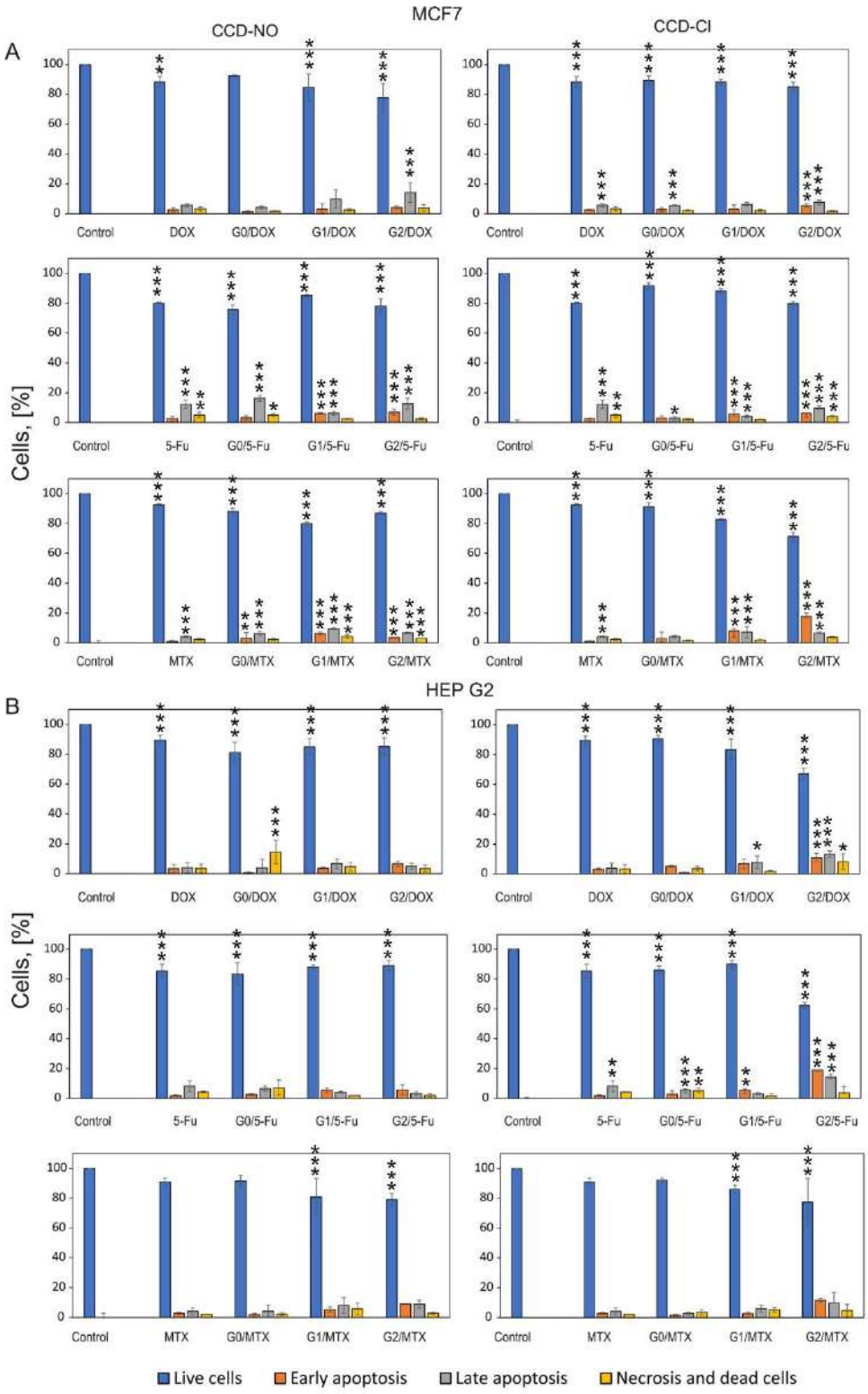

Figure S2. The percentages of cells in different phases and apoptosis profile evaluated by flow cytometry and measured using annexin V/propidium iodide staining in PBS 10 mmol/L, pH-7.4. MCF7 – (A) and HEP G2 – (B) cells were interacted with dendrimers, anticancer drugs or dendrimer drug complexes. Incubation time 24 h. The concentrations of drugs were as follows: DOX, 0.1 μmol/L (DOX/Dendrimer molar ratio 1:7); 5-FU, 1 μmol/L (5-FU/Dendrimer molar ratio 1:1); MTX, 2 nmol/L (MTX/Dendrimer molar ratio:

1:32). Results are means ± SD, from a min. of 3 independent experiments. Statistically significant differences vs. control *p <0.05, **p < 0.01, ***p < 0.001.

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Łódź, dnia 24.05.2023 r.

Oświadczenie

1. Oświadczam, że mój udział w pracy Hołota M., Magiera J., Michlewska S., Kubczak M., Sanz del Olmo N., García-Gallego S., Ortega P., de la Mata F.J., Ionov M., Bryszewska M. In Vitro Anticancer Properties of Copper Metallodendrimers. Biomolecules. 2019 Apr 18;9(4):155. doi:10.3390/biom9040155, polegał na:
   - Wykonaniu pomiarów średnicy hydrodynamicznej oraz potencjału zeta,
   - określeniu aktywności hemolitycznej dendrymerów,
   - określeniu cytotoksyczności dendrymerów wobec komórek linii 1301 i HL60,
   - wykonaniu części pomiarów anizotropii fluorescencji,
   - wykonaniu widm dichroizmu kołowego,
   - współwykonaniu eksperymentów dotyczących określenia cytotoksyczności dendrymerów wobec jednojądrzastych komórek krwi obwodowej,
   - współuczestniczeniu w interpretacji uzyskanych wyników,
   - zebraniu literatury do napisania manuskryptu,
   - przygotowaniu pierwotnej wersji manuskryptu,
   - współuczestniczeniu w odpowiedzi na recenzje.

   Osobą współwykonującą wymienione powyżej eksperymenty był mgr Jakub Magiera – magistrant Katedry Biofizyki Ogólnej w latach 2018-2019, który wykonał pojedyncze powtórzenia w ramach nauki technik badawczych pod moją opieką.

   Mój wkład w przygotowanie pracy w formie publikacji stanowi 53%.

2. Oświadczam, że mój udział w pracy Sanz del Olmo N., Hołota M., Michlewska S., Gómez R., Ortega P., Ionov M., de la Mata F.J., Bryszewska M. Copper (II) Metallodendrimers Combined with Pro-Apoptotic siRNAs as a Promising Strategy Against Breast Cancer Cells. Pharmaceutics. 2020 Aug 2;12(8):727. Doi: 10.3390/pharmaceutics12080727, polegał na:
   - wykonaniu części pomiarów średnicy hydrodynamicznej i potencjału zeta,
   - wykonaniu części pomiarów widm dichrozimu kołowego,

- wykonaniu pomiarów polaryzacji fluorescencji,
- współwykonaniu analiz z zastosowaniem agarozowej elektroforezy żelowej,
- współwykonaniu badań cytotoksyczności,
- wykonaniu analiz internalizacji z zastosowaniem cytometrii przepływowej,
- wykonaniu analizy statystycznej,
- współuczestniczeniu w interpretacji uzyskanych wyników,
- współuczestniczeniu w zebraniu literatury do napisania manuskryptu,
- współuczestniczeniu w przygotowaniu pierwotnej wersji manuskryptu,
- współuczestniczeniu w odpowiedzi na recenzje.

Osobą współwykonującą wymienione powyżej eksperymenty była dr Natalia Sanz del Olmo z Katedry Chemii Organicznej i Chemii Nieorganicznej Uniwersytetu w Alcalá de Henares (Hiszpania), przebywająca w Katedrze Biofizyki Ogólnej w ramach stażu badawczego (wrzesień-listopad 2018 i maj 2019). Dr Natalia Sanz del Olmo wykonała część eksperymentów w ramach nauki technik biofizycznych i biologicznych pod moją opieką.

Mój wkład w przygotowanie pracy w formie publikacji stanowi 30%.

3. Oświadczam, że mój udział w pracy Hołota M., Michlewska S., García-Gallego S., Sanz del Olmo N., Ortega P., Bryszewska M., de la Mata F.J., Ionov M. Combination of Copper Metallodendrimers with Conventional Antitumor Drugs to Combat Cancer in In Vitro Models. Int J Mol Sci. 2023 Feb 17;24(4):4076. doi: 10.3390/ijms24044076, polegał na:
   - współwykonaniu części pomiarów średnicy hydrodynamicznej oraz potencjału zeta,
   - współwykonaniu badań cytotoksyczności,
   - wykonaniu eksperymentów dotyczących wytwarzania reaktywnych form tlenu w komórkach,
   - wykonaniu eksperymentów dotyczących zmian mitochondrialnego potencjału błonowego,
   - wykonaniu eksperymentu z zastosowaniem cytometrii przepływowej,
   - współuczestniczeniu w interpretacji uzyskanych wyników,
   - wykonaniu analizy statystycznej,
   - zebraniu literatury do napisania manuskryptu,
   - przygotowaniu pierwotnej wersji manuskryptu,
   - współuczestniczeniu w odpowiedzi na recenzje.

Osobą współwykonującą wymienione powyżej eksperymenty była dr Natalia Sanz del Olmo z Katedry Chemii Organicznej i Chemii Nieorganicznej Uniwersytetu w Alcalá de Henares (Hiszpania), przebywająca w Katedrze Biofizyki Ogólnej w ramach stażu badawczego (wrzesień-listopad 2018 i maj 2019). Dr Natalia Sanz del Olmo wykonała część eksperymentów w ramach nauki technik biofizycznych i biologicznych pod moją opieką.

Mój wkład w przygotowanie pracy w formie publikacji stanowi 50%.

Marian Hołota

WYDZIAŁ BIOLOGII
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e-mail: maksim.ionov@biol.uni.lodz.pl

Prof. dr hab. Maksim Ionov

Łódź, dnia 23.05.2023 r.

Oświadczenie

1. Oświadczam, że mój udział w pracy Hołota M., Magiera J., Michlewska S., Kubczak M., Sanz del Olmo N., García-Gallego S., Ortega P., de la Mata F.J., Ionov M., Bryszewska M. In Vitro Anticancer Properties of Copper Metallodendrimers. Biomolecules. 2019 Apr 18;9(4):155. doi:10.3390/biom9040155, polegał na projektowaniu i kierowaniu badaniami opisanymi w pracy oraz korekcie manuskryptu. Mój wkład w przygotowanie pracy w formie publikacji stanowi 3%.

2. Oświadczam, że mój udział w pracy Sanz del Olmo N., Hołota M., Michlewska S., Gómez R., Ortega P., Ionov M., de la Mata F.J., Bryszewska M. Copper (II) Metallodendrimers Combined with Pro-Apoptotic siRNAs as a Promising Strategy Against Breast Cancer Cells. Pharmaceutics. 2020 Aug 2;12(8):727. doi: 10.3390/pharmaceutics12080727, polegał na projektowaniu i kierowaniu badaniami opisanymi w pracy oraz korekcie manuskryptu. Mój wkład w przygotowanie pracy w formie publikacji stanowi 3%.

3. Oświadczam, że mój udział w pracy Hołota M., Michlewska S., García-Gallego S., Sanz del Olmo N., Ortega P., Bryszewska M., de la Mata F.J., Ionov M. Combination of Copper Metallodendrimers with Conventional Antitumor Drugs to Combat Cancer in In Vitro Models. Int J Mol Sci. 2023 Feb 17;24(4):4076. doi: 10.3390/ijms24044076, polegał na projektowaniu i kierowaniu badaniami opisanymi w pracy oraz korekcie manuskryptu. Mój wkład w przygotowanie pracy w formie publikacji stanowi 3%.

WYDZIAŁ BIOLOGII
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UNIWERSYTET ŁÓDZKI

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Dr Sylwia Michlewska

Łódź, dnia 24-05-2023r

Oświadczenie

1. Oświadczam, że mój udział w pracy Hołota M., Magiera J., Michlewska S., Kubczak M., Sanz del Olmo N., García-Gallego S., Ortega P., de la Mata F.J., Ionov M., Bryszewska M. In Vitro Anticancer Properties of Copper Metallodendrimers. Biomolecules. 2019 Apr 18;9(4):155. doi:10.3390/biom9040155, polegał na zaplanowaniu części eksperymentów, wykonaniu analiz z zastosowaniem transmisyjnej mikroskopii elektronowej oraz współprzygotowaniu i edycji manuskryptu. Mój wkład w przygotowanie pracy w formie publikacji stanowi 15%.

2. Oświadczam, że mój udział w pracy Sanz del Olmo N., Hołota M., Michlewska S., Gómez R., Ortega P., Ionov M., de la Mata F.J., Bryszewska M. Copper (II) Metallodendrimers Combined with Pro-Apoptotic siRNAs as a Promising Strategy Against Breast Cancer Cells. Pharmaceutics. 2020 Aug 2;12(8):727. doi: 10.3390/pharmaceutics12080727, polegał na zaplanowaniu części eksperymentów i analizie danych oraz wykonaniu badań z zastosowaniem transmisyjnej mikroskopii elektronowej, mikroskopii konfokalnej, oraz współprzygotowaniu i edycji manuskryptu. Mój wkład w przygotowanie pracy w formie publikacji stanowi 23%.

3. Oświadczam, że mój udział w pracy Hołota M., Michlewska S., García-Gallego S., Sanz del Olmo N., Ortega P., Bryszewska M., de la Mata F.J., Ionov M. Combination of Copper Metallodendrimers with Conventional Antitumor Drugs to Combat Cancer in In Vitro Models. Int J Mol Sci. 2023 Feb 17;24(4):4076. doi: 10.3390/ijms24044076, polegał na zaplanowaniu eksperymentów i analizie danych oraz wykonaniu badań z zastosowaniem mikroskopii konfokalnej oraz współprzygotowaniu i edycji manuskryptu. Mój wkład w przygotowanie pracy w formie publikacji stanowi. Mój wkład w przygotowanie pracy w formie publikacji stanowi 25%.

WYDZIAŁ BIOLOGII
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Prof. dr hab. Maria Bryszewska

Łódź, dnia 22.05.2023

Oświadczenie

1. Oświadczam, że mój udział w pracy Hołota M., Magiera J., Michlewska S., Kubczak M., Sanz del Olmo N., García-Gallego S., Ortega P., de la Mata F.J., Ionov M., Bryszewska M. In Vitro Anticancer Properties of Copper Metallodendrimers. Biomolecules. 2019 Apr 18;9(4):155. doi:10.3390/biom9040155, polegał na kierowaniu projektem i finansowaniu badań uwzględnionych w pracy, oraz korekcie ostatecznej wersji manuskryptu. Mój wkład w przygotowanie pracy w formie publikacji stanowi 3%.

2. Oświadczam, że mój udział w pracy Sanz del Olmo N., Hołota M., Michlewska S., Gómez R., Ortega P., Ionov M., de la Mata F.J., Bryszewska M. Copper (II) Metallodendrimers Combined with Pro-Apoptotic siRNAs as a Promising Strategy Against Breast Cancer Cells. Pharmaceutics. 2020 Aug 2;12(8):727. doi: 10.3390/pharmaceutics12080727, polegał na kierowaniu projektem i finansowaniu badań uwzględnionych w pracy, oraz korekcie ostatecznej wersji manuskryptu. Mój wkład w przygotowanie pracy w formie publikacji stanowi 3%.

3. Oświadczam, że mój udział w pracy Hołota M., Michlewska S., García-Gallego S., Sanz del Olmo N., Ortega P., Bryszewska M., de la Mata F.J., Ionov M. Combination of Copper Metallodendrimers with Conventional Antitumor Drugs to Combat Cancer in In Vitro Models. Int J Mol Sci. 2023 Feb 17;24(4):4076. doi: 10.3390/ijms24044076, polegał na kierowaniu projektem i finansowaniu badań uwzględnionych w pracy, oraz korekcie ostatecznej wersji manuskryptu. Mój wkład w przygotowanie pracy w formie publikacji stanowi 3%.

M. Bryszewska

Łódź, dnia 15.05.2023r.

Mgr Jakub Magiera

Aktualna afiliacja:
Katedra Genetyki i Podstaw Hodowli Zwierząt
Wydział Medycyny Weterynaryjnej i Nauk o Zwierzętach
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Afiliacja w roku opublikowania pracy:
Katedra Biofizyki Ogólnej,
Wydział Biologii i Ochrony Środowiska
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ul. Pomorska 141/143, 90-236 Łódź

e-mail: jakubmagiera92@gmail.com

Oświadczenie

Oświadczam, że mój udział w pracy Hołota M., Magiera J., Michlewska S., Kubczak M., Sanz del Olmo N., García-Gallego S., Ortega P., de la Mata F.J., Ionov M., Bryszewska M. In Vitro Anticancer Properties of Copper Metallodendrimers. Biomolecules. 2019 Apr 18;9(4):155. doi:10.3390/biom9040155, polegał na wykonaniu części pomiarów: anizotropii fluorescencji, widm dichroizmu kołowego oraz określenia cytotoksyczności dendrymerów wobec jednojądrzastych komórek krwi obwodowej. Mój wkład w przygotowanie pracy w formie publikacji stanowi 7%.

Jakub Magiera

WYDZIAŁ BIOLOGII
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Mgr Małgorzata Kubczak

Łódź, dnia 23.05.2023

Oświadczenie

Oświadczam, że mój udział w pracy Hołota M., Magiera J., Michlewska S., Kubczak M., Sanz del Olmo N., García-Gallego S., Ortega P., de la Mata F.J., Ionov M., Bryszewska M. In Vitro Anticancer Properties of Copper Metallodendrimers. Biomolecules. 2019 Apr 18;9(4):155. doi:10.3390/biom9040155, polegał na wykonaniu analizy statystycznej wyników przedstawionych badań. Mój wkład w przygotowanie pracy w formie publikacji stanowi 3%.

Kubczak

Name: Prof. Francisco Javier de la Mata

Affiliation: Departamento de Química Orgánica y Química Inorgánica
Facultad de Farmacia
Universidad de Alcalá,
Alcalá de Henares, Spain

Date:

Statement of contribution

To whom it may concern

I am co-author of following publications:

1. Hołota M., Magiera J., Michlewska S., Kubczak M., Sanz del Olmo N., García-Gallego S., Ortega P., de la Mata F.J., Ionov M., Bryszewska M. In Vitro Anticancer Properties of Copper Metallodendrimers. Biomolecules. 2019 Apr 18;9(4):155. doi:10.3390/biom9040155. I contributed to the general supervising of the synthesis and characterization of the dendrimers used in this work and participated in the editing of the final version manuscript. Overall contribution - 3%.

2. Sanz del Olmo N., Hołota M., Michlewska S., Gómez R., Ortega P., Ionov M., de la Mata F.J., Bryszewska M. Copper (II) Metallodendrimers Combined with Pro-Apoptotic siRNAs as a Promising Strategy Against Breast Cancer Cells. Pharmaceutics. 2020 Aug 2;12(8):727. doi: 10.3390/pharmaceutics12080727. I contributed to the general supervising of the synthesis and characterization of the dendrimers used in this work and participated in the editing of the final version manuscript. Overall contribution - 3%.

3. Hołota M., Michlewska S., García-Gallego S., Sanz del Olmo N., Ortega P., Bryszewska M., de la Mata F.J., Ionov M. Combination of Copper Metallodendrimers with Conventional Antitumor Drugs to Combat Cancer in In Vitro Models. Int J Mol Sci. 2023 Feb 17;24(4):4076. doi: 10.3390/ijms24044076. I contributed to the general supervising of the synthesis and characterization of the dendrimers used in this work and participated in the editing of the final version manuscript. Overall contribution - 3%.

DE LA MATA DE LA MATA FRANCISCO JAVIER - DNI 08969382A

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Edificio de Farmacia

Universidad de Alcalá

May 23rd, 2023

Statement of contribution

To whom it may concern

I am co-author of following publications:

Hołota M., Magiera J., Michlewska S., Kubczak M., Sanz del Olmo N., García-Gallego S., Ortega P., de la Mata F.J., Ionov M., Bryszewska M. In Vitro Anticancer Properties of Copper Metallodendrimers. Biomolecules. 2019 Apr 18;9(4):155. doi:10.3390/biom9040155. I was involved in the preparation of the dendrimers used in this work and participated in the editing of the final version manuscript. My contribution to the manuscript preparation is 5%.

Sanz del Olmo N., Hołota M., Michlewska S., Gómez R., Ortega P., Ionov M., de la Mata F.J., Bryszewska M. Copper (II) Metallodendrimers Combined with Pro-Apoptotic siRNAs as a Promising Strategy Against Breast Cancer Cells. Pharmaceutics. 2020 Aug 2;12(8):727. doi: 10.3390/pharmaceutics12080727. I was involved in the preparation of the dendrimers used in this work and participated in the editing of the final version manuscript. My contribution to the manuscript preparation is 5%.

Hołota M., Michlewska S., García-Gallego S., Sanz del Olmo N., Ortega P., Bryszewska M., de la Mata F.J., Ionov M. Combination of Copper Metallodendrimers with Conventional Antitumor Drugs to Combat Cancer in In Vitro Models. Int J Mol Sci. 2023 Feb 17;24(4):4076. doi: 10.3390/ijms24044076. I was involved in the preparation of the dendrimers used in this work and participated in the editing of the final version manuscript. My contribution to the manuscript preparation is 5%.

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Universidad de Alcalá

Name: Dr Natalia Sanz del Olmo, PhD

Affiliation: Departamento de Química Orgánica y Química Inorgánica
Facultad de Farmacia
Universidad de Alcalá,
Alcalá de Henares, Spain

Date: 15/05/2023

Statement of contribution

To whom it may concern

I am co-author of following publications:

1. Hołota M., Magiera J., Michlewska S., Kubczak M., Sanz del Olmo N., García-Gallego S., Ortega P., de la Mata F.J., Ionov M., Bryszewska M. In Vitro Anticancer Properties of Copper Metallodendrimers. Biomolecules. 2019 Apr 18;9(4):155. doi:10.3390/biom9040155. I was involved in the preparation of the dendrimers used in this work. My contribution to the manuscript preparation is 3%.

2. Sanz del Olmo N., Hołota M., Michlewska S., Gómez R., Ortega P., Ionov M., de la Mata F.J., Bryszewska M. Copper (II) Metallodendrimers Combined with Pro-Apoptotic siRNAs as a Promising Strategy Against Breast Cancer Cells. Pharmaceutics. 2020 Aug 2;12(8):727. doi: 10.3390/pharmaceutics12080727. I was involved in the preparation of the dendrimers used in this work, participated in the experiments performing, analysis of obtained results and manuscript writing. My contribution to the manuscript preparation is 30%.

3. Hołota M., Michlewska S., García-Gallego S., Sanz del Olmo N., Ortega P., Bryszewska M., de la Mata F.J., Ionov M. Combination of Copper Metallodendrimers with Conventional Antitumor Drugs to Combat Cancer in In Vitro Models. Int J Mol Sci. 2023 Feb 17;24(4):4076. doi: 10.3390/ijms24044076. I was involved in the preparation of the dendrimers used in this work and participated in the biophysical measurements and cytotoxicity studies presented in the publication. My contribution to the manuscript preparation is 8%.

Signature

Name: Prof. Sandra García Gallego

Affiliation: Departamento de Química Orgánica y Química Inorgánica
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Universidad de Alcalá,
Alcalá de Henares, Spain

Date: 24/05/2023

Statement of contribution

To whom it may concern

I am co-author of following publications:

1. Hołota M., Magiera J., Michlewska S., Kubczak M., Sanz del Olmo N., García-Gallego S., Ortega P., de la Mata F.J., Ionov M., Bryszewska M. In Vitro Anticancer Properties of Copper Metallodendrimers. Biomolecules. 2019 Apr 18;9(4):155. doi:10.3390/biom9040155. I was involved in the preparation of the dendrimers used in this work and participated in the editing of the manuscript. My contribution to the manuscript preparation is 3%.

2. Hołota M., Michlewska S., García-Gallego S., Sanz del Olmo N., Ortega P., Bryszewska M., de la Mata F.J., Ionov M. Combination of Copper Metallodendrimers with Conventional Antitumor Drugs to Combat Cancer in In Vitro Models. Int J Mol Sci. 2023 Feb 17;24(4):4076. doi: 10.3390/ijms24044076. I was involved in the preparation of the dendrimers used in this work and participated in the editing of the manuscript. My contribution to the manuscript preparation is 3%.

Signature 

Łódź, dnia 30 maja 2023 r.

Oświadczenie

Niniejszym oświadczamy, że deklaracja dotycząca wkładu autora w powstanie artykułów podpisana przez Sandrę García Gallego jest zgodna z wolą autora.

Zeskanowane oświadczenie dotyczące wkładu autora w powstanie publikacji zostało załączone do rozprawy doktorskiej z powodu opóźnienia w uzyskaniu wersji oryginalnej.

Kandydat do stopnia doktora:

Promotor:

Name: Prof. Rafael Gómez Ramírez

Affiliation: Departamento de Química Orgánica y Química Inorgánica
Facultad de Farmacia
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Alcalá de Henares, Spain

Date:

Statement of contribution

To whom it may concern

I am co-author of following publication:

Sanz del Olmo N., Hołota M., Michlewska S., Gómez R., Ortega P., Ionov M., de la Mata F.J., Bryszewska M. Copper (II) Metallodendrimers Combined with Pro-Apoptotic siRNAs as a Promising Strategy Against Breast Cancer Cells. Pharmaceutics. 2020 Aug 2;12(8):727. doi: 10.3390/pharmaceutics12080727. I contributed to the general supervising of the synthesis of the dendrimers used in this work. Overall contribution - 3%.

Signature