WYDZIAŁ BIOLOGII
i OCHRONY ŚRODOWISKA
Uniwersytet Łódzki

Stacjonarne Studia Doktoranckie
Genetyki Molekularnej, Cytogenetyki
i Biofizyki Medycznej

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Dendrymery polipropylenoiminowe jako nośniki przeciwnowotworowych nukleotydów adenozynowych

Poly(propyleneimine) dendrimers as carriers of anticancer adenosine nucleotides

Praca doktorska

wykonana w Katedrze Biofizyki Ogólnej
Instytutu Biofizyki Uniwersytetu Łódzkiego

pod kierunkiem
prof. dr hab. Barbary Klajnert-Maculewicz

Łódź, 2019

Składam serdeczne podziękowania
Pani prof. dr hab. Barbarze Klajnert-Maculewicz
za okazane zaufanie, cierpliwość oraz nieocenioną pomoc
w trakcie przygotowywania niniejszej rozprawy doktorskiej.

Dziękuję również ***pracownikom oraz doktorantom Katedry Biofizyki Ogólnej***
i Katedry Biofizyki Molekularnej Uniwersytetu Łódzkiego
za życzliwość, wsparcie, miłą atmosferę pracy oraz merytoryczną pomoc
w trakcie wykonywania badań.

Spis treści

Załączniki

1. Źródła finansowania badań prowadzonych w ramach pracy doktorskiej

Projekt OPUS „Komórkowe i molekularne mechanizmy działania kompleksów dendrymerów PPI z lekami przeciwnowotworowymi – analogami nukleozydowymi”, 2014/13/B/NZ3/04643

COST Action CA17140 „Nano2Clinic. Cancer Nanomedicine – from the bench to the bedside”

2. Współpraca

Katedra Biofizyki Molekularnej, Wydział Biologii i Ochrony Środowiska, Uniwersytet Łódzki

Katedra Chemii Fizycznej, Wydział Chemii, Uniwersytet Łódzki

Jagiellońskie Centrum Innowacji

Leibniz Institute of Polymer Research Dresden, Drezno, Niemcy

SUPSI-DTI IDSIA-Dalle Molle Institute for Artificial Intelligence, Manno, Szwajcaria

3. Spis publikacji wchodzących w skład rozprawy doktorskiej

1. **Gorzkiewicz M.**, Klajnert-Maculewicz B. (2017), Dendrimers as nanocarriers for nucleoside analogues, European Journal of Pharmaceutics and Biopharmaceutics, 114:43-56. **IF 4.491, MNiSW 40**
2. **Gorzkiewicz M.**, Buczkowski A., Appelhans D., Voit B., Pułaski Ł., Pałecz B., Klajnert-Maculewicz B. (2018), Poly(propyleneimine) glycodendrimers non-covalently bind ATP in a pH- and salt-dependent manner – model studies for adenosine analogue drug delivery, International Journal of Pharmaceutics, 544(1):83-90. **IF 3.862, MNiSW 40**
3. **Gorzkiewicz M.**, Jatczak-Pawlik I., Studzian M., Pułaski Ł., Appelhans D., Voit B., Klajnert-Maculewicz B. (2018), Glycodendrimer nanocarriers for direct delivery of fludarabine triphosphate to leukemic cells: improved pharmacokinetics and pharmacodynamics of fludarabine, Biomacromolecules, 19(2):531-543. **IF 5.738, MNiSW 45**
4. **Gorzkiewicz M.**, Deriu M.A., Studzian M., Janaszewska A., Grasso G., Pułaski Ł., Appelhans D., Danani A., Klajnert-Maculewicz B. (2019), Fludarabine-specific molecular interactions with maltose-modified poly(propyleneimine) dendrimer enable effective cell entry of the active drug form: comparison with clofarabine, Biomacromolecules, 20(3):1429-1442. **IF 5.667, MNiSW 45**
5. **Gorzkiewicz M.**, Appelhans D., Boye S., Lederer A., Voit B., Klajnert-Maculewicz B. (2019), Effect of the structure of therapeutic adenosine analogues on stability and surface electrostatic potential of their complexes with poly(propyleneimine) dendrimers, Macromolecular Rapid Communications, 40(15):1900181. **IF 4.078, MNiSW 40**

Sumaryczny współczynnik wpływu (ang. *Impact Factor*, IF) publikacji wchodzących w skład rozprawy doktorskiej: 23.836

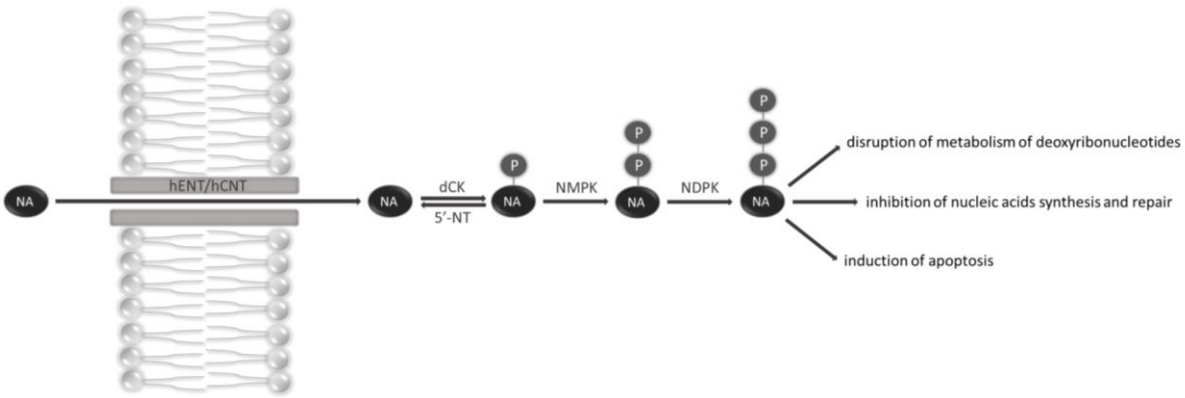
Łączna liczba punktów według listy czasopism punktowanych MNiSW: 210

4. Omówienie celu naukowego i uzyskanych wyników

4.1. Wprowadzenie

Związki z grupy analogów nukleozydów (ang. *nucleoside analogues*, NAs) były jednymi z pierwszych leków wprowadzonych do terapii przeciwnowotworowych. W przypadku nowotworów układu krwiotwórczego najczęściej stosuje się analogi adenozyny i cytydyny. Substancje te wykazują różnorodną aktywność cytotoksyczną, obejmującą zaburzenia metabolizmu naturalnych nukleotydów, hamowanie procesów replikacji i naprawy DNA oraz indukcję apoptozy.

Ze względu na hydrofilowy charakter NAs, ich komórkowy wychwyt na drodze dyfuzji prostej jest ograniczony. Wnikają one do wnętrza komórki dzięki transportowi za pośrednictwem białek błonowych, tzw. transporterów nukleozydów (ang. *nucleoside transporters*, NTs). Wewnątrz komórki analogi nukleozydów są stopniowo fosforylowane do mono-, dwu- i trójfosforanów. Należy podkreślić, iż w większości przypadków tylko lek przekształcony do formy trójfosforanu wykazuje aktywność cytotoksyczną względem komórek nowotworowych. Ponieważ NTs mogą przenosić przez błonę tylko defosforylowane substraty, niemożliwe jest dostarczenie z ich udziałem leku w formie aktywnej[1] (Rysunek 1).



Rys. 1. Metabolizm i mechanizm działania przeciwnowotworowych analogów nukleozydowych. NA: analog nukleozydowy; P: grupa fosforanowa; hENT/hCNT: transportery nukleozydów; dCK: kinaza deoksycytydynowa; NMPK: kinaza monofosforanowa; NDPK: kinaza difosforanowa; 5'-NT: 5'-nukleotydaza (źródło: Gorzkiewicz M., Klajnert-Maculewicz B. (2017), Eur. J. Pharm. Biopharm., 114:43-56).

[1] Galmarini C.M. et al. (2002), Lancet Oncol., 3(7):415-424.

W przypadku terapii z zastosowaniem NAs komórki nowotworowe mogą nabywać lekooporność w trakcie podawania leku, opierającą się w dużej mierze na obniżonej ekspresji transporterów błonowych i zahamowanej aktywności wewnątrzkomórkowych kinaz[2]. Możliwą strategią chemioterapii jest dostarczenie aktywnej, trójfosforanowej postaci leku w połączeniu z nośnikiem bezpośrednio do komórki, co pozwoli ominąć ograniczenia związane z nieefektywnym transportem, degradacją enzymatyczną bądź nieefektywną aktywacją na drodze fosforylacji wewnątrzkomórkowej. Ponadto zadaniem nośnika jest zmniejszenie niepożądanych oddziaływań leku z komórkami prawidłowymi[3].

W celu przezwyciężenia mechanizmów oporności i poprawienia komórkowego wychwytu leków prowadzone są badania dotyczące możliwości zastosowania dendrymerów jako nośników substancji aktywnych. Dendrymery to polimery o regularnej, trójwymiarowej strukturze z trzema charakterystycznymi elementami: rdzeniem, wnętrzem tworzonym przez promieniście rozchodzące się dendrony i terminalnymi grupami funkcyjnymi. Rozmiar cząsteczki określany jest przez jej generację, czyli liczbę powtarzających się warstw rozgałęzionych monomerów przyłączonych do rdzenia. Dzięki zoptymalizowanym metodom syntezy dendrymery mają dobrze zdefiniowaną budowę i są homogenne pod względem masy cząsteczkowej. Ich zastosowaniu w medycynie sprzyja dobra rozpuszczalność i przenikalność przez błony komórkowe oraz wysoka reaktywność i poliwalentna struktura pozwalające na łączenie dendrymeru z cząsteczkami terapeutycznymi. Liczne badania dotyczące potencjału dostarczania leków przez dendrymery wskazują, że mogą one transportować dużą liczbę cząsteczek aktywnych, zapewniając ich zwiększoną rozpuszczalność, wydłużony okres półtrwania we krwi i kontrolowaną dystrybucję, jednocześnie chroniąc je przed biologiczną i chemiczną degradacją oraz pokonując komórkowe mechanizmy oporności. Wszystkie te cechy sprawiają, że dendrymery są bardzo dobrymi kandydatami na nośniki leków[4].

Dendrymery kationowe (takie jak polipropylenoiminowe, PPI) są szczególnie interesujące ze względu na ich zdolność do tworzenia stabilnych, niekowalencyjnych kompleksów z nukleotydami[5] oraz ich ochrony przed degradacją enzymatyczną[6].

[2] Galmarini C.M. et al. (2001), Leukemia, 15(6):875-890.
[3] Hajdo L. et al. (2010), Drug Dev. Res., 71(7):383-394.
[4] Mintzer M.A., Grinstaff M.W. (2011), Chem. Soc. Rev., 40(1):173-190.
[5] Szulc A. et al. (2015), Int. J. Pharm., 495(2):940-947.
[6] Szulc A. et al. (2012), New J. Chem., 36(8):1610-1615.

Ponieważ jednak dendrymery te charakteryzują się wysoką cytotoksycznością związaną z niespecyficznymi oddziaływaniami z błonami komórkowymi, uszkodzeniem mitochondriów i generowaniem reaktywnych form tlenu[7,8], istotne jest zmniejszenie ich dodatniego ładunku powierzchniowego, aby umożliwić zastosowanie kliniczne. Można to osiągnąć przez kowalencyjną modyfikację powierzchniowych grup aminowych cząsteczkami obojętnymi, takimi jak reszty cukrowe[9]. Glikozylacja może przynieść dodatkowe korzyści w przypadku dostarczania leków przeciwbiałaczkowych, ponieważ komórki krwi zazwyczaj wykazują nadekspresję powierzchniowych receptorów lektynowych o wysokim powinowactwie do ligandów cukrowych[10]. Może to umożliwić specyficzny dokomórkowy transport dendrymerów o powierzchni zmodyfikowanej cukrami, zwiększając wydajność dostarczania leku.

4.2. Cel pracy i hipoteza badawcza

Głównym celem niniejszej pracy była charakterystyka tworzenia i stabilności kompleksów nukleotydów adenozynowych z dendrymerami polipropylenoiminowymi generacji 4 (w formie niezmodyfikowanej lub częściowo zmodyfikowanej powierzchniowo cukrami) oraz ocena możliwości zastosowania tych polimerów jako nośników leków przeciwnowotworowych z grupy analogów nukleozydowych.

Hipoteza badawcza zakłada, że dendrymery PPI mogą efektywnie oddziaływać z terapeutycznymi nukleotydami i transportować aktywne, trójfosforanowe formy leków bezpośrednio do wnętrza komórek, zwiększając tym samym skuteczność i swoistość ich działania oraz umożliwiając pokonanie mechanizmów oporności.

4.3. Metodyka badań

Badania prowadzono przy użyciu następujących materiałów i technik badawczych:

1. Analiza tworzenia, oznaczenie stechiometrii i stabilności kompleksów dendrymerów polipropylenoiminowych generacji 4 w formie niezmodyfikowanej (PPI G4) oraz o powierzchni w około 30% zmodyfikowanej resztami maltozy (PPI-Mal OS G4) lub

[7] Thomas T.P. et al. (2009), Biomacromolecules, 10(12):3207-3214.
[8] Ziemba B. et al. (2012), J. Biomed. Mater. Res. A, 100(11):2870-2880.
[9] Janaszewska A. et al. (2012), New J. Chem., 36(2):428-437.
[10] Jain K. et al. (2012), Biomaterials, 33(16):4166-4186.

maltotriozy (PPI-Mal III OS G4) z modelowym nukleotydem adenozynowym, 5'-trójfosforanem adenozyny (ATP) – izotermiczne miareczkowanie kalorymetryczne (ang. *isothermal titration calorimetry*, ITC), miareczkowanie z pomiarem potencjału zeta.

2. Oznaczenie cytotoksyczności fludarabiny w formie nukleozydu (Ara-FA), trójfosforanu (Ara-FATP) oraz ich kompleksów z dendrymerem PPI-Mal OS G4 w ludzkich liniach komórkowych hodowanych w warunkach *in vitro*: CCRF (ostrej białaczki limfoblastycznej T-komórkowej, ang. *acute lymphoblastic T-cell leukemia*), THP-1 (ostrej białaczki monocytowej, ang. *acute monocytic leukemia*) i U937 (chłoniaka histiocytarnego, ang. *histiocytic lymphoma*) – technika resazurynowa.

3. Oznaczenie ekspresji transporterów nukleozydów specyficznych dla fludarabiny (hENT1, hENT2, hCNT3) na poziomie mRNA w badanych liniach komórkowych – metoda RT-PCR w czasie rzeczywistym.

4. Oznaczenie cytotoksyczności fludarabiny w formie nukleozydu, trójfosforanu oraz ich kompleksów z dendrymerem PPI-Mal OS G4 w badanych liniach komórkowych po inkubacji ze specyficznym inhibitorem transportera hENT1, nitrobenzylotioinozyną (NBMPR) – technika resazurynowa.

5. Oznaczenie zaburzeń homeostazy komórkowej w komórkach linii U937 po inkubacji z fludarabiną w formie nukleozydu, trójfosforanu oraz kompleksem Ara-FATP-PPI-Mal OS G4:

- zahamowanie syntezy DNA i RNA – oznaczenie jakościowe (mikroskopia konfokalna) i ilościowe (test przesiewowy o wysokiej zawartości danych, ang. *high-content screening*, HCS), test inkorporacji bromourydyny i bromodeoksyurydyny,
- spadek potencjału mitochondrialnego – spektrofluorymetryczny test płytkowy z zastosowaniem barwnika JC-1,
- aktywność kaspazy-3 – spektrofluorymetryczny test płytkowy z zastosowaniem fluorogennego substratu kaspazy-3, Ac-DEVD-AFC,
- poziom eksternalizacji fosfatydyloseryny – spektrofluorymetryczny test płytkowy z zastosowaniem aneksyny V.

6. Analiza dokomórkowego transportu fludarabiny w formie nukleozydu, trójfosforanu oraz ich kompleksów z dendrymerem PPI-Mal OS G4 w komórkach linii U937, w obecności lub nieobecności inhibitora hENT1 (NBMPR) – synteza znakowanego izotopowo

trójfosforanu fludarabiny (^{3}H-Ara-FATP) z komercyjnie dostępnego nukleozydu (^{3}H-Ara-FA), pomiar radioaktywności wewnątrzkomórkowej.

7. Oznaczenie cytotoksyczności klofarabiny w formie nukleozydu (CAFdA), trójfosforanu (CAFdATP) oraz ich kompleksów z dendrymerami PPI G4 oraz PPI-Mal OS G4 w komórkach linii U937 – technika resazurynowa.

8. Analiza kinetyki tworzenia kompleksów fludarabiny i klofarabiny w formie nukleozydów i trójfosforanów z dendrymerami PPI G4 i PPI-Mal OS G4 – technika powierzchniowego rezonansu plazmonowego (ang. *surface plasmon resonance*, SPR).

9. Charakterystyka oddziaływań fludarabiny i klofarabiny w formie trójfosforanów z dendrymerami PPI G4 i PPI-Mal OS G4 na poziomie molekularnym – komputerowe modelowanie molekularne. Komputerowe modele kompleksów nukleotyd-dendrymer zostały opracowane i scharakteryzowane przez zespół z SUPSI-DTI IDSIA-Dalle Molle Institute for Artificial Intelligence (Manno, Szwajcaria).

10. Ocena powierzchniowego potencjału elektrostatycznego kompleksów fludarabiny i klofarabiny w formie trójfosforanów z dendrymerami PPI G4 i PPI-Mal OS G4 – pomiar potencjału zeta.

11. Ocena stabilności kompleksów fludarabiny i klofarabiny w formie nukleozydów i trójfosforanów z dendrymerami PPI G4 i PPI-Mal OS G4 w różnych warunkach środowiskowych – ultrafiltracja, frakcjonowanie przepływowe w asymetrycznym polu sił przepływu (ang. *asymmetric flow field-flow fractionation,* AF4).

4.4. Omówienie wyników

Pierwszy etap pracy obejmował oznaczenie stechiometrii i stabilności kompleksów za pomocą izotermicznego miareczkowania kalorymetrycznego (ITC) z wykorzystaniem ATP jako modelowego nukleotydu adenozynowego. W badaniach zastosowano dendrymer PPI G4 oraz jego analogi o powierzchni w około 30% zmodyfikowanej resztami maltozy lub maltotriozy. Glikodendrymery PPI częściowo opłaszczone cukrami (tzw. glikodendrymery typu „open shell” (OS)) zostały wybrane jako potencjalny nośnik terapeutycznych nukleotydów ze względu na swoją wysoką biokompatybilność oraz zachowany dodatni ładunek powierzchniowy, umożliwiający oddziaływania z negatywnie naładowanymi resztami fosforanowymi nukleotydów.

Na podstawie pomiarów ITC wyznaczono stałe tworzenia kompleksów (K), stechiometrię badanych oddziaływań oraz zmiany wartości funkcji termodynamicznych opisujących daną reakcję. Porównano oddziaływania w wodzie oraz w buforze HEPES i buforze fosforanowym przy dwóch wartościach pH (7,4 oraz 5,8). Przeprowadzone doświadczenia wykazały, że dendrymery PPI mogą wiązać ATP i tworzyć stabilne kompleksy poprzez oddziaływania elektrostatyczne między zjonizowanymi resztami fosforanowymi nukleotydu i powierzchniowymi grupami aminowymi dendrymeru. Wniosek ten został dodatkowo poparty przez fakt, że w doświadczeniu kontrolnym z zastosowaniem adenozyny jako liganda nie odnotowano efektów termicznych wskazujących na tworzenie kompleksu, oraz przez różnice w wydajności kompleksowania niezmodyfikowanego dendrymeru PPI i jego wariantów zmodyfikowanych cukrami. Udowodniono, że proces tworzenia kompleksu nukleotyd-dendrymer PPI jest spontaniczny, napędzany entalpią i zależy od składu buforu i jego pH (wraz ze spadkiem pH wzrasta liczba miejsc wiążących dendrymeru PPI ze względu na protonację pierwszo- i trzeciorzędowych amin w jego strukturze).

Wyniki otrzymane dzięki ITC – liczbę cząsteczek ATP kompleksowanych przez dendrymer – potwierdzono przeprowadzając miareczkowanie z pomiarem potencjału zeta. Pozwoliło to wyznaczyć optymalny stosunek liczby cząsteczek trójfosforanu do dendrymeru w kompleksie (20:1 dla dendrymeru PPI G4 oraz 10:1 dla dendrymerów zmodyfikowanych maltozą i maltotriozą w pH 7,4), co zostało wykorzystane w dalszych badaniach[11].

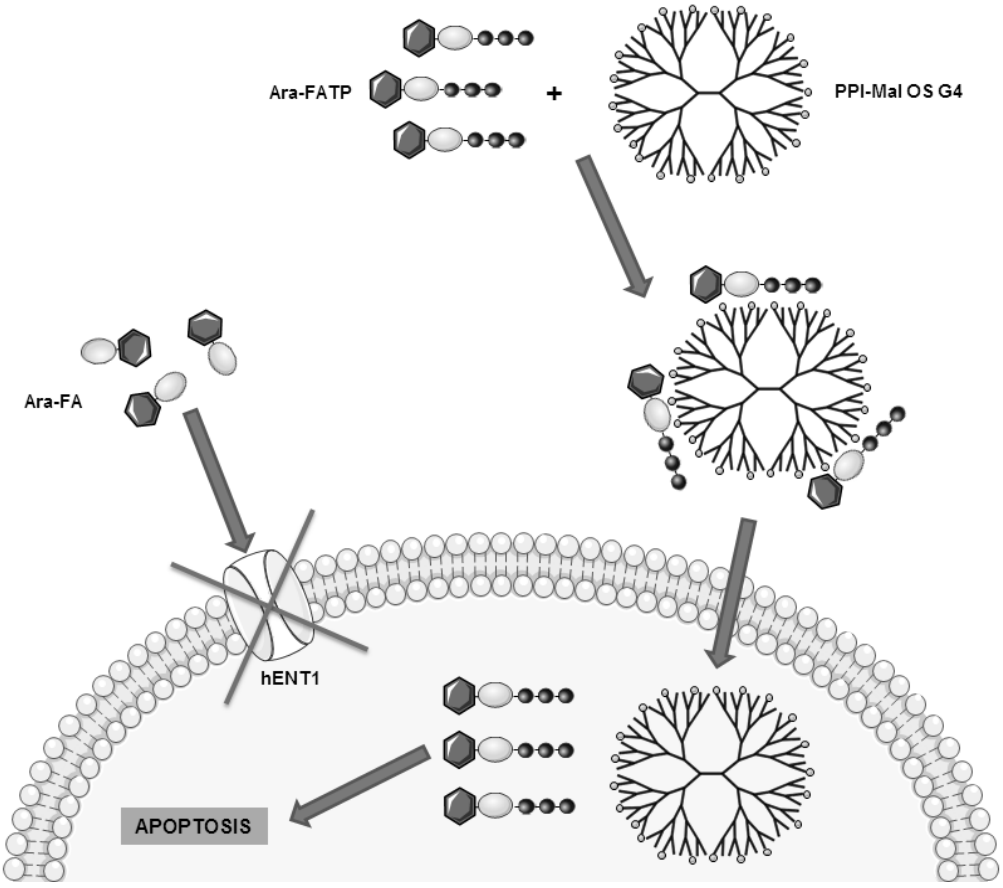
Ponieważ nie zaobserwowano istotnych różnic w parametrach termodynamicznych i stechiometrii kompleksów tworzonych przez glikodendrymery, w dalszej części pracy postanowiono skupić się na dendrymerze maltozowym, dla którego już wcześniej wykazano zdolność do dostarczania trójfosforanu cytarabiny do komórek nowotworowych[12].

Kolejnym etapem badań była ocena potencjału dokomórkowego transportu przez dendrymer PPI-Mal OS G4 aktywnego, trójfosforanowego metabolitu fludarabiny, analogu deoksyadenozyny wykorzystywanego w leczeniu różnych typów nowotworów krwi. Udało się wykazać, że Ara-FATP ma ograniczoną toksyczność wobec badanych

[11] Gorzkiewicz M. et al. (2018), Int. J. Pharm., 544(1):83-90.
[12] Szulc A. et al. (2016), Int. J. Pharm., 513(1-2):572-583.

komórek w porównaniu do wolnego nukleozydu, podczas gdy kompleksowanie z dendrymerem (który sam w badanym zakresie stężeń nie wpływa na przeżywalność komórek) znacznie zwiększa jego aktywność w stopniu porównywalnym z działaniem Ara-FA. Zgodnie z oczekiwaniami, dendrymer nie wpływał na aktywność cytotoksyczną formy nukleozydowej.

Wrażliwość badanych linii komórkowych na fludarabinę okazała się zgodna z poziomem ekspresji transportera hENT1 (ang. *human equilibrative nucleoside transporter 1*). Stosując specyficzny inhibitor tego białka, nitrobenzylotioinozynę (NBMPR), udowodniono, że transport za pośrednictwem hENT1 jest etapem limitującym cytotoksyczną aktywność fludarabiny, natomiast kompleksowanie z dendrymerem pozwala Ara-FATP zabijać komórki nawet przy zahamowanej aktywności białka transportującego. Wskazuje to, że zastosowanie dendrymeru PPI-Mal OS G4 do dostarczania terapeutycznych nukleotydów mogłoby pozwolić na pokonanie naturalnie występującej oporności na leki związanej ze zmniejszoną aktywnością transportera błonowego (Rysunek 2).



Rys. 2. Schemat obrazujący dokomórkowy transport fludarabiny w formie nukleozydu (Ara-FA) oraz trójfosforanu (Ara-FATP) w kompleksie z dendrymerem PPI-Mal OS G4, w obecności inhibitora hENT1 (źródło: Gorzkiewicz M. et al. (2018), Biomacromolecules, 19(2):531-543, zmodyfikowano).

W końcowym etapie, stosując szereg testów do oceny eksternalizacji fosfatydyloseryny, zmian potencjału mitochondrialnego, aktywności kaspazy-3 i syntezy DNA/RNA wykazano, że tworzenie kompleksu nie zmienia specyficznego wewnątrzkomórkowego mechanizmu działania trójfosforanu fludarabiny, związanego z hamowaniem wydłużania łańcuchów kwasów nukleinowych i prowadzącego do apoptozy na drodze wewnątrzpochodnej[13].

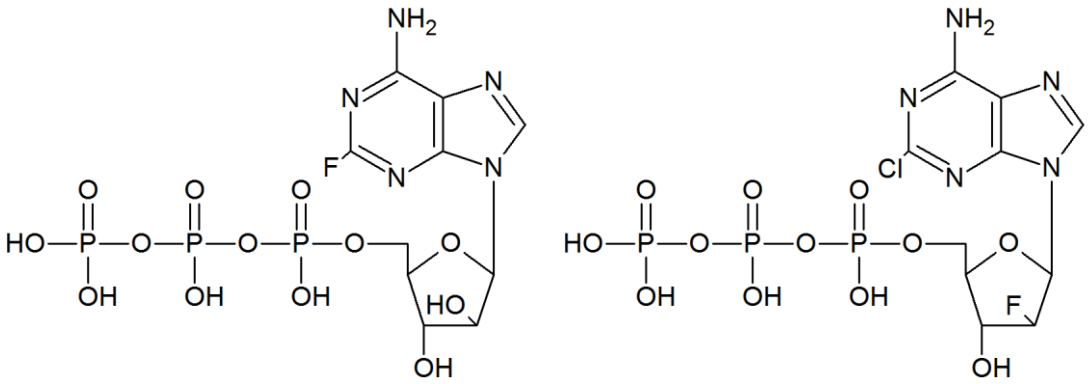
Ponieważ przeprowadzone doświadczenia nie są bezpośrednim dowodem autonomicznego, niezależnego od hENT1 dokomórkowego transportu nukleotydu przez dendrymer, w kolejnym etapie badań zaprojektowano i zoptymalizowano metodę enzymatycznej syntezy znakowanego izotopowo trójfosforanu fludarabiny z komercyjnie dostępnego nukleozydu. Umożliwiło to obserwację zmian wewnątrzkomórkowego stężenia leku w czasie. Wykazano, że dendrymer PPI-Mal OS G4 nie wpływa na komórkowy wychwyt ^{3}H-Ara-FA, jednocześnie wydajnie transportując jego trójfosforanową pochodną do wnętrza komórek linii U937. Zastosowanie inhibitora hENT1 (NBMPR) skutecznie zahamowało dokomórkowy transport ^{3}H-Ara-FA, ^{3}H-Ara-FATP oraz mieszaniny ^{3}H-Ara-FA-PPI-Mal OS G4. W tych samych warunkach dendrymer zachował zdolność przezbłonowego transportu ^{3}H-Ara-FATP. Obserwacje te dostarczyły ostatecznego dowodu na prawdziwość hipotezy sformułowanej na podstawie testów cytotoksyczności: dendrymer PPI-Mal OS G4 posiada zdolność alternatywnego, niezależnego od hENT1 dokomórkowego transportu nukleotydów.

W dalszych badaniach zastosowano klofarabinę, lek z grupy analogów nukleozydowych drugiej generacji, aby zweryfikować hipotezę, że dendrymer PPI-Mal OS G4 może służyć jako uniwersalny nośnik terapeutycznych nukleotydów adenozynowych. Różnice strukturalne między fludarabiną i klofarabiną są ograniczone do dwóch atomów: fludarabina posiada atom fluoru w pozycji 2 zasady adeninowej, który w przypadku klofarabiny został zastąpiony atomem chloru. Ponadto w strukturze klofarabiny wprowadzony został dodatkowy atom fluoru w pozycji 2’ pierścienia arabinofuranozylowego (Rysunek 3). Zgodnie z oczekiwaniami, testy cytotoksyczności wykazały, że klofarabina w formie nukleozydu jest bardziej toksyczna niż jej forma nukleotydowa. Kompleksowanie z dendrymerem nie wpływało na aktywność CAFdA, natomiast kompleks CAFdATP-PPI-Mal OS G4 wykazywał znacząco zmniejszony efekt

[13] Gorzkiewicz M. et al. (2018), Biomacromolecules, 19(2):531-543.

cytotoksyczny w porównaniu z wolnym trójfosforanem. Efekt ten pogłębił się po zastosowaniu niezmodyfikowanego dendrymeru (PPI G4) jako nośnika.

Biorąc pod uwagę znaczące różnice w cytotoksyczności kompleksów fludarabina-dendrymer i klofarabina-dendrymer, podjęto próbę ich charakterystyki przy zastosowaniu techniki powierzchniowego rezonansu plazmonowego (SPR) i komputerowego modelowania molekularnego. W przypadku oznaczeń SPR, analiza powinowactwa pozwoliła wykazać brak oddziaływań między Ara-FA i CAFdA a dendrymerami PPI G4 i PPI-Mal OS G4, a także wyznaczyć stałe dysocjacji (K_D) kompleksów badanych dendrymerów z trójfosforanowymi formami leków. Zaobserwowano, że CAFdATP wykazuje wyższe powinowactwo do dendrymerów PPI w porównaniu z Ara-FATP, co może skutkować różnicami w szybkości uwalniania obu leków z kompleksów.



Rys. 3. Struktura chemiczna trójfosforanu fludarabiny (A) i klofarabiny (B).

Komputerowe modele molekularne opracowane przez zespół prof. Danani z SUPSI-DTI IDSIA-Dalle Molle Institute for Artificial Intelligence w Szwajcarii potwierdziły, że reszty fosforanowe nukleotydów są odpowiedzialne za główne oddziaływania z powierzchniowymi grupami aminowymi dendrymerów PPI. Jednak w przypadku trójfosforanu fludarabiny zarówno część nukleozydowa (w około 25%), jak i reszty fosforanowe (w około 75%) biorą udział w oddziaływaniach z powierzchnią dendrymeru, co może ułatwiać uwalnianie leku z kompleksu. Co więcej, CAFdATP wykazywał znacznie większą tendencję do maskowania dodatniego powierzchniowego potencjału elektrostatycznego dendrymeru w porównaniu z Ara-FATP. Ponieważ dodatnio naładowane nanocząstki mają większą zdolność do przekraczania bariery błon

komórkowych niż obojętne lub ujemnie naładowane[14], ta właściwość trójfosforanu klofarabiny może ograniczać komórkowy wychwyt kompleksu CAFdATP-dendrymer PPI, przyczyniając się tym samym do jego zmniejszonej aktywności cytotoksycznej[15].

Wyniki oznaczeń SPR i modelowania komputerowego zostały zweryfikowane dzięki zastosowaniu (1) pomiarów potencjału zeta w celu oceny powierzchniowego potencjału elektrostatycznego kompleksów lek-dendrymer, oraz (2) dwóch technik separacyjnych: ultrafiltracji i frakcjonowania przepływowego w asymetrycznym polu sił przepływu (AF4), różniących się natężeniem przepływu i działaniem sił ścinających, w celu scharakteryzowania stabilności kompleksów w różnych warunkach środowiskowych.

Pomiary potencjału zeta potwierdziły wyniki symulacji *in silico*: CAFdATP wykazywał znacznie silniejszą tendencję do maskowania dodatniego ładunku powierzchniowego dendrymerów PPI niż Ara-FATP. Trójfosforan klofarabiny tworzył również stabilniejsze kompleksy z dendrymerami PPI niż trójfosforan fludarabiny (więcej cząsteczek CAFdATP pozostawało związanych z dendrymerem w tych samych warunkach środowiskowych). Ty samym potwierdzono także wyniki oznaczeń SPR o wyższym powinowactwie trójfosforanu klofarabiny do dendrymerów PPI. Wskazuje to na prawdziwość hipotezy o ograniczonym uwalnianiu CAFdATP z kompleksu, co może dodatkowo zmniejszyć potencjał dostarczania tego leku przez dendrymery PPI. Stabilność kompleksów była zależna od stosunku molowego nukleotyd-dendrymer oraz pH, będąc największą w środowisku kwaśnym. Ponadto zwiększenie siły jonowej (poprzez zastosowanie buforu z dodatkiem NaCl w stężeniu fizjologicznym) powodowało spadek stabilności kompleksów[16].

4.5. Wnioski

Na podstawie wyników badań przeprowadzonych w ramach niniejszej rozprawy doktorskiej można sformułować następujące wnioski:

- dendrymery PPI, w tym również glikodendrymery typu „open shell", mogą tworzyć stabilne kompleksy z nukleotydami adenozynowymi dzięki niekowalencyjnym oddziaływaniom między resztami fosforanowymi nukleotydów i powierzchniowymi

[14] Honary S., Zahir F. (2013), Trop. J. Pharm. Res., 12(2):255-273.
[15] Gorzkiewicz M. et al. (2019), Biomacromolecules, 20(3):1429-1442.
[16] Gorzkiewicz M. et al. (2019), Macromol. Rapid. Commun., 40(15):1900181.

grupami aminowymi dendrymerów. Proces ten silnie zależy od warunków środowiskowych, szczególnie pH i obecności jonów nieorganicznych;

- dendrymer PPI generacji 4 o powierzchni częściowo zmodyfikowanej maltozą (PPI-Mal OS G4) może służyć jako efektywny nośnik dla trójfosforanowej formy fludarabiny, zwiększając jej dokomórkowy transport, a tym samym jej cytotoksyczność i pokonując oporność związaną z obniżoną ekspresją lub aktywnością hENT1 dzięki autonomicznej drodze wejścia do komórki. Wskazuje to na mechanistyczne wyjaśnienie tego zjawiska, w którym nietoksyczny dendrymer PPI-Mal OS G4 nie zmienia swoistej aktywności fludarabiny, jednocześnie zwiększając jej komórkowy wychwyt i umożliwiając bezpośrednią, niezależną od wewnątrzkomórkowej fosforylacji toksyczność dzięki niekowalencyjnym oddziaływaniom nukleotyd-dendrymer;
- potencjalnie silniejsze oddziaływania trójfosforanu klofarabiny z dendrymerami PPI i jego zdolność do maskowania dodatniego ładunku powierzchniowego tych polimerów może ograniczyć zdolność kompleksów do wewnątrzkomórkowego dostarczania i uwalniania aktywnej formy leku;
- modyfikacje wprowadzone w strukturze analogów adenozynowych mogą odpowiadać za różnice w oddziaływaniach z dendrymerami PPI, wpływając na właściwości kompleksów, a tym samym na ich dokomórkowy transport i uwalnianie leku, co może skutkować różnym efektem terapeutycznym kompleksów;
- stabilność kompleksów nukleotyd-dendrymer silnie zależy od warunków środowiskowych, szczególnie pH i siły jonowej, co należy wziąć pod uwagę podczas projektowania badań *in vivo* oraz co może stanowić trudność w zastosowaniu badanych układów w terapiach przeciwnowotworowych.

5. Streszczenie w języku polskim

Leki z grupy analogów adenozynowych należą do antymetabolitów o szerokim zastosowaniu w leczeniu białaczek i chłoniaków. Jednakże związki te dla swojej aktywności cytotoksycznej wymagają ułatwionego transportu dokomórkowego za pośrednictwem specyficznych transporterów błonowych oraz fosforylacji przez kinazy wewnątrzkomórkowe, skutkującej tworzeniem aktywnych form trójfosforanowych. Tak złożony metabolizm może prowadzić do zmniejszenia efektu terapeutycznego i wystąpienia oporności komórkowej związanej z obniżeniem ekspresji transporterów lub aktywności wewnątrzkomórkowych kinaz. W celu pokonania tych ograniczeń i zwiększenia skuteczności terapii przeciwnowotworowej z wykorzystaniem analogów adenozynowych, zaproponowano bezpośredni dokomórkowy transport aktywnych, trójfosforanowych form leków za pomocą nanonośników.

Liczne badania potwierdziły, że rozgałęzione dendrymery o ściśle określonej strukturze są dobrymi kandydatami na nośniki leków. Dowiedziono, że dendrymery mogą przenosić dużą liczbę cząsteczek aktywnych, jednocześnie zapewniając ich zwiększoną rozpuszczalność i kontrolowaną biodystrybucję. Ponadto niewielki rozmiar i kulisty kształt tych polimerów sprzyjają ich wnikaniu do komórek i długotrwałemu krążeniu we krwi.

Dendrymery kationowe mogą tworzyć stabilne niekowalencyjne kompleksy z lekami naładowanymi ujemnie, jednak ich toksyczność ogranicza bezpośrednie zastosowanie kliniczne. Dlatego też w celu zmniejszenia dodatniego ładunku powierzchniowego dendrymerów wprowadza się kowalencyjne modyfikacje ich powierzchni, na przykład za pomocą reszt cukrowych. Ponieważ analogi adenozynowe są najczęściej stosowane jako leki przeciwbiałaczkowe, wykorzystanie glikodendrymerów jako nośników jest w tym przypadku szczególnie uzasadnione: komórki białaczkowe wykazują zwykle nadekspresję powierzchniowych receptorów lektynowych o wysokim powinowactwie do ligandów cukrowych, umożliwiając specyficzną endocytozę i ograniczając działania niepożądane leków.

Głównym celem niniejszej pracy była charakterystyka kompleksów nukleotydów adenozynowych z dendrymerami polipropylenoiminowymi generacji 4 (PPI G4) oraz ocena możliwości zastosowania ich w terapii przeciwnowotworowej.

Pierwszy etap badań obejmował zastosowanie izotermicznego miareczkowania kalorymetrycznego i miareczkowania z pomiarem potencjału zeta do oznaczenia

stechiometrii i parametrów termodynamicznych oddziaływań między dendrymerami PPI (w formie niezmodyfikowanej lub częściowo zmodyfikowanej resztami maltozy lub maltotriozy) i 5'-trójfosforanem adenozyny (ATP), zastosowanym jako modelowy nukleotyd adenozynowy. Wykazano, że dendrymery PPI mogą skutecznie oddziaływać z nukleotydami i tworzyć stabilne kompleksy poprzez oddziaływania elektrostatyczne między zjonizowanymi grupami fosforanowymi trójfosforanu i aminowymi dendrymeru. Proces kompleksowania jest spontaniczny, napędzany entalpią i zależy od składu i pH buforu. Wyznaczone optymalne stosunki liczby cząsteczek nukleotydu do dendrymeru w kompleksie zastosowano w dalszych badaniach.

Kolejne doświadczenia z zastosowaniem kompleksu dendrymeru PPI o powierzchni zmodyfikowanej maltozą (PPI-Mal OS G4) i trójfosforanu fludarabiny (Ara-FATP), przeprowadzone na ludzkich liniach komórek białaczkowych hodowanych w warunkach *in vitro* (CCRF, THP-1, U937) wykazały, że Ara-FATP ma ograniczoną aktywność cytotoksyczną w stosunku do badanych komórek w porównaniu do formy nukleozydowej leku (Ara-FA), natomiast kompleksowanie z glikodendrymerem znacznie zwiększa jego toksyczność. Ponadto wykazano, że transport za pośrednictwem białka hENT1 jest etapem ograniczającym toksyczność Ara-FA, podczas gdy kompleksowanie z PPI-Mal OS G4 pozwala Ara-FATP zabijać komórki nawet w obecności inhibitora hENT1. Wskazuje to, że zastosowanie glikodendrymerów jako nośników leków może pozwolić na pokonanie lekooporności związanej ze zmniejszoną aktywnością transporterów błonowych. W końcowym etapie tej części badań udowodniono, że tworzenie kompleksu nie zmienia specyficznego wewnątrzkomórkowego mechanizmu działania Ara-FATP, związanego z hamowaniem syntezy DNA i RNA oraz indukowaniem apoptozy na drodze wewnątrzpochodnej.

Ponieważ doświadczenia te nie dostarczyły bezpośredniego dowodu na niezależny od hENT1 dokomórkowy transport nukleotydu przez dendrymer, w kolejnym etapie badań opracowano metodę syntezy znakowanego izotopowo trójfosforanu fludarabiny. Pozwoliło to ostatecznie udowodnić zdolność kompleksu nukleotyd-glikodendrymer do wewnątrzkomórkowego dostarczenia leku bez udziału błonowych transporterów nukleozydów.

Biorąc pod uwagę tak obiecujące wyniki, w dalszym toku badań zastosowano klofarabinę (CAFdA), lek z grupy analogów adenozynowych drugiej generacji, w celu

weryfikacji hipotezy, że dendrymery PPI mogą służyć jako uniwersalne nośniki terapeutycznych nukleotydów. Wykorzystując technikę powierzchniowego rezonansu plazmonowego i modelowanie molekularne w celu scharakteryzowania właściwości kompleksów lek-dendrymer, wykazano, że trójfosforan klofarabiny (CAFdATP) charakteryzuje się znacząco różnymi oddziaływaniami z dendrymerami PPI w porównaniu z Ara-FATP, co prowadzi do odmiennego efektu terapeutycznego kompleksu (zmniejszonej, a nie zwiększonej aktywności cytotoksycznej). Na tej podstawie postawiono hipotezę, że silniejsze oddziaływania CAFdATP z dendrymerami PPI i jego zdolność do maskowania dodatniego ładunku powierzchniowego tych polimerów mogą ograniczyć potencjał kompleksów do przenikania przez błonę komórkową i wewnątrzkomórkowego uwalniania leku.

W końcowym etapie badań zastosowano pomiary potencjału zeta, ultrafiltrację i frakcjonowanie przepływowe w asymetrycznym polu sił przepływu w celu określenia powierzchniowego potencjału elektrostatycznego i stabilności kompleksów nukleotyd-dendrymer. Zgodnie z oczekiwaniami, CAFdATP wykazał znacznie silniejszą tendencję do maskowania dodatniego ładunku powierzchniowego dendrymerów PPI niż Ara-FATP i do tworzenia stabilniejszych kompleksów niezależnie od warunków środowiskowych. Wyniki te wskazują na prawdziwość hipotezy dotyczącej potencjalnie zmniejszonego wychwytu komórkowego i wewnątrzkomórkowego uwalniania CAFdATP z kompleksu, co może ograniczyć potencjał dostarczania tego leku przez dendrymery PPI.

Podsumowując, przeprowadzone badania wskazują na możliwość zastosowania dendrymerów PPI jako nośników dla adenozynowych leków nukleotydowych w celu zwiększenia ich stężenia wewnątrzkomórkowego oraz aktywności przeciwnowotworowej. Należy jednak podkreślić fakt, że stabilność i właściwości kompleksów nukleotyd-dendrymer silnie zależą od warunków środowiskowych, a także od struktury chemicznej leku, co należy wziąć pod uwagę podczas projektowania doświadczeń zarówno *in vitro*, jak i *in vivo*.

6. Streszczenie w języku angielskim

Adenosine analogue drugs constitute a class of antimetabolites with a wide range of applications in the therapy of leukemias and lymphomas. However, for their cytotoxic activity, these compounds require facilitated transfer through the cell membrane by specialized nucleoside transporters and activation by intracellular kinases leading to formation of active triphosphate forms. This complex metabolism may lead to diminished therapeutic outcome and emergence of drug resistance due to downregulation of transporter expression or decreased activity of intracellular kinases. For the circumvention of these obstacles and enhancement of efficacy of anticancer chemotherapy with adenosine analogues, the direct intracellular delivery of active triphosphates by nanocarrier systems has been proposed.

Numerous studies confirmed that highly-branched dendrimers of well-defined structure are superior to other nanoparticles with regard to drug delivery. Dendrimers provide high loading capacity, as well as improved solubility and biodistribution of drugs. Their nanometric size and globular shape favour cell entry and prolong blood circulation time.

While cationic dendrimers form stable non-covalent complexes with anionic drugs, their inherent toxicity hampers their direct clinical application. Thus, surface covalent modifications, including glycosylation, have been introduced in order to reduce positive charge of the dendrimers. Since adenosine analogues are most often used as antileukemics, the use of glycodendrimers is especially justified: leukemic cells usually overexpress surface lectin receptors with high affinity for carbohydrate ligands, enabling receptor-mediated endocytosis and decreasing the detrimental side effects on healthy tissues.

The present work aimed at characterisation of complexes of adenosine nucleotides and poly(propyleneimine) dendrimers of the 4th generation (PPI G4), and the assessment of their applicability in anticancer therapy.

In the first stage of research, isothermal titration calorimetry and zeta potential titration have been applied for the determination of stoichiometry and thermodynamic parameters of interactions between PPI dendrimers (unmodified or partially surface-modified with maltose or maltotriose) and adenosine-5’-triphosphate (ATP) as a model adenosine nucleotide. It has been shown that PPI dendrimers possess the ability to

efficiently interact with nucleoside triphosphates and to form stable complexes via electrostatic interactions between the ionized phosphate and amino groups on the nucleotide and the dendrimer, respectively. The complexation process is spontaneous, enthalpy-driven and depends on buffer composition and pH. The determined optimal nucleotide-dendrimer ratios have been used in subsequent studies.

Further experiments in *in vitro*-cultured leukemic cell lines (CCRF, THP-1, U937) using complexes of maltose-modified PPI dendrimer (PPI-Mal OS G4) and fludarabine triphosphate (Ara-FATP) have shown that Ara-FATP has limited cytotoxicity towards investigated cells compared to the nucleoside form (Ara-FA), but the complexation with glycodendrimer significantly increases its activity. Moreover, it has been shown that the transport via hENT1 transporter is a limiting step for the toxicity of Ara-FA, while complexation with PPI-Mal OS G4 allows Ara-FATP to kill cells even in the presence of hENT1 inhibitor. Thus, the use of glycodendrimers for drug delivery may allow to circumvent the cellular resistance associated with decreased transporter activity. Finally, it has been demonstrated that complex formation does not change the specific intracellular action of Ara-FATP, preserving its ability to inhibit nucleic acid synthesis and induce apoptosis via the intrinsic pathway.

Since these experiments do not provide a direct proof of the hENT1-independent intracellular transport of the nucleotide by the dendrimer, in the next stage of research a novel synthesis technique for radioactively-labelled fludarabine triphosphate has been devised, allowing to demonstrate the ability of nucleotide-glycodendrimer complex to deliver the drug into the cells without the involvement of membrane nucleoside transporters.

Considering these promising results, in the further course of studies, clofarabine (CAFdA), an adenosine analogue drug of the second generation, has been applied to verify the hypothesis that PPI dendrimers may serve as universal carriers for therapeutic nucleotides. Using surface plasmon resonance and molecular modelling to elucidate the properties of drug-dendrimer complexes, it has been shown that clofarabine triphosphate (CAFdATP) exhibits significantly different molecular interactions with PPI dendrimers in comparison to Ara-FATP, leading to different therapeutic outcome of the complex (decreased rather than increased cytotoxicity). It has been hypothesized that stronger interactions of CAFdATP with PPI dendrimers and its ability to mask surface positive

charge of these macromolecules may decrease the capacity of the complexes to cross cell membrane and release the drug.

In the final stage of studies, zeta potential measurements, ultrafiltration, and asymmetric flow field-flow fractionation have been applied for the determination of surface electrostatic potential and stability of nucleotide-dendrimer formulations. Indeed, CAFdATP has shown a significantly higher tendency to mask positive surface charge of PPI dendrimers than Ara-FATP, and to form stronger complexes regardless of environmental conditions. These results support the hypothesis of potentially reduced cellular uptake and intracellular release of CAFdATP from the complex, which may decrease the delivery potential of PPI macromolecules in case of this drug.

In conclusion, the performed studies indicate the potential of the application of PPI dendrimers as carriers for adenosine nucleotide drugs in order to enhance their intracellular concentration, leading to improved therapeutic outcome. However, it is crucial to note that the stability and properties of nucleotide-dendrimer complexes strongly depend on the environmental conditions and the chemical structure of the drug, which should be taken into account during the design of both *in vitro* and *in vivo* studies.

7. Dorobek naukowy

7.1. Spis publikacji niewchodzących w skład rozprawy doktorskiej

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Sumaryczny współczynnik wpływu (IF) publikacji stanowiących całkowity dorobek naukowy: 67.474
Łączna liczba punktów według listy czasopism punktowanych MNiSW: 680

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7.3. Doniesienia konferencyjne

7.3.1. Wystąpienia ustne

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7.4. Działalność naukowa

7.4.1. Projekty

7.4.1.1. Koordynacja projektów naukowych

1. Projekt PRELUDIUM „Dendrymer PAMAM jako nośnik mesalazyny dla zwiększenia efektywności terapii nieswoistych zapaleń jelit – badania *in vitro*" (2018/31/N/NZ7/00374), finansowany przez Narodowe Centrum Nauki.

7.4.1.2. Udział w projektach naukowych

1. Projekt „Badanie mechanizmów molekularnych na styku organizm ludzki-patogen-czynniki środowiska (InterMolMed)" (POIG.01.01.02-10-107/09), finansowany w ramach Programu Operacyjnego Innowacyjna Gospodarka.
2. Projekt „Innowacyjny preparat przeciwko rakowi wątrobokomórkowemu (HCC)" (PBS1/A9/16/2012), finansowany przez Narodowe Centrum Badań i Rozwoju w ramach Programu Badań Stosowanych.
3. Projekt „Opracowanie polowego testu diagnostycznego wykrywającego zakażenie pszczoły miodnej patogenem *Nosema ceranae*" (PBS1/B8/6/2012), finansowany przez Narodowe Centrum Badań i Rozwoju w ramach Programu Badań Stosowanych.

4. Projekt OPUS „Modulacja funkcji bariery krew-mózg przez nanocząstki – czynniki zagrożenia środowiskowego i nanoterapeutyki” (2012/07/B/NZ4/01770), finansowany przez Narodowe Centrum Nauki.
5. Projekt OPUS „Komórkowe i molekularne mechanizmy działania kompleksów dendrymerów PPI z lekami przeciwnowotworowymi – analogami nukleozydowymi” (2014/13/B/NZ3/04643), finansowany przez Narodowe Centrum Nauki.
6. Projekt HARMONIA „Auto-fluoryzujące dendrymery – badania spektrofluorymetryczne i komórkowe” (2014/14/M/NZ3/00498), finansowany przez Narodowe Centrum Nauki.
7. Projekt OPUS „Dendrymery fosforowe jako nośniki fotouczulaczy – badania *in vivo*” (2017/25/B/NZ7/01304), finansowany przez Narodowe Centrum Nauki.

7.4.2. Staże zagraniczne

1. University of Paris-Sud 11, INSERM UMR-S757, Orsay, Francja (5.01-5.04.2015).
2. Leibniz Institute of Polymer Research Dresden, Drezno, Niemcy (8-14.01.2017, 7.03-6.07.2018, 25.02-22.03.2019).

7.4.3. Zgłoszenia patentowe

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7.4.4. Nagrody

1. Stypendium START Fundacji na rzecz Nauki Polskiej 2019.
2. Stypendium Ministra Nauki i Szkolnictwa Wyższego za wybitne osiągnięcia dla studentów i doktorantów 2018.
3. Stypendium Ministra Nauki i Szkolnictwa Wyższego za wybitne osiągnięcia dla studentów i doktorantów 2017.

Kopie publikacji wchodzących w skład rozprawy doktorskiej

European Journal of Pharmaceutics and Biopharmaceutics 114 (2017) 43–56

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Review article

Dendrimers as nanocarriers for nucleoside analogues

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ARTICLE INFO

Article history:
Received 1 August 2016
Revised 2 October 2016
Accepted in revised form 14 December 2016
Available online 13 January 2017

ABSTRACT

Dendrimers constitute a class of hyperbranched macromolecules with several potential applications due to their unique properties such as a well-defined structure, multivalency and biocompatibility. These polymers became one of the most promising drug nanocarriers, providing improved solubility of therapeutics, high loading capacity and controllable biodistribution pattern. In addition, the use of dendrimers as drug delivery devices in cancer therapies may help to overcome the resistance mechanisms by transporting activated drug molecules directly to cancer cells.

In the recent years, dendrimers were intensively studied for delivery of nucleoside analogues (NAs), essential elements of antiviral therapies, as well as treatments of leukemia, lymphoma and various types of solid tumors. These agents act as antimetabolites, competing with physiological nucleosides, and interacting with intracellular enzymes and nuclear acids to induce cytotoxicity. However, efficiency of NAs-based therapies is often limited by factors like fast metabolism, disadvantageous biodistribution, low solubility and various side effects. In case of treatment of leukemia, target cells usually develop drug resistance, which reduces the activity of nucleoside analogues even further. Thus, drug carrier systems are studied to improve the efficacy and specificity of action of these compounds.

In this review, we summarize available data concerning the possibility of application of dendrimers as delivery devices for nucleoside analogues and their active, triphosphate forms.

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http://dx.doi.org/10.1016/j.ejpb.2016.12.030
0939-6411/

1. Introduction

Nucleoside analogues (NAs) are a group of antimetabolites most commonly used against haematological malignancies. Their primary mechanism of cytotoxicity involves inhibition of important nucleotide metabolism enzymes and disruption of synthesis of nucleic acids, leading to induction of apoptosis. Most of the nucleoside analogues share common metabolic pathway: they are transported through a cell membrane by nucleoside transporters (NTs) and phosphorylated by cellular kinases to their active, triphosphate forms.

Due to several resistance mechanisms including fast metabolism, as well as unfavorable biodistribution, low solubility and specificity of interaction with target cells, anti-tumor therapies based on nucleoside analogues have limited efficiency. What is more, since the only efficient pathway of cellular transport requires NTs, the administration of drugs as triphosphates is impossible. Thus, there is a need for efficient drug carrier systems that could improve the efficacy and specificity of anticancer drugs.

Dendrimers are monodisperse, polyvalent, usually globular macromolecules with a regular and highly branched structure. These polymers possess several unique physicochemical features that make them particularly attractive from a medical point of view. Due to their well-defined, three-dimensional architecture, as well as high reactivity, biocompatibility and solubility, they may act as efficient nanocarriers for many therapeutic compounds, transporting them in an encapsulated or covalently conjugated form.

Application of dendrimers as drug delivery devices may shield NAs from biological and chemical degradation, help to overcome various resistance mechanisms and enable the delivery of activated forms of these therapeutics. Therefore, it is necessary to thoroughly investigate the formation of active dendrimer-NAs complexes and conjugates, their pharmacokinetics and pharmacodynamics, as well as interaction with healthy and cancer cells both *in vitro* and *in vivo*.

2. Dendrimers – properties and application in drug delivery

Modern approaches in nanotechnology enable the manufacturing of chemical compounds with a wide range of applications, from single nanoparticles to more complicated structures of inorganic and biological macromolecules, as well as different forms of polymers. Multi-functional macromolecules with nanometric size may in the nearest future become the basis of medicine, in many cases even replacing conventional drugs.

To date, among all studied nanostructures, dendrimers are the best characterized ones. These sphere-shaped polymers possess monodisperse, symmetrical structure, which gives a number of unique properties [1]. Three main components may be distinguished in their architecture: a central core, branches that are composed of repeated monomeric units radially attached to the core, and functional end groups on the surface (Fig. 1). All three elements perform critical functions, affecting the size, shape and properties of the dendrimer. The size of the macromolecule is determined by its generation, which refers to the number of recurring layers of branched monomers attached to the core. The core itself is usually called "generation 0". Composition of dendrons generates free spaces in the structure of dendrimers, which enable a variety of applications. The properties of dendrimer's interior depend on the type of monomer used for synthesis [2–4].

Lower generation dendrimers possess more asymmetric and open structure in comparison to dendrimers of higher generation. With an increase of the generation above the 4th, dendrimers adopt globular structure [5]. They are characterized by low density of the core region, which increases regularly towards the surface. When a critical branched state is achieved, the surface chemical groups form a closed membrane-like structure, and dendrimers cannot be synthesized further because of the lack of space. This phenomenon is called a "starburst effect" [6].

The concept of synthesis of highly branched polymeric compounds was proposed at the beginning of the 1940s by Stockmayer and Flory [7]. First low molecular weight dendrimeric amines (called "octopus molecules") were obtained in the early 1970s by the team of Vögtle. In 1978 the same group of scientists described the synthesis of these compounds, this time referring to them as "cascade molecules" [8]. This publication initiated the work of Tomalia and his team on the synthesis of the first macromolecular branched polymers [9]. Tomalia also suggested use of the term "dendrimer" to describe a new group of compounds.

Since their first synthesis in the early 1980s, the unique properties of dendrimers have been thoroughly tested for use in medicine, e.g. for drug and gene delivery or diagnostics [10]. Dendrimers offer plenty of advantages in comparison to other architectural forms of polymers, since characteristics of branched macromolecules are quite different from conventional, linear compounds [11]. These differences are mainly due to the methods of synthesis. The classical polymerization process usually generates polydisperse products of different molecular weights, while dendrimers can be synthesized in a controlled manner, which provides homogeneous macromolecules of well-defined structure and uniform molecular weight [9,12,13]. It also gives a possibility to design compounds with specific chemical properties, by appropriate selection of monomers and terminal groups.

Thanks to the globular shape and internal cavities, dendrimers exhibit encapsulation abilities, so that they can be used as

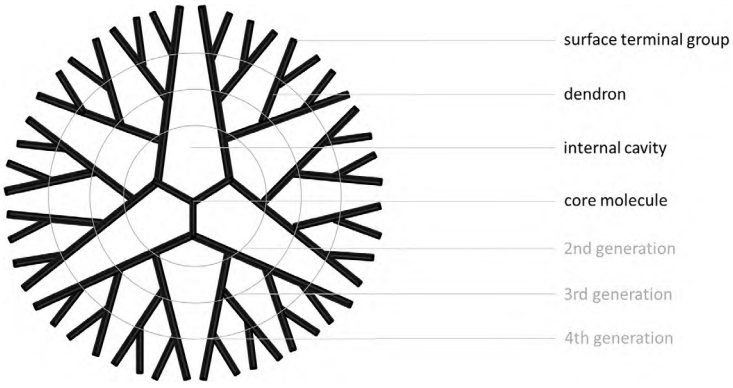

Fig. 1. The structure of dendrimer molecule.

"host-guest" molecules, binding and transporting various compounds in their interior. Dendrimer's surface terminal groups are primarily responsible for the physicochemical and biological features of the macromolecule. Their number depends on the core and dendrons used for the synthesis, and increases exponentially with the generation [14]. In most cases, terminal groups are highly reactive, and may be subjected to further modifications. Post-modification of the surface of the macromolecule is carried out either to change the physicochemical properties of the compound, or to generate particular activity, e.g. catalytic or therapeutic. Large number of surface functional groups is also responsible for high solubility and biopermeability of dendrimers [15]. However, the character and charge of surface groups influence the cytotoxic activity of these polymers. Regardless of molecular structure and surface composition, cationic dendrimers are more hemolytic and cytotoxic than neutral and anionic dendrimers, due to their non-specific affinity to negatively charged cell membranes. Their toxicity is generation-dependent and increases with the number of surface groups [16,17]. In contrast, negatively charged and neutral dendrimers do not exhibit cytotoxic activity *in vitro* [18]. To enable therapeutic and diagnostic applications, it is necessary to reduce the toxicity of positively charged dendrimers. For this purpose, chemical modifications of terminal groups are performed, which include e.g. PEGylation (attachment of poly(ethylene glycol) (PEG) chains) [19–21] and glycosylation [22–25]. In addition, surface modifications including attachment of folate, dextran [26], tuftsin [27], antibodies [28], etc. enhance the targeting potential, significantly prolong blood half-life, improve biocompatibility and biodistribution pattern of dendrimers [29].

Unique properties of dendrimers are responsible for their tremendous potential and advantage over different carrier systems, particularly in the field of drug delivery. The three-dimensional structure with multifunctional terminal groups and numerous internal cavities allow dendrimers to carry therapeutic compounds either encapsulated within the dendritic scaffold or attached to the surface chemical moieties (bound covalently or complexed by electrostatic and van der Waals interactions) (Fig. 2). Such complexes are characterized by high stability and efficiency of intracellular transport. Dendrimers improve solubility of drugs, extend their blood circulation time by reducing their elimination with the urine, maintain drug concentrations in the plasma above the minimal therapeutic dose, and ensure their protection from environmental conditions. The use of dendrimers as drug carriers can contribute to circumvent the resistance mechanisms, as well as facilitate the transport of the drug directly to target cells without affecting normal cells of the organism [3,4,30]. All these features make dendrimers particularly finest candidate for applications in biomedical field.

3. Nucleotide and nucleoside analogues

Nucleoside analogues (NAs) have been in clinical use for almost 50 years. Developed as antiviral drugs, they were among the first chemotherapeutic agents to be introduced for the medical treatment of cancer. Nowadays this group of antimetabolites is commonly used against haematological malignancies, including leukaemias and lymphomas, as well as solid tumors.

This family of compounds includes a variety of purine and pyrimidine nucleoside derivatives with numerous cytotoxic activities. The use of nucleoside analogues as antiviral agents mainly involves human immunodeficiency virus (HIV) (e.g. azidothymidine, stavudine, dideoxy-inosine and -cytidine) and herpes simplex virus treatment (e.g. acyclonucleosides) [31]. Research is also conducted on the application of these drugs in the treatment of hepatitis B [32] and hepatitis C [33] viral infections. Antiviral nucleoside analogues differ in terms of mechanism of action in comparison to orally available drugs, which target the viral polymerase. Their activity is primarily based on the inhibition of DNA replication process. This includes inhibition of primer synthesis, as well as inhibitory effect on plus- and minus-strand DNA elongation [34].

Nucleoside analogues are successfully applied in the treatment of acute myeloid leukemia (AML) and lymphoproliferative disorders. For haematological malignancies therapy, adenosine analogues (e.g. cladribine, fludarabine, pentostatin) and cytidine analogues (e.g. cytarabine, decitabine) are most commonly used. Moreover, gemcitabine (usually in combination with other drugs, e.g. cisplatin), and uracil analogues (fluorouracil, capecitabine) show anticancer effect in solid tumors [35–38]. These compounds exhibit a great variety of cytotoxic activities, which include: disruption of the metabolism of natural deoxyribonucleotides (e.g. by inhibiting ribonucleotide reductase activity), inhibition of replication and repair processes of DNA (e.g. by inhibiting DNA polymerase activity, or being incorporated into the DNA and RNA and altering them) and induction of apoptosis, either through mitochondrial pathway or death receptor signaling pathway. Mode of action of synthetic nucleotides is diverse and depends on their structure, stability, specificity of nucleoside metabolic enzymes and interaction of nucleoside/nucleotide with cell components and proteins involved in cellular metabolic processes [36,37].

Adenosine triphosphate analogues are potent inhibitors of ribonucleotide reductase (RR), a critical enzyme in the process of

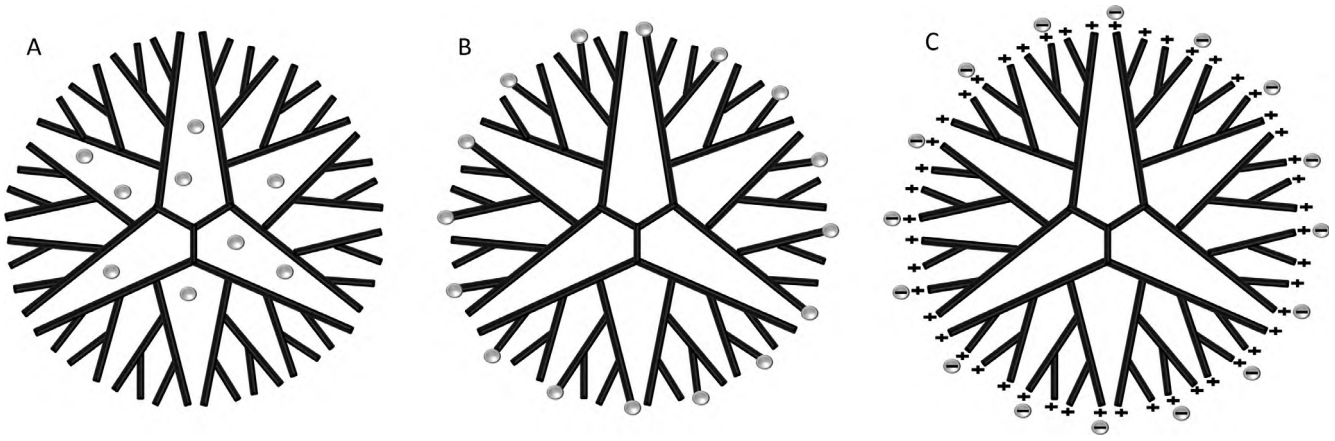

Fig. 2. Possible ways of drug-dendrimer binding: A. Encapsulation inside the dendritic scaffold; B. Covalent bonding; C. Non-covalent interactions with charged functional groups.

de novo synthesis of deoxynucleoside diphosphates (dNDPs). Inhibition of RR ultimately leads to imbalance in the pool of dNTPs [39], which facilitates incorporation of nucleoside analogues triphosphate derivatives into DNA [36]. Insertion of nucleoside analogues into DNA may lead to inhibition of chain elongation. Such an effect is caused either by NAs that lack 3′-OH groups of sugar moieties, which are necessary to form a phosphodiester bond, or those with sugar moiety conformations different than 2′-deoxyribose ring, e.g. arabinose. Unusual conformation of sugar moiety is a factor that hampers formation of a phosphodiester bond, but does not exclude it. The presence of the nucleoside analogue with altered conformation of sugar residue in the DNA affects its structural integrity, and may promote inaccurate replication and induction of mutations [40–42].

As for the process of replication itself, NAs can inhibit the activity of primase and polymerase α, preventing the initiation of DNA synthesis, as well as other polymerases, which may lead to the arrest of DNA repair mechanisms (polymerases β and ε) and inhibition of synthesis of mitochondrial DNA (polymerase γ) [43,44]. Furthermore, adenosine analogues, through inhibition of S-adenosyl homocysteine hydrolase, influence the concentration of S-adenosyl methionine and lead to alteration in DNA methyltransferase (DNMT1) activity, which results in changes of CpG islands methylation status [45].

Nucleoside analogues may also activate the process of apoptosis, either through intrinsic pathway involving mitochondria, or through the activation of death receptor-mediated extrinsic pathways [40].

All nucleoside analogues share common intracellular transport and metabolic pathways, which include active transfer through the cell membrane and activation by intracellular kinases leading to formation of active triphosphate forms (Fig. 3). As hydrophilic molecules, NAs cannot penetrate the cell membrane by passive diffusion, and require specialized nucleoside transporters (NTs) facilitating cell entry. These proteins are generally classified into two groups: concentrative (CNTs) and equilibrative (ENTs) nucleoside transporters. The human equilibrative NTs (hENTs) are present in virtually all tissue types. hENT1, hENT2 and hENT3 recognize a wide range of purine and pyrimidine nucleosides. Additionally, hENT2 efficiently transports nucleobases. hENT4 is exclusively selective for adenosine, and possesses ability to transport a variety of organic cations. The human concentrative NTs (hCNTs) family consisting of three members (hCNT1, 2, and 3) is capable of translocating both pyrimidine and purine nucleosides against a concentration gradient by a sodium-dependent mechanism. The hCNT1 exhibits permeant selectivity for pyrimidine nucleosides but may also transport adenosine, while the hCNT2 protein transports purine nucleosides and uridine. hCNT3 is capable of transporting a wide range of substrates. It is important to note that human NTs can transport compounds only in their dephosphorylated form [46–48].

Inside the cell, NAs are progressively phosphorylated to mono-, di-, and triphosphates. Of the four human deoxynucleoside kinases, deoxycytidine kinase (dCK) has the broadest range of substrate specificity. This enzyme is responsible for the first step of NAs phosphorylation to the monophosphates. The regulation of activity of dCK is the rate-limiting step in the process of activation of nucleoside analogue drugs. In normal cells, dCTP through negative feedback inhibits the activity of deoxycytidine kinase, which leads to inhibition of phosphorylation of both natural deoxynucleosides and their analogues. dCK activity is about 3 to 5 times higher in cancer cells compared to normal cells [49].

The next step of phosphorylation, catalyzed by nucleoside monophosphate kinases (NMPKs), proceeds with equal efficiency for all natural and synthetic nucleoside monophosphates. The last stage, performed by nucleoside diphosphate kinases (NDPKs), may occur with different capacity, because the specificity of the enzyme depends on the conformation of the sugar moiety [50].

Anticancer efficiency of NAs might be limited by various factors, e.g. fast metabolism, disadvantageous biodistribution, low solubility and low specificity of interactions with cancer cells. Moreover, the actual molecular mechanism of toxicity depends on the type of drug and the nature of the cells, hampering the development of an effective therapy. The complicated metabolism of nucleoside analogues causes their exposure to wide range of potential resistance mechanisms. The major factor in NAs resistance is associated with decreased expression of nucleoside transporters, leading to inefficient uptake and accumulation of therapeutic agents inside the cancer cells. Decreased level of deoxycytidine kinase and other kinases is also observed in resistant cells, resulting in inadequate conversion of nucleosides to their active, triphosphate forms. What is more, nucleoside analogues are designed to mimic endogenous metabolites and as such they are in danger of catabolic degradation by natural enzymatic mechanisms acting both in blood serum and inside the cells. These includes 5′-nucleotidase (5′-NT), which dephosphorylates both natural and cytotoxic mononucleotides, and cytidine deaminase (CDA) (responsible for conversion of cytidine and deoxycytidine to uridine and deoxyuridine, respectively), which increased levels have been reported in resistant cells. Resistance against NAs might be caused also by alterations of proteins

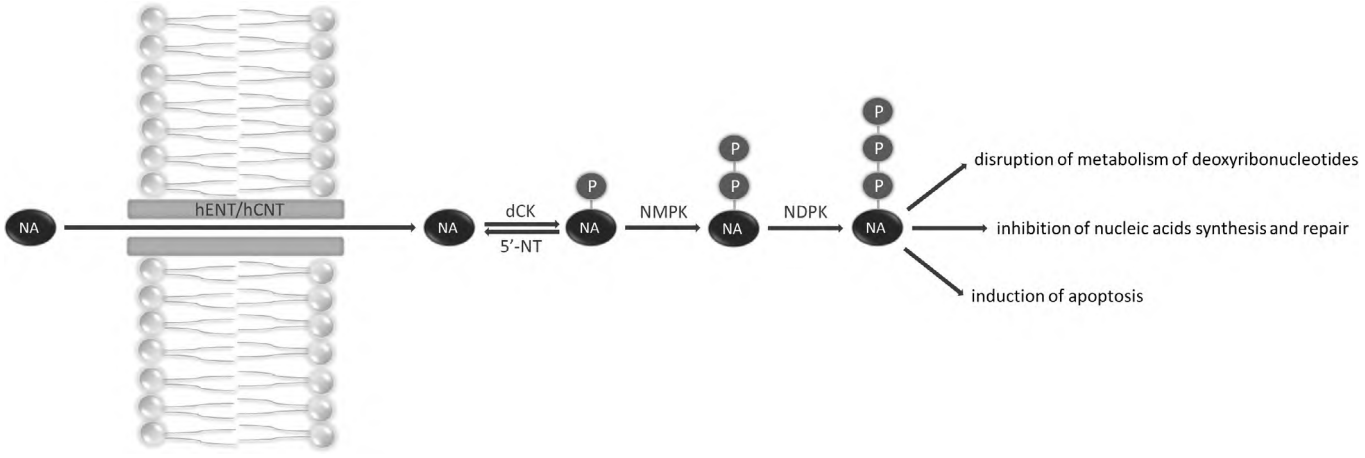

Fig. 3. Metabolism and mechanisms of action of nucleoside analogues. NA: nucleoside analogue; P: phosphate group; hENT/hCNT: human equilibrative/concentrative nucleoside transporter; dCK: deoxycytidine kinase; NMPK: monophosphate kinase; NDPK: diphosphate kinase; 5′-NT: 5′-nucleotidase.

Table 1
Complex formation between dendrimers and non-therapeutic nucleotides.

Nucleotide	Dendrimer	References
AMP	PPI-Mal OS G4, PPI-Mal OS G5	[60]
	PEI G3	[65]
ADP	PPI-Mal OS G4, PPI-Mal OS G5	[60]
	PEI G3	[65]
ATP	PPI-Mal OS G4, PPI-Mal OS G5	[60]
	VPD	[62]
	PEI G3	[65]
	PEI with various oligosaccharide shells	[66]
	PEI-Mal	[67]
Mant-ATP	PPI G4, PPI G5, PPI-Mal OS G4, PPI-Mal OS G5	[61]
	VPD	[62]
CTP	PPI G3, PPI G4, PPI-Mal OS G3, PPI-Mal OS G4, PPI-Mal DS G3, PPI-Mal DS G4	[63]
GMP	PAMAM G5	[64]

implicated in interactions with nucleoside analogues to exert cytotoxic effect, e.g. DNA polymerases, ribonucleotide reductase and CTP synthase. Finally, drug resistance may be due to the defective induction of apoptosis [36,51,52].

4. Dendrimers as nanocarriers for nucleoside analogues

In order to overcome all of above-mentioned resistance mechanisms and to enhance cellular uptake of NAs, numerous studies on the use of drug delivery systems have been conducted. Application of nanocarriers with their own autonomous modes of cellular entry and cargo release may help circumvent problems related to the difficult and rate-limiting transport of drug molecules into the cell, at the same time shielding them from degradation and catabolism. The use of drug carriers may also enable delivery of the active triphosphate forms of therapeutics, thereby preventing the inefficient phosphorylation of nucleoside analogues inside the target cells. Additionally, this approach may prevent undesired interactions of drugs with healthy tissues and decrease toxic side effects of chemotherapy. The variety of nanoparticles proposed as drug delivery devices offers several ways to improve the efficiency of nucleoside analogues in anticancer therapy [53–55].

As mentioned before, dendrimers can be used as drug carriers in two ways: by the covalent bonding of therapeutic compounds, or by forming stable complexes, based on electrostatic forces, between ionized moieties of drugs and dendrimers. The latter method includes both surface interactions and encapsulation within dendritic scaffold. Currently conducted research is aimed at biophysical and biochemical characterization of the complexes and conjugates of dendrimers with nucleotides and nucleosides analogues, as well as evaluation of their effects on mammalian cells *in vitro* and *in vivo*.

4.1. Dendrimers-NAs non-covalent complexes

4.1.1. Kinetics of formation of complexes between dendrimers and non-therapeutic nucleotides

In order to explore the possibility of using different kinds of dendrimers as carriers for active forms of nucleoside analogue drugs, a series of tests using naturally occurring nucleotides and their derivatives has been conducted (Table 1).

To date, probably the most complex and comprehensive research on the possibility of formation of complexes between dendrimers and nucleosides has been conducted by Szulc et al. Their studies indicate that poly(propylene imine) (PPI) dendrimers (Fig. 4A) modified with maltose and maltotriose (PPI-Mal and PPI-Mal III) may be promising drug delivery systems for triphosphate forms of nucleoside analogue drugs. PPI dendrimers modified with sugar moieties have been chosen because of their non-toxicity, non-immunogenicity, biopermeability and ability to stay in blood circulation for the time needed to exhibit the desired effect [23–25,56,57]. Thanks to the crowded maltose shell, modified dendrimers possess greater interaction area, as well as lower flexibility and higher probability of defined cavities within the scaffold, which can improve the stability of drug-dendrimer complexes and increase loading capacity of polymers. What is more, sugar units may enable interactions with drug molecules by electrostatic and hydrogen bonding [23], as well as specific carbohydrate-protein interactions that are integral to receptor-mediated endocytosis [58]. Some cancer cells overexpress various types of surface lectin receptors, with high affinity for carbohydrate ligands. Therefore, sugar-modified dendrimers may serve as carriers for targeted delivery of anticancer agents. Several studies indicate that dendrimers with oligosaccharide shell may be capable of interacting with the lectin receptors, which enable efficient translocation through the cell membrane [22,59].

Using FPLC, NMR, spectrofluorimetric methods and isothermal titration calorimetry (ITC), Szulc et al. characterized different nucleotide-dendrimer complexes. The complexation of the 4th (G4) and the 5th (G5) generation dendrimers with physiological nucleotides (AMP, ADP and ATP) has been investigated. ATP was the only nucleotide that was efficiently bound by PPI-Mal dendrimers. The number of nucleotides complexed with dendrimers depended on sodium chloride (NaCl) concentration and pH of the solution. For instance, at pH 7.0 and 150 mmol/L NaCl, the ATP: PPI-Mal G5 ratio equaled to 2:1. The highest efficiency of complexation was achieved at pH 7.0 and 50 mmol/L NaCl - under such conditions, one molecule of PPI-Mal G5 bound approximately 15 molecules of ATP. Complexes were formed immediately upon mixing of nucleotides with dendrimers and were stable for 48 h. Most importantly, they protected nucleotides from the enzymatic degradation by alkaline phosphatase. This outcome indicates that NAs could be delivered to the cell in an active phosphorylated form without a need to be activated by kinases, thereby avoiding drug resistance [60].

Further investigation involved 2′-/3′-O-(N′-methylanthraniloyl)-ATP (Mant-ATP), a spectrofluorimetric dye which spectrum strongly depends on the characteristics of microenvironment. It had been shown that the intensity of the fluorescence emission increased significantly when dendrimers were added to the solution of Mant-ATP. The effect was strongest for modified PPI-Mal dendrimers, less marked for unmodified PPI dendrimers in an acidic environment, and weakest for unmodified PPI dendrimers at pH 7.4. Also, PPI G5 bound more Mant-ATP molecules than PPI G4. Experiments confirmed that complexation of PPI dendrimers with nucleoside analogues is generation- and pH-dependent. Acidic environment enhanced electrostatic interactions and caused changes in conformation of dendrimer, thereby facilitating dendrimer-dye binding [61].

As it has not been completely clear whether the drugs are bound more in the interior or on the exterior of dendrimers, this aspect has also been studied. For unmodified PPI dendrimers at pH 7.4, the number of Mant-ATP molecules attached to the surface was not dependent on the generation. In contrast, approximately three times more molecules were incorporated into the higher generation dendrimer interior. The dye was efficiently bound inside the scaffold only in case of PPI-Mal G4. No Mant-ATP was found in the interior of PPI-Mal G5 dendrimers, probably due to the dense surface oligosaccharide layer. Moreover, encapsulation was not observed for unmodified PPI dendrimers under acidic conditions. At low pH all terminal amino groups of unmodified dendrimer were protonated, and therefore electrostatic interactions between cationic dendrimers and negatively charged Mant-ATP molecules were stronger on the surface of the polymer, at the same

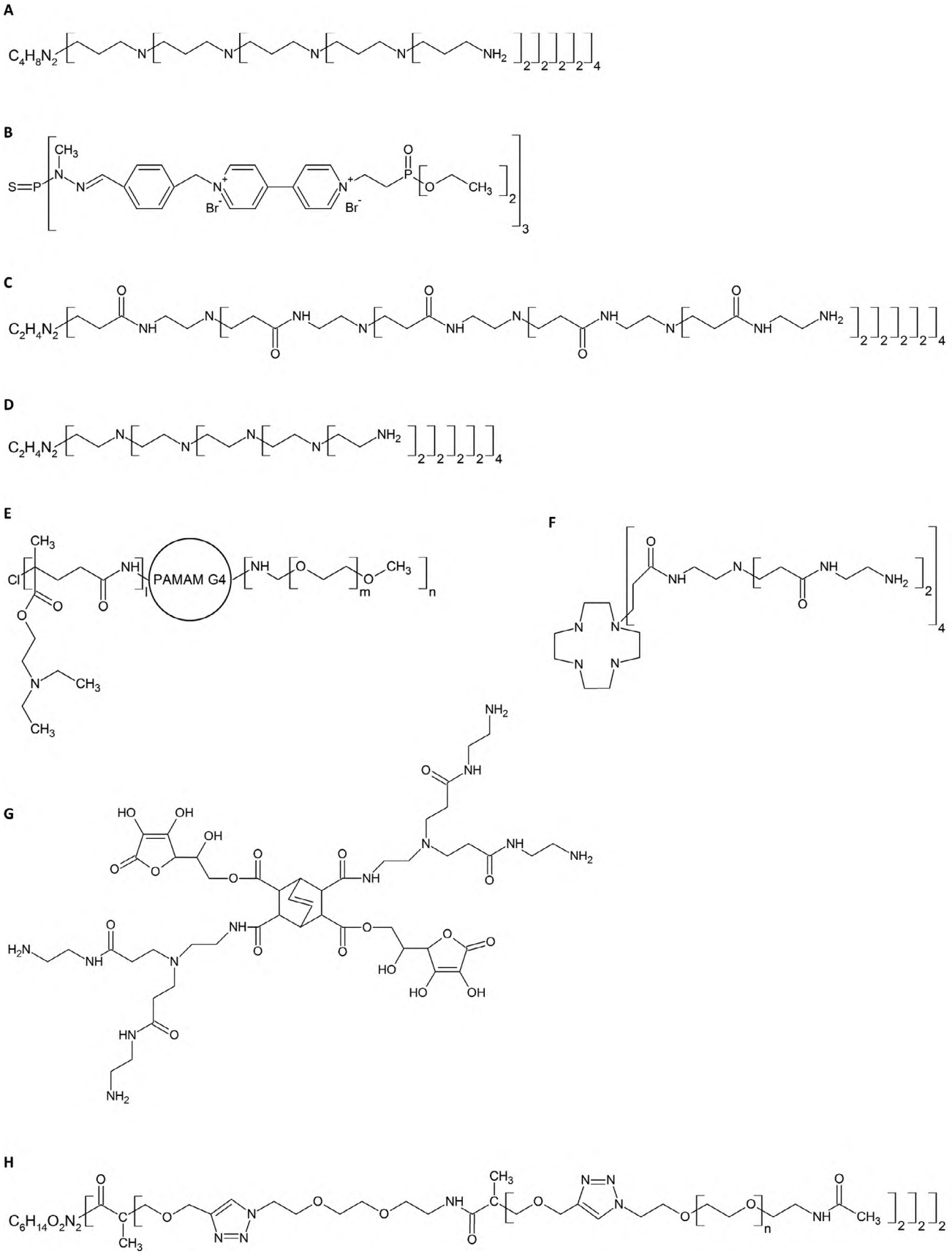

Fig. 4. Chemical structures of dendrimers: A. Poly(propylene imine) (PPI) G4 dendrimer; B. Viologen phosphorus dendrimer (VPD); C. Poly(amido amine) (PAMAM) G4 dendrimer; D. Poly(ethylene imine) (PEI) G4 dendrimer; E. Poly(2-(N,N-diethylamino)ethyl methacrylate) with methoxy-poly(ethylene glycol)-poly(amido amine) dendrimer (PPD); F. Non-classical G2 dendrimer with a cyclic core of 1,4,7,10-tetraazacyclododecane; G. Non-classical G2 dendrimer with core structure based on ascorbic acid and dicarboxylic acid; H. Oligo(ethylene glycol) (OEG)-based G2 dendrimer.

time decreasing the affinity between Mant-ATP and the interior of the polymer [61].

The evaluation of the stoichiometry and mechanism of formation of stable complexes between cationic viologen phosphorus dendrimers (VPDs) (Fig. 4B) and ATP brought similar results. Efficiency of complex formation depended on various factors such as the type of solvent, pH, environment polarity, as well as other ions or molecules participating in the reaction. Dendrimer-nucleotide complexes were stabilized mainly by non-covalent electrostatic and aromatic-aromatic interactions. NMR titration method showed that VPDs bound approximately 2 ATP molecules. Surprisingly, the interactions of VPDs with Mant-ATP showed a decrease in fluorescence in contrast to previous findings. Observed phenomenon was probably the result of interactions of the dendrimer with an aromatic ring of Mant-ATP, which is located close to the dye's chromophore [62].

Experiments with cytidine triphosphate (CTP) and unmodified, partially modified (PPI-Mal OS) and completely modified (PPI-Mal DS) with maltose poly(propylene imine) dendrimers of the 3rd and the 4th generation showed that complexes formed spontaneously by mixing CTP and dendrimers, and efficiency of this process depended on pH and ion concentration. Binding of PPI dendrimers with CTP was generation-dependent, being more efficient for higher generations of dendrimers. Interestingly, complete surface modification of dendrimers with maltose decreased their ability to bind CTP. However, partially modified PPI G4 still showed a satisfactory efficiency of complex forming. Although the partial maltose modification of dendrimers reduces the number of binding sites or binding constants, it was sufficient to cover amine groups responsible for hemolysis, without affecting the ability to bind triphosphates [63].

Reports of other research groups support the possibility of stable complex formation between dendrimers and nucleotides. Hu et al. investigated the binding efficiency of guanosine monophosphate (GMP) by poly(amido amine) (PAMAM) dendrimer (Fig. 4C) of the 5th generation. NMR spectra indicated the electrostatic interactions between amine groups of the dendrimer and phosphate groups of GMP. Those interactions significantly depended on pH, and were the strongest under moderately acidic conditions. GMP binding occurred both on the surface and in the interior of dendrimer, preventing its aggregation and thereby enhancing stability of complexes [64]. Potentiometric titrations indicated strong interactions between protonated forms of poly (ethylene imine) (PEI) dendrimers (Fig. 4D) of the 3rd generation (G3) and the anionic species of ATP, ADP and AMP over the wide range of pH (2.5–11.0). Surprisingly, studied dendrimers showed a binding preference for ADP over AMP and ATP in a pH-dependent manner - the affinity for ADP was rising above pH 5.0, while the dendrimer-ATP complexes were stable only below pH 9.0. What is more, the dendrimer was able to affect dephosphorylation of the nucleotide depending on the pH of the solution. In alkaline environment, dephosphorylation was enhanced compared to the spontaneous process, while under acidic conditions, insertion of the nucleotide phosphate chain into the dendritic scaffold protected ATP from the spontaneous degradation, thereby inhibiting the process. The presence of PEI G3 at pH 9.0 caused an approximate 6-fold enhancement of the dephosphorylation, while at pH 3.0 the spontaneous cleavage of ATP was slowed down by about 30%. Such phenomenon is in agreement with the various ATP adducts formed at the different pH values [65].

Poly(ethylene imine) dendrimers with various oligosaccharide shells were able to complex from 25 up to 100 ATP molecules. The interaction between the polymer and ATP molecules was influenced by molar mass and degree of modification of the PEI surface with oligosaccharide units. Authors assume that under certain pH conditions the complexation of sugar-modified PEI dendrimers with nucleotides is primarily mediated by electrostatic interactions. The accessibility of amine groups for ATP was hampered in case of the maltotriose functionalized PEI (PEI-Mal III), in contrast to PEI with different but not very dense maltose (PEI-Mal) and maltoheptaose (PEI-Mal VII) modification. The complexation resulted in over 3-fold increase of ATP uptake in SKOV-3 cells, in comparison to the free nucleotide [66]. Those studies contributed to creation of poly-N-isopropylacrylamide (PNIPAAm) hydrogel loaded with non-covalently linked dendrimer-ATP complexes. For this purpose, PEI-Mal dendrimers have been chosen, due to their ability to bind nucleoside triphosphates and to interact with boronic acid units of hydrogel thanks to the presence of oligosaccharide shell. PEI-Mal dendrimers exhibited the ability to bind up to 128 molecules of ATP per 1 polymer molecule, and the process of complexation was time-dependent: after 15 min more than 60% of the ATP molecules were bound with dendrimers, while after more than 3 h complexation reached 75–80%. Such dendrimer hydrogel hybrids are meant to prevent the destruction of dendrimer-drug complexes and to introduce targeting capabilities for the drug delivery by controlled, pH-dependent release of therapeutic molecules [67].

4.1.2. Complexes of dendrimers and 5-fluorouracil (5-FU)

Among the therapeutic nucleoside analogues tested for the possibility of complex formation with dendrimers, much attention is paid to 5-fluorouracil (5-FU) (Fig. 5). For over 30 years, 5-fluorouracil has proven to be one of the most effective drugs for several common malignancies. Similarly to other nucleoside analogues, 5-FU requires intracellular activation in order to exert its cytotoxic effects. 5-fluorodeoxyuridine monophosphate (5-FdUMP) binds covalently with thymidylate synthase (TS), with resultant inhibition of this enzyme and depletion of 2′-deoxithymidine triphosphate pool, thereby interfering with DNA biosynthesis and repair. Another possible mechanism of cytotoxicity involves incorporation of 5-fluorodeoxyuridine triphosphate (5-FdUTP) into DNA, leading to inhibition of DNA synthesis and function. In addition, 5-fluorouridine triphosphate (5-FUTP) is incorporated into nuclear and cytoplasmic RNA species, which disrupt standard RNA function and protein synthesis. Unfortunately, following administration, the drug is rapidly eliminated from the organism, with a half-life of approximately 8–20 min. Application of nanocarriers can improve biodistribution of 5-FU, increase its plasma concentrations and extend the time of blood circulation [68,69].

The drug is a diprotic acid with pKa 8 and 13. At pH 7.4, about 20% of 5-FU molecules is ionized [70], which enables electrostatic interactions and formation of non-covalent bonds with carrier molecules.

PAMAM dendrimers are the most frequently tested nanocarriers for 5-FU. A single molecule of PAMAM G4 contains about 250 potential bonding sites, including 64 surface primary amine groups, as well as 62 internal tertiary amine groups and 124 amide groups [71].

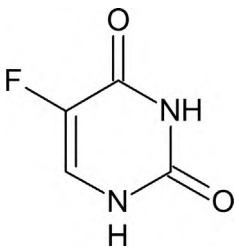

Fig. 5. The structure of 5-fluorouracil.

The research of Buczkowski et al. showed that both the generation and the type of surface terminal groups of dendrimer had impact on the binding process between 5-FU and PAMAM macromolecules. For instance, the results of spectroscopic and calorimetric measurements indicate spontaneous binding of 5-fluorouracil by both cationic PAMAM G3 and hydroxyl PAMAM-OH G3 dendrimer in aqueous solutions. PAMAM G3 dendrimer bound about 25 ± 8 drug molecules, the majority of which (24 ± 3) interacted with amide groups, while the remaining ones (5 ± 1) - with terminal amine groups. PAMAM-OH G3 dendrimer bound significantly less molecules of 5-FU (6.0 ± 1.6), mainly through tertiary amine groups [72].

The molecule of PAMAM G4 at 20°C has about 12 active sites that efficiently bind 5-FU (equilibrium constant K ≅ 5600) and about 37 active sites that interact 5-FU to a lesser extent (equilibrium constant K ≅ 150). Interestingly, NMR measurements showed that the internal tertiary amine groups of this dendrimer do not bind 5-fluorouracil. The authors speculated that the remaining 37 active sites with a lower affinity may correspond to the surface non-protonated amine and amide groups, which can interact with 5-FU through hydrogen bonds [73]. The complexation of the drug with PAMAM G4 is an exothermic process, which is followed by a favorable change in entropy. NMR measurements confirmed that the dendrimer macromolecule binds 5-FU by interaction with surface amine groups, and that PAMAM G4 has about 30 active sites binding the drug [74]. These results were consistent with previous reports - the separation method showed that about 30 molecules of fluorouracil were complexed with PAMAM G4. Interestingly, spectrophotometric measurements proved that the complete saturation of PAMAM G4 dendrimer in aqueous solution at room temperature was achieved for 90 molecules of 5-FU [71].

Exothermic and spontaneous complexation of 5-fluorouracil with PAMAM G5 dendrimer and its hydroxyl analogue PAMAM-OH G5 in aqueous solution has also been confirmed. Cationic PAMAM G5 complexed approximately three times more molecules of 5-FU (n = 100) in comparison to PAMAM-OH G5 (n = 30), which probably resulted from stronger electrostatic interaction of unmodified dendrimer's protonated surface amine groups. The interaction of surface hydroxyl groups of PAMAM-OH G5 with the drug, possibly through hydrogen bonds, was significantly weaker [75].

The possibility of clinical application of such complexes and their activity *in vitro* have been examined. PAMAM G4 were used for facilitating skin penetration by 5-FU. The studies were carried out by applying the drug together with the dendrimer or by pretreating the skin with dendrimer before the drug application. Pre-treatment with PAMAM G4 in lipophilic solvents (mineral oil and isopropyl myristate) increased the skin permeation of 5-FU. The outcome was due to altering the skin barrier by dendrimer, generally through transepidermal water loss and decrease of skin resistance, which caused the enhancement in skin permeation of the drug by 4-fold in mineral oil and 2.5-fold in isopropyl myristate. On the other hand, dendrimer applied simultaneously with the drug increased the drug's permeability by decreasing its solubility in the vehicle [70].

In order to achieve synergism in cancer cells, PAMAM G5 dendrimer stabilized silver nanoparticles (DsAgNPs) were used to encapsulate 5-fluorouracil. Studies performed *in vitro* confirmed the continuous release of 5-FU from nanocomposites. What is more, complexes were found to have synergistic antiproliferative effect, and to induce oxidative stress in A549 and MCF-7 cell lines. Examination of gene expression indicated triggering of the p53-mediated caspase cascade, leading to induction apoptosis [76].

PAMAM G5-5-FU complexes combined with antisense microRNA 21 (as-miR-21) were also tested for growth suppression of breast cancer cells, giving positive results. The codelivery of as-miR-21 significantly improved the cytotoxicity and chemosensitivity of 5-FU, increased the apoptotic percentage of the MCF-7 cells and decreased their migration ability [77].

In order to improve the 5-FU complexing properties of dendrimers, their surface has been modified with various compounds. PAMAM dendrimers modified with phospholipid showed 53% drug loading. The complexes were relatively stable for at least 1 month at room temperature and at 40°C. *In vivo* studies performed in albino rats showed that the phospholipid coated drug-dendrimer complexes were more effective than free 5-FU during oral administration. The lymphatic uptake was also increased indicating absorption of the developed delivery devices through the lymphatic pathway [78].

PAMAM dendrimers up to the 4th generation were modified with folic acid and folic acid-PEG-NHS (N-hydroxysuccinimide) conjugates. Such constructs were evaluated for anticancer 5-FU delivery potential in tumor-bearing mice. Studies indicated that approximately 31% of fluorouracil was loaded in folate-PEG-dendrimer conjugates. Such nanocarriers were biocompatible and effective in tumor targeting compared to non-PEGylated dendrimers. Modification of dendrimers via PEG-folic acid reduced their hemolytic toxicity, prolonged drug release and increased accumulation in the tumor [79].

Moreover, the PEGylation of PAMAM G4 was found to increase their drug-loading capacity, at the same time reducing the drug release rate. *In vitro* and blood-level studies in albino rats showed that PEGylated dendrimers were suitable for prolonged delivery of 5-FU, without the induction of haematological disorders [80].

A new derivative of PAMAM G4, poly(2-(N,N-diethylamino) ethyl methacrylate) (PDEA) with methoxy-poly(ethylene glycol)-poly(amido amine) (PPD) (Fig. 4E) was synthesized. PDEA is a pH-sensitive polymer containing tertiary amine groups. In weakly acidic environment, the PDEA chains of PPD were hydrophilic and extended, so the drug could easily enter or exit the nanostructure. Under neutral or weakly alkaline conditions, the PDEA chains were hydrophobic and contracted, allowing efficient encapsulation of the drug, reaching 92,5%. Release of 5-FU from constructed nanocarriers was highly pH-dependent, and it was faster under acidic conditions. Thanks to the PEGylation, the 5-FU-loaded nanocarrier exhibited prolonged half-life after intravenous administration, as well as tumor-targeting activity in mice models [81].

4.1.3. Complexes of dendrimers and gemcitabine (dFdC)

Gemcitabine (2′,2′-difluoro 2′-deoxycytidine, dFdC) (Fig. 6A) has been developed as an alternative for cytarabine (cytosine arabinoside, 1- β-D-arabinofuranosylcytosine, Ara-C) (Fig. 6B) in order to increase its activity spectrum. It has been shown that dFdC is effective against a variety of solid tumors, including pancreas cancer and neoplastic changes in breast, ovary, urinary bladder and

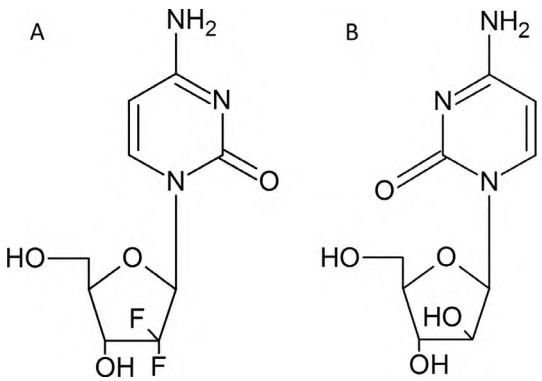

Fig. 6. The structures of nucleoside analogues: A. Gemcitabine; B. Cytarabine.

lungs. It may also have therapeutic application in haematological malignancies. Despite structural and pharmacological similarities to Ara-C, gemcitabine is characterized by unique cellular metabolism and mechanism of action. The drug is more lipophilic in comparison to cytarabine, making it a better substrate for nucleoside membrane transporters. What is more, of all known substrates for dCK, it has the greatest affinity to gemcitabine. These features result in more efficient accumulation, longer retention in tumor cells and enhanced cytotoxicity of gemcitabine triphosphate (dFdCTP). The cytotoxic activity of gemcitabine includes several mechanisms linked to DNA synthesis. Gemcitabine triphosphate competes with deoxycytidine triphosphate (dCTP) as an inhibitor of DNA polymerase. The diphosphate form (dFdCDP) inhibits ribonucleoside reductase, resulting in depletion of deoxyribonucleotide pools necessary for DNA synthesis, and thereby increasing the effects of dFdCTP and facilitating its incorporation into DNA strand during synthesis. Cell resistance to gemcitabine includes standard mechanisms observed in case of other nucleoside analogues: down-regulation of expression of NTs, target enzymes, enzymes involved in the metabolism of the drug, as well as alterations in the apoptotic pathways [36,37,40,82,83].

Palecz et al. studied the thermodynamics of complex formation of cationic PAMAM G4, neutral PAMAM-OH G4 and anionic PAMAM-COONa G3.5 with dFdC hydrochloride in aqueous solutions. ITC measurements showed that the number of complexed gemcitabine molecules is the highest for PAMAM-COONa G3.5 (44 ± 6) in comparison to PAMAM G4 (25 ± 8) and PAMAM-OH G4 (20 ± 9). The constants of drug bonding with the dendrimer macromolecules increased in the following order: PAMAM-OH G4 < PAMAM G4 < PAMAM-COONa G3.5. This outcome indicates that the electrostatic interactions, having a character of ionic pairs, are the main forces stabilizing the drug-dendrimer complexes. The studied interactions had spontaneous and exothermal character [84].

dFdC was found to be efficiently transported to lung tumor cells by mannosylated poly(propylene imine) (PPI-Man) dendrimers of the 4th generation. The drug was loaded into PPI and PPI-Man dendrimers using equilibrium dialysis method. The complexation of gemcitabine by PPI-Man was found to be almost twice as high as in case of unmodified PPI, which may be the result of increased number of functional groups that can participate in these interactions. PPI-Man-dFdC complexes showed the lowest IC50 value in A549 cell line. The pharmacokinetic and tissue distribution studies in albino rats indicated that the mean residence time and deposition of PPI-Man-dFdC complexes in lungs was significantly higher in comparison to free drug and PPI-dFdC complexes. The drug release was pH-dependent, and faster under acidic conditions [85].

Furthermore, gemcitabine and retinoic acid loaded PAMAM dendrimer-coated magnetic iron oxide nanoparticles were tested for the effects on pancreatic cancer cell lines and pancreatic stellate cells (PCSs). The loading efficiency of 10 μM gemcitabine equaled 83% in PBS buffer. This concentration was found to be the highest and most efficient for drug loading. Gemcitabine release studies were performed in acetate buffer, which imitated endosomal conditions. Release of the drug after the first 10 h reached approximately 72%. As for the *in vitro* studies, application of gemcitabine and retinoic acid complexed with magnetic nanoparticles caused the reversal of drug resistance by increasing its accumulation in cells. It was also found that gemcitabine released from the nanoparticles efficiently killed pancreatic cancer cell lines and PSC cells [86].

4.1.4. Complexes of dendrimers and anti-HIV drugs

In order to overcome the problems of short half-life, poor bioavailability and adverse side effects associated with plasma concentrations of antiviral therapeutics, dendrimers are also tested for encapsulation of nucleoside analogues used to prevent and treat acquired immunodeficiency syndrome (AIDS), caused by the human immunodeficiency virus (HIV).

Stavudine (2′,3′-didehydro-2′,3′-dideoxythymidine, d4T) (Fig. 7A), a nucleoside analogue reverse-transcriptase inhibitor, was complexed with PEGylated PAMAM G4 and G5 dendrimers using dialysis method. The studied dendrimer exhibited significantly extended drug release profile (96 h for G4 and 120 h for G5 dendrimers) in comparison to non-PEGylated PAMAM dendrimers (24 and 36 h for G4 and G5 dendrimers, respectively) [87]. Similar results were obtained for lamivudine (2′,3′-dideoxy-3′-thiacytidine, 3TC) (Fig. 7B), an antiretroviral reverse-transcriptase inhibitor for HIV/AIDS and chronic hepatitis B [88]. Lamivudine was also complexed with PPI-Man G5 dendrimers by equilibrium dialysis method. The entrapment of 3TC in PPI-Man was significantly higher than in case of unmodified PPI, which again was due to the presence of oligosaccharide shell, resulting in an increased number of functional groups available for complexation as well as steric obstacle preventing the drug dissociation. *In vitro*, PPI-Man dendrimers prolonged the release up to 144 h in comparison to unmodified polymers, which released the drug by 24 h. Furthermore, significant increase in cellular uptake of PPI-Man-3TC complexes was observed, which was 21 and 8,3 times higher than in case of a free drug and unmodified PPI after 48 h of incubation, respectively. Enhanced cellular uptake resulted in higher anti-HIV activity of lamivudine-loaded dendrimers [22].

Since only triphosphates of nucleoside analogues exhibit antiviral activity, the attempts were made to deliver the active forms of drugs into HIV-1 infected cells in order to improve efficiency of chemotherapy. For this purpose, nanocarriers of activated nucleoside reverse transcriptase inhibitors (Nano-NRTI) have been elaborated, including PAMAM-COOH G5 dendrimers modified with

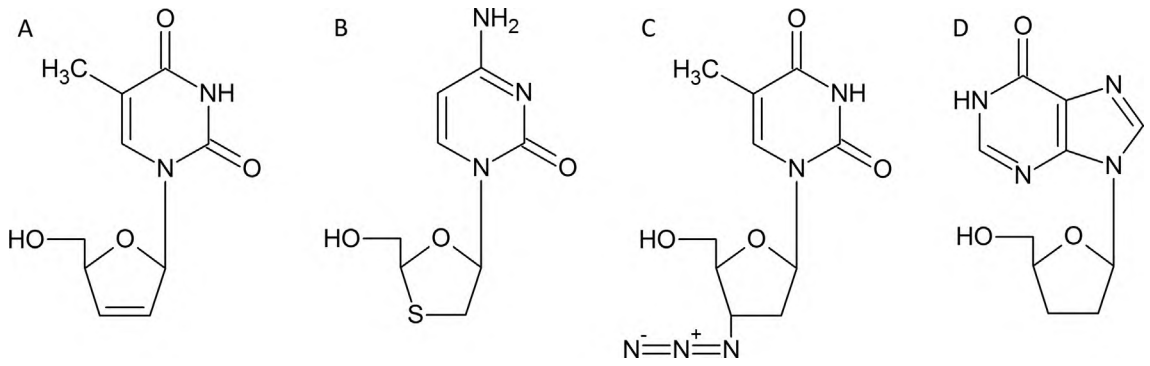

Fig. 7. The structures of anti-HIV nucleoside analogues: A. Stavudine; B. Lamivudine; C. Zidovudine; D. Didanosine.

PEG-PEI conjugates. Nano-NRTIs were prepared by mixing aqueous solutions of zidovudine (3′-azido-3′-deoxythymidine, AZT) (Fig. 7C) and didanosine (2′,3′-dideoxyinosine, ddI) (Fig. 7D) triphosphate forms with nanocarriers, followed by freeze-drying. Such complexes were then examined for intracellular accumulation, cytotoxicity, and antiviral activity in monocyte-derived macrophages (MDMs) infected with macrophage-tropic viral strain HIV-1 ADA. Triphosphate-loaded nanocarriers were efficiently captured by MDMs and showed sufficient drug release after 1 h. All Nano-NRTIs showed high efficacy of HIV-1 inhibition, without toxic impact on macrophages [89].

A summary of the studies on the use of non-covalent dendrimer-drug complexes in antiviral and anticancer therapy is given in Table 2.

4.1.5. Comments on complexation studies

Cited above, numerous studies of the mechanism and kinetics of formation of complexes between dendrimers and various types of nucleosides and nucleotides, as well as *in vitro* research indicate that dendrimers can serve as an effective nanocarriers for therapeutic nucleoside analogues without the necessity of creation of covalent chemical bonds between their particles. However, the differences in the amount of nucleosides/nucleotides complexed with dendrimers of the same type, obtained by various research groups are puzzling. This phenomenon can be explained by the sensitivity of the methods used. Some of them (e.g. ITC), being more sensitive, may detect not only the direct interactions between the molecules and dendrimer, but also the particles found in the close proximity of the polymer, giving inconsistent results. Moreover, due to the presence of phosphate residues, the nucleotide form possesses negative charge, allowing stronger interactions with the positively charged dendrimers compared to the drug in the nucleoside form. When analyzing the scientific data, in some cases the results do not meet that theory. To some extent this can be explained by different methods of complexation. For example, the equilibrium dialysis method, enabling the disposal of molecules that do not interact with the dendrimer, may provide more accurate results than direct mixing of dendrimer with the drug, which can affect the equilibrium constant of complex formation.

The nucleoside/nucleotide analogue drugs interact with dendrimers mainly due to the electrostatic forces, and therefore proper surface modifications and their density should be chosen, in order to maintain a sufficient amount of electric charges while reducing cytotoxicity of the polymer. The impact of modified dendrimers on cell cultures, e.g. the effect of the sugar moieties on the activation of the inflammatory response pathways should be studied as well. It is also unclear whether the observed increase in cytotoxic activity of the drug after addition of the dendrimer is due to improved transport or the synergistic action of the two molecules. These aspects may considerably affect the biomedical applications of dendrimers, thus it is necessary to comprehensively examine their biocompatibility, not only at the level of cytotoxicity. It should be noted that the transport efficiency of the drug is not the only characteristic that determines the application of dendrimers in medicine.

Special attention should be paid to the stability of complexes at different conditions. The drug-dendrimer interactions based on electrostatic forces strongly depend on pH and the presence of various ions, which may affect the transport efficacy *in vivo*. It is important to study the stability of complexes in the environment that simulates *in vivo* conditions, for example in culture medium.

4.2. Dendrimers-NAs conjugates

Scientific data on the use of dendrimers as carriers of covalently bound nucleoside analogues is much poorer than in the case of non-covalent complexes (Table 3). Nevertheless, thanks to the permanent chemical bonding between the drug molecule and nanocarrier, such constructs may prove to be more stable and thus more efficient than complexes based on electrostatic interactions. During the preparation of drug-dendrimer conjugates, which main task is to transport the therapeutic molecule directly to the target

Table 2
Complex formation between dendrimers and therapeutic nucleotides.

		Dendrimer	References
anticancer drugs	5-fluorouracil	PAMAM G4	[70,71,73,74]
		PAMAM G3, PAMAM-OH G3	[72]
		PAMAM G5	[76,77]
		PAMAM G5, PAMAM-OH G5	[75]
		PAMAM modified with phospholipid	[78]
		PAMAM modified with folic acid and folic acid-PEG-NHS conjugates	[79]
		PAMAM G4 modified with PEG	[80]
		PPD (PAMAM G4 modified with methoxy-PEG and PDEA)	[81]
	Gemcitabine	PAMAM G4, PAMAM-OH G4, PAMAM-COONa G3.5	[84]
		PPI G4, PPI-Man G4	[85]
		PAMAM	[86]
anti-HIV drugs	Stavudine	PAMAM G4, PAMAM G5	[87]
	Lamivudine	PAMAM G4, PAMAM G5	[88]
		PPI G5, PPI-Man G5	[22]
	Zidovudine, didanosine	PAMAM-COOH G5 modified with PEG-PEI conjugates	[89]

Table 3
Dendrimer-drug conjugates.

		Dendrimer	References
anticancer drugs	5-fluorouracil	Dendrimer with a cyclic core of 1,4,7,10-tetraazacyclododecane, G2	[90]
		Dendrimer with core structure based on ascorbic acid and dicarboxylic acid, G1 and G2	[91]
	Cytarabine	PAMAM-OH G4	[92]
	Gemcitabine	Oligo(ethylene glycol)-based dendrimer, G4	[93]
anti-HIV drugs	(−)-β-D-(2R,4R)-dioxolane-thymine	PAMAM G2, G3, G5 and G6	[96]

cells, the selection of appropriate linker is extremely important. Such linker should be subjected to specific digestion inside the cell, thereby allowing release of the drug.

4.2.1. Conjugates with therapeutic NAs

Interestingly, the first reports on dendrimer-NAs conjugates apply to non-classical polymers. In order to create a nanocarrier covalently bound with 5-fluoruracil, a new class of dendritic polymers with a cyclic core of 1,4,7,10-tetraazacyclododecane and four poly(amido amine) branches (Fig. 4F) was synthesized. Part of the surface amine groups of the dendrimers were acylated using acetic anhydride to form a linker for 5-FU. The release of fluorouracil in phosphate buffer solution (37°C, pH 7.4) was investigated. Under such conditions, conjugates underwent spontaneous hydrolysis, leading to the release of the drug over several days. The release was found to be generation-dependent [90].

Conjugates with 5-FU were also created using another non-classical dendrimers with core structure based on ascorbic acid and dicarboxylic acid (Fig. 4G). Ascorbic acid was added in order to reduce 5-FU-associated side effects and to inhibit the angiogenesis of tumor cells. Higher generation of such dendrimers were obtained through attachment of methyl acrylate and amide chains. The *in vitro* cytotoxicity was evaluated using mouse mammary carcinoma (FM3A), mouse leukemia (P338), and human histiocytic lymphoma (U937) as cancer cell lines, and mouse liver cells (AC2F) as a normal cell line. Interestingly, conjugates significantly reduced the toxicity of 5-fluorouracil against normal cell line, indicating that such constructs can decrease potential side effects. What is more, higher dendrimer generation conjugate (G2-5-FU) showed higher antitumor activity compared to G1-5-FU conjugate, probably due to the slow hydrolysis rate and the released amount of 5-FU. The *in vivo* tests on mice bearing sarcoma 180 tumor cell line showed that treatment with dendrimer-fluorouracil conjugate resulted in prolonged survival time in comparison to free drug therapy. Also, G2-5-FU conjugates exhibited outstanding antiangiogenic properties [91].

In order to overcome cell resistance mechanisms, which include deactivation by CDA, leading to the formation of a biologically inactive metabolite, spongouridine (1-β-D-arabinofuranosyluracil, Ara-U), as well as low lipophilicity and rapid clearance from the organism, a conjugate of PAMAM-OH G4 dendrimer and Ara-C was elaborated. Ara-C is mainly used in the treatment of acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL) and in numerous lymphomas. Ara-C cytotoxicity is believed to be the result of DNA polymerase inhibition or incorporation of cytarabine triphosphate (Ara-CTP) into DNA [36,37]. The covalent bond was created between the primary hydroxyl group of cytarabine and dendrimer. It was shown that conjugates continuously released the drug over 14 days in PBS, and that the release was faster in human plasma. Furthermore, thanks to the covalent bonding between PAMAM-OH G4 and Ara-C, the deamination of Ara-C to Ara-U was significantly delayed. Conjugates showed four times higher cytotoxic activity against A549 cell line compared to free Ara-C after 72 h of treatment [92].

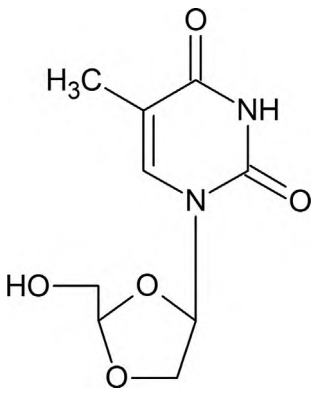

Fig. 8. The structure of (−)-B-D-(2R,4R)-dioxolane-thymine (DOT).

Oligo(ethylene glycol) (OEG)-based G4 dendrimers (Fig. 4H) were constructed and bound covalently with gemcitabine. *In vitro* and *in vivo* studies showed that both OEG-dFdC and PAMAM-dFdC conjugates had similar cytotoxicity in comparison to the free drug. However, the OEG-dFdC conjugates with the longest peripheral PEG tails exhibited extended circulation time and more efficient tumor accumulation, thereby having significantly higher antitumor activity than the gemcitabine-conjugated PAMAM dendrimers [93].

PAMAM G2, G3, G5 and G6 dendrimers were conjugated with (−)-β-D-(2R,4R)-dioxolane-thymine (DOT) (Fig. 8) via ester or phosphate linkers for the application in anti-HIV therapy. DOT exhibited modestly potent antiviral activity against most of the HIV-1 mutants resistant to commonly use nucleoside analogues [94,95], probably due to low efficiency of intracellular transport, poor initial phosphorylation by kinase or fast degradation of the triphosphate form. Therefore, an attempt to connect the drug to the dendrimer in order to improve the drug delivery to target cells and increase the potency of DOT was made. Synthesized conjugates were evaluated for anti-HIV activity against HIV-1 LAI viral strain in primary human peripheral blood mononuclear cells (PBMCs), as well as for cytotoxicity in uninfected PBMCs, CEM (T-lymphoblastoid cell line) and Vero (African green monkey kidney cell line). PAMAM-DOT conjugates exhibited a greater antiviral activity in comparison to free DOT. Unfortunately, as might have been expected, PAMAM dendrimers also exhibited increased cytotoxicity against normal cells [96].

4.2.2. Alternative use of nucleotides/nucleotides-dendrimer conjugates

Unlike nanocarriers that are intended to release their drug cargo at the site of action, some PAMAM-nucleosides conjugates were designed to act as the cell surface G protein-coupled receptors' (GPCRs) agonists, without drug release. Such constructs were elaborated to improve the activity and stability of ligand in biological systems, and to enhance its pharmacokinetic and pharmacodynamic properties compared to the monomeric ligands. The research involved both adenosine receptors (ARs) and purinergic P2Y receptors that are expressed at various levels and in diverse combinations in nearly every tissue [97–100].

In order to synthesize polymer-bound adenine nucleotides with high coenzymatic activities, adenine nucleotides (ATP and ADP) were covalently bound with PAMAM dendrimers. Conjugates were obtained either by binding adenine nucleotides directly with PAMAM-COOH dendrimer, or by modifying the nucleotides by introduction of a spacer arm containing carboxylic terminal group. Both types of conjugates were prepared using carbodiimide activation technique. The amount of adenine nucleotides bound to the dendrimer was found to be pH- and temperature-dependent. Nucleotides conjugated with the nanocarrier were coenzymically active against acetate kinase, glucokinase and hexokinase, with high degree of stability [101].

5. Conclusion

Scientific data on the use of dendrimers as drug delivery devices show that these macromolecules, usually after surface modification, can serve as effective nanocarriers of nucleoside analogues both in complexed and conjugated form. Results suggest that such transport system hold potential to increase the efficiency and

reduce the side effects of antiviral and anticancer therapy. Further *in vivo* studies are required to investigate mechanisms of cell death induction and the impact of dendrimer-NAs complexes and conjugates on living organisms.

Acknowledgments

This work was supported by National Science Centre, Poland (project UMO-2014/13/B/NZ3/04643 "Cellular and molecular mechanism of action of PPI dendrimer complexes with nucleoside analogue anticancer drugs").

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International Journal of Pharmaceutics 544 (2018) 83–90

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Poly(propyleneimine) glycodendrimers non-covalently bind ATP in a pH- and salt-dependent manner – model studies for adenosine analogue drug delivery

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ARTICLE INFO

Keywords:
Poly(propyleneimine) (PPI) dendrimers
Glycodendrimers
Nucleoside analogues (NAs)
Adenosine-5′-triphosphate (ATP)
Complexation

ABSTRACT

Adenosine analogue drugs (such as fludarabine or cladribine) require transporter-mediated uptake into cells and subsequent phosphorylation for anticancer activity. Therefore, application of nanocarrier systems for direct delivery of active triphosphate forms has been proposed. Here, we applied isothermal titration calorimetry and zeta potential titration to determine the stoichiometry and thermodynamic parameters of interactions between 4th generation poly(propyleneimine) dendrimers (unmodified or sugar-modified for increased biocompatibility) and ATP as a model adenosine nucleotide. We showed that glycodendrimers have the ability to efficiently interact with nucleoside triphosphates and to form stable complexes via electrostatic interactions between the ionized phosphate and amino groups on the nucleotide and the dendrimer, respectively. The complexation process is spontaneous, enthalpy-driven and depends on buffer composition (strongest interactions in organic buffer) and pH (more binding sites in acidic pH). These properties allow us to consider maltose-modified dendrimers as especially promising carriers for adenosine analogues.

1. Introduction

One of the promising approaches enabling the improvement of conventional anticancer chemotherapy involves the application of carrier systems which are meant to eliminate the adverse side effects and provide targeted transport of the drug directly to the cancer cells. In order to achieve this goal, the drug delivery agents must be able to hold an adequate amount of the therapeutic substance and to overcome obstacles like drug resistance, disadvantageous biodistribution and rapid clearance from the bloodstream. The drug delivery systems (DDS) should be characterized by prolonged blood half-life, specific tumor accumulation, efficient intracellular transport and controlled drug release (Kakde et al., 2011). Many of these requirements are met by various nanoparticles (De Jong and Borm, 2008), which have been additionally reported to protect the drugs from degradation, improve their solubility and modulate pharmacokinetic properties, allowing to overcome several limitations of standard anticancer therapy (Emeje et al., 2012).

Nucleoside analogue drugs are very promising antimetabolites with wide range of applications in the therapy of various types of leukaemias and lymphomas. Adenosine analogues such as fludarabine, cladribine and clofarabine have complex pharmacokinetics: in order to exert cytotoxic activity, they require conversion to triphosphate active forms by intracellular kinases. However, neither nucleoside nor nucleotide forms can passively penetrate the cell membrane, instead relying upon the transport of nucleoside prodrugs by specialized nucleoside transporters (Galmarini et al., 2002). This leads to delayed therapeutic effects and emergence of drug resistance due to downregulation of transporter expression or decreased activity of intracellular kinases (Jordheim and Dumontet, 2007; Zhang et al., 2007). These obstacles could be overcome by direct delivery of active, triphosphate forms of adenosine analogues to cancer cells by nanocarrier DDS.

Recent studies have confirmed that perfectly branched dendrimers are superior to other nanoparticles in case of drug delivery. Modern methods of synthesis of these polymers ensure their well-defined structure, monodispersity, multivalency and biocompatibility (Klajnert

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https://doi.org/10.1016/j.ijpharm.2018.03.063
Received 12 February 2018; Received in revised form 30 March 2018; Accepted 31 March 2018
Available online 10 April 2018
0378-5173/

and Bryszewska, 2001; Tomalia et al., 1985). Dendrimers provide high loading capacity, as well as improved solubility and biodistribution of the drugs (Kojima et al., 2000). Their nanometric size and globular shape favour cell entry and extend blood circulation time. While cationic, amino group-containing poly(propyleneimine) (PPI) dendrimers form stable non-covalent complexes with anionic drugs, including nucleotides (Gorzkiewicz and Klajnert-Maculewicz, 2017), their inherent toxicity precludes their direct clinical application. On the other hand, sugar modification of these dendrimers was demonstrated to greatly increase their biocompatibility and bioavailability (Klajnert et al., 2008; Janaszewska et al., 2012; Ziemba et al., 2012). Since adenosine analogues are most often used as antileukaemics, the use of glycodendrimers is especially justified: leukaemic cells usually overexpress surface lectin receptors which bind carbohydrate ligands with high affinity, enabling receptor-mediated endocytosis. Sugar-modified dendrimers have the potential of active interaction with cancer cells, allowing to decrease the detrimental effects on healthy tissues (Jain et al., 2012; Mangold and Cloninger, 2006; Yau et al., 2015).

Drug-dendrimer interactions are mainly driven by electrostatic and van der Waals forces, so the stability of such complexes is largely dependent on the environmental conditions, ionic strength and pH. This may considerably reduce the capacity of dendrimers to function as DDS *in vivo*. Moreover, the character and density of surface modifications may affect the formation of complexes and decrease the number of bound drug molecules.

Therefore, in order to support the rationale for their use in innovative antileukaemic therapy, it is vital to thoroughly investigate the formation of complexes between glycodendrimers and nucleotides, as well as their stability in different environmental conditions. We have previously confirmed that sugar-modified poly(propyleneimine) dendrimers can form stable complexes with pyrimidine nucleotides (Szulc et al., 2015) and can deliver their anticancer analogues to cancer cells *in vitro* (Szulc et al., 2016). Since drug-dendrimer interactions strongly depend on their chemical structure, we have now decided to analyse the biophysics of complex formation for ATP as model for active form of clinically used adenosine analogues (e.g. fludarabine or clofarabine). We applied isothermal titration calorimetry (ITC) and zeta potential titration for the characterization of dendrimer-triphosphate complexation stoichiometry, kinetics and thermodynamics. For this purpose, we used poly(propyleneimine) dendrimer of 4th generation (PPI G4) and its glycodendrimer variants partially modified with maltose (PPI-Mal OS G4) and maltotriose (PPI-Mal III OS G4) in order to evaluate the impact of the type and degree of surface modification with sugar moieties on the capacity of complex formation and stability. Moreover, we verified the impact of environmental conditions (pH and buffer composition) on these parameters. Our results show that ATP binds efficiently to glycodendrimers with stoichiometry and thermodynamic parameters consistent with surface electrostatic interactions. Lower pH and HEPES buffer increase binding capacity and complex stability, respectively.

2. Materials and methods

2.1. Materials

Poly(propyleneimine) dendrimer of the 5th generation (MW 7167.97 g/mol) with 64 terminal amino groups (Fig. 1) was obtained from SyMO-Chem (Eindhoven, The Netherlands) and specified as 4th generation (PPI G4) following the uniform nomenclature description of polyamine dendrimers for poly(propyleneimine) and poly(amidoamine) dendrimers (Tomalia and Rookmaker, 2009).

PPI dendrimers of the 4th generation with primary surface amino groups partially modified with maltose (PPI-Mal OS G4, MW 21113 g/mol) or maltotriose (PPI-Mal III OS G4, MW 29145 g/mol) (open shell glycodendrimers, Fig. 1) were prepared as described previously, and their molecular weights were determined by an established ^{1}H NMR approach (Fischer et al., 2010). Adenosine and adenosine-5′-triphosphate (ATP) were obtained from Sigma Aldrich. Different buffers were used to study the effect of environment on dendrimer-ATP complex formation: phosphate buffer (10 mM, pH 7.4 or 5.8), HEPES buffer (10 mM, pH 7.4 or 5.8) and double-distilled water.

2.2. Isothermal titration calorimetry (ITC)

The thermal effects of interactions between PPI dendrimers and adenosine or ATP were determined using a VP-ITC calorimeter (MicroCal, 2004) for titration under isothermal conditions.

Three independent measurements were carried out for each dendrimer:

- the 5 μM aqueous solution of the dendrimer in the measurement chamber was titrated with 5 mM aqueous solution of ATP sodium salt from the syringe,
- the 8 μM HEPES buffer (pH 7.4) solution of the dendrimer in the measurement chamber was titrated with 8 mM solution of ATP sodium salt in HEPES buffer (pH 7.4) from the syringe,
- the 8 μM phosphate buffer (pH 7.4) solution of the dendrimer in the measurement chamber was titrated with 8 mM solution of ATP sodium salt in phosphate buffer (pH 7.4) from the syringe,
- the 8 μM phosphate buffer (pH 5.8) solution of the dendrimer in the measurement chamber was titrated with 8 mM solution of ATP sodium salt in phosphate buffer (pH 5.8) from the syringe.

The higher concentrations of the dendrimers and ATP enabled the precise measurements of lower thermal effects of mixing the phosphate buffer solutions compared to aqueous solutions.

The titration using adenosine as a ligand was performed only for the unmodified PPI G4 dendrimer in aqueous solution.

For background correction, water/HEPES/phosphate buffer (in the cell) were titrated with adenosine/ATP in water/HEPES/phosphate buffer (in the syringe) at the same concentrations, and the background was subtracted from the final curves. The thermal effect of diluting the dendrimer solution under the titration conditions was neglected.

For all measurements, the titrant portions were added to 1.4275 mL of the dendrimer solution via 287.37 μL syringe. The first portion of titrant was added in a volume of 4 μL, and the next 22 portions were added in volumes of 8 μL each at 600 s intervals. During the titration, the reaction mixture was kept at a constant temperature of 25 °C and stirred at a constant speed of 416 rpm.

It has been observed that the attachment of the ATP molecules occurs immediately after the addition of the first portion to the dendrimer solution. In subsequent titrations, additional nucleotide molecules were attached to the dendrimer, as evidenced by lower peaks on the thermogram. The absence of the thermal effect in the final titration step was considered to be the last stage when the ligand binding sites in the dendrimer molecule were completely saturated. Analyzing the course of thermal effects Q of direct interactions of dendrimers with the ligand molecules as a function of the macromolecule concentration M_t and its ligand X_t in the cell with a working volume of V_0, the model of a single type of bonding centers in the macromolecule was used to calculate the parameters of binding: the number of bonding centers in the macromolecule n and equilibrium constant K, enthalpy ΔH and entropy ΔS of ligand binding to the dendrimer. The Origin MicroCal program dedicated to the calorimeter calculates the parameters of binding by the method of non-linear multi-parameter regression, using the equation for ITC (MicroCal, 2004):

$$Q = \frac{nM_t\Delta HV_0}{2}\left[1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} - \sqrt{\left(1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t}\right)^2 - \frac{4X_t}{nM_t}}\right]$$

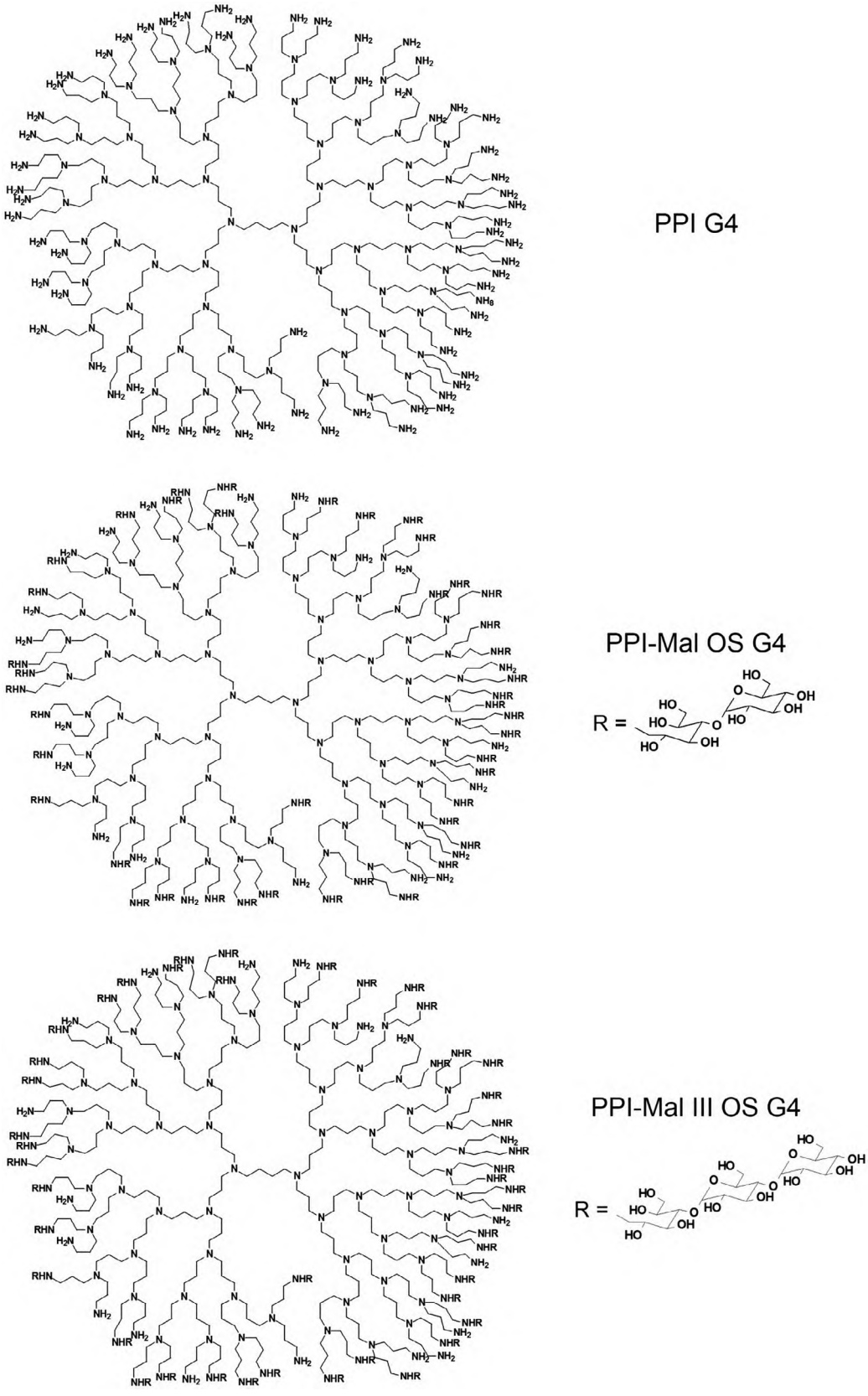

Fig. 1. Chemical structure of the dendrimers under evaluation. PPI-Mal OS G4 has 43 Mal units chemically coupled to 4th generation PPI scaffold, while PPI-Mal III OS G4 has 45 Mal III units. Mal = maltose and Mal III = maltotriose.

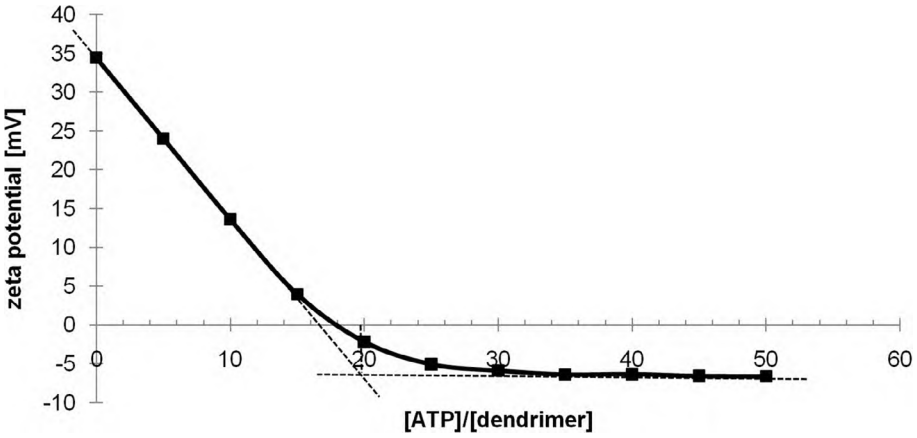

Fig. 2. Stoichiometry determination for ATP-PPI G4 complex in aqueous solution. Initial linear dependence of dendrimer zeta potential on ATP/dendrimer mixture stoichiometry was extrapolated to the intersection with eventual zeta potential value of fully saturated dendrimer and binding stoichiometry was calculated from the intersection point.

2.3. Zeta potential measurements

Measurements of zeta potential were performed with the use of Zetasizer Nano ZS (Malvern Instruments Ltd., UK). All samples were placed in the folded capillary cell (DTS 1070, Malvern) and measured at 25 °C. The data were analyzed using the Malvern software.

Water and HEPES (pH 7.4 or 5.8) solutions containing studied dendrimers in a constant concentration (10 μM) were prepared, and their zeta potential has been measured. The dendrimer solutions were subsequently titrated with ATP solution at concentration increasing in 50 μM steps. The dendrimer-ATP solutions were incubated at room temperature for 5 min and the zeta potential was measured. The experiments were conducted at the final concentrations of ATP ranging from 0 to 500 μM.

The analysis of the binding curves for all studied systems enabled the evaluation of the stoichiometry of complexes. Fig. 2 shows an example of the stoichiometry determination for ATP-PPI G4 complex in aqueous solution.

3. Results

Since our experimental goal was to evaluate the applicability of glycodendrimers as DDS for adenosine analogues, we tested binding of ATP to three different dendrimers: unmodified, maltose- and maltotriose-modified PPI. Dendrimer concentration ranged between 5 and 10 μM (exact concentration was dictated by technical considerations), and all experiments were performed at 25 °C as this is the temperature at which the complex will most probably be formed in a pharmaceutical setting. We tested the kinetics and thermodynamics of ATP-dendrimer binding in three different buffer compositions: unbuffered water, inorganic buffer (phosphate) and organic buffer (HEPES). In some experiments, we tested the impact of decreasing buffer pH from physiological (7.4) to slightly acidic, corresponding to acidified intracellular compartments after complex endocytosis.

3.1. Isothermal titration calorimetry

Using the isothermal titration calorimetry technique, we determined the thermal effects of titration of 8 μM solutions of PPI G4, PPI-Mal OS G4 and PPI-Mal III OS G4 dendrimers with 8 mM solution of ATP sodium salt in 10 mM HEPES buffer, pH 7.4 (Fig. 3). The parameters of binding ATP molecules to bonding centers of PPI dendrimers (the number of bonding centers in the macromolecule n, equilibrium constant K, enthalpy ΔH, entropy ΔS and Gibbs free energy ΔG) are listed in Table 1.

The results obtained from these experiments show that the process of binding of ATP molecules by the PPI dendrimers has a spontaneous character ($K > 1$, $\Delta G < 0$) and is accompanied by an advantageous

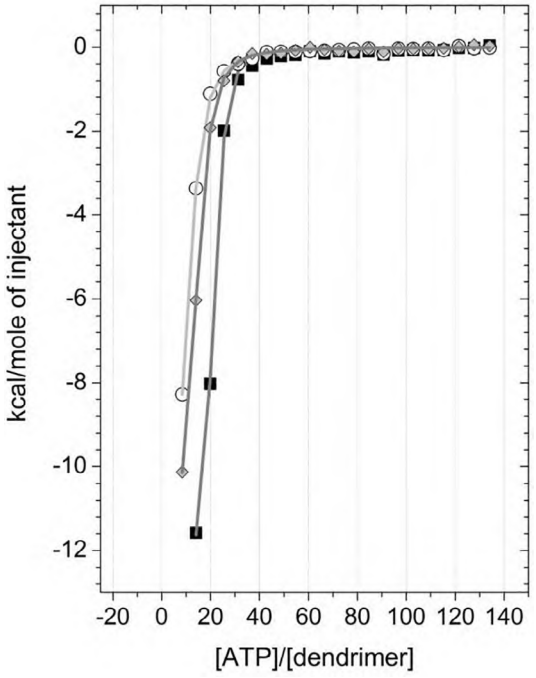

Fig. 3. Titration curves for ITC experiments performed in HEPES buffer pH 7.4 at room temperature (25 °C): thermal effects for the calorimetric titration of 8 μM solutions of PPI G4 (■), PPI-Mal OS G4 (○) and PPI-Mal III OS G4 (♦) dendrimers with 8 mM solution of ATP sodium salt demonstrate rapid and strong binding.

change in enthalpy ($\Delta H < 0$). The exothermic values of enthalpy suggest the advantage of the direct effects of ATP-dendrimer interactions over the effects of their partial dehydration. The high values of the equilibrium constant ($K > 10^3$) indicate that such complexes are relatively stable (Buczkowski et al., 2012; Jones and Atkins, 2000). The negative values of entropy show that the saturation of the dendrimer's active sites with the ATP molecules is accompanied by an increase in the degree of order in the thermodynamic system. This phenomenon may also indicate the surface mechanism of the ligand binding. Unmodified PPI G4 dendrimer binds more ATP molecules in comparison to maltose- and maltotriose-modified derivatives. Moreover, glycodendrimers bind ATP with a lower equilibrium constant compared to the unmodified dendrimer.

We repeated the same experiments in unbuffered aqueous solution (using lower dendrimer and ATP concentration due to stronger thermal effects), obtaining analogous results (Table 1). We observed significantly lower equilibrium constants and lower values of entropy

Table 1
Thermodynamic parameters of interactions between ATP and dendrimers.

Dendrimer	n	K	ΔH [cal/mol]	ΔS [cal/K * mol]	ΔG [cal/mol]
HEPES buffer pH 7.4					
PPI G4	17 ± 2	273000 ± 54000	−12700 ± 200	−17.7 ± 1.1	−7416 ± 117
PPI-Mal OS G4	9 ± 2	94400 ± 14000	−14100 ± 600	−24.5 ± 2.3	−6787 ± 88
PPI-Mal III OS G4	12 ± 3	129000 ± 19000	−12000 ± 200	−16.9 ± 1.0	−6972 ± 87
Water					
PPI G4	21 ± 2	42000 ± 10000	−26800 ± 6800	−69 ± 23	−6310 ± 140
PPI-Mal OS G4	8 ± 2	22300 ± 9900	−25900 ± 9100	−67 ± 31	−5930 ± 260
PPI-Mal III OS G4	9 ± 3	25000 ± 10000	−17000 ± 9200	−37 ± 32	−6000 ± 240
Phosphate buffer pH 7.4					
PPI G4	21	3340 ± 440	−15000 ± 4800	−34 ± 16	−4807 ± 78
PPI-Mal OS G4	8	2000 ± 380	−35000 ± 4900	−102 ± 17	−4500 ± 110
PPI-Mal III OS G4	9	2830 ± 300	−19200 ± 4000	−49 ± 14	−4709 ± 63
Phosphate buffer pH 5.8					
PPI G4	32 ± 1	20600 ± 2800	−6090 ± 860	−0.7 ± 3.2	−5890 ± 81
PPI-Mal OS G4	25	2910 ± 980	−12200 ± 1600	−25.1 ± 6.0	−4730 ± 200
PPI-Mal III OS G4	25	3780 ± 840	−10800 ± 2000	−19.9 ± 7.1	−4880 ± 130

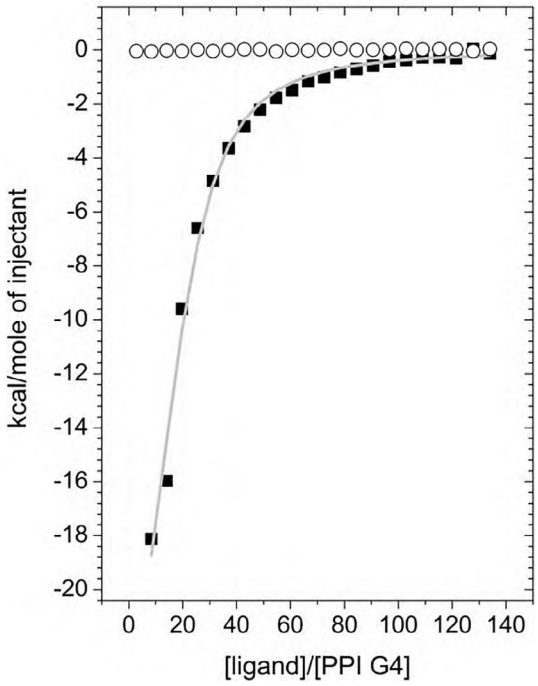

Fig. 4. Titration curves for ITC experiments performed in aqueous solutions at room temperature (25 °C): thermal effects for the calorimetric titration of 5 μM solution of PPI G4 dendrimer with 5 mM solution of adenosine (○) and ATP sodium salt (■), demonstrating a requirement for the phosphate residues for binding to dendrimer.

change for all dendrimers in comparison to HEPES buffer, while stoichiometry remained largely unchanged. In order to verify the postulate that higher stoichiometry of ATP binding to unmodified PPI dendrimer stems from more non-covalent electrostatic binding sites for phosphate anion moieties in the nucleotide, we compared ATP binding to the nucleoside form (adenosine – Fig. 4) in aqueous solution. As expected, there were no thermal effects of adenosine addition, indicating a lack of complex formation.

Analogous titrations of 8 μM dendrimer solutions with 8 mM ATP sodium salt solution were performed in 10 mM phosphate buffer. Thermodynamic parameters (Table 1) indicate much weaker interactions during complex formation than both in HEPES buffer and in water. This is true both for thermodynamic affinity (measured by equilibrium constant) and system order (measured by entropy change). Due to this low affinity, we had to approximate equivalent stoichiometry to previous conditions rather than derive it directly from non-linear regression; however, resulting curve fitting validated this procedure and good fits were obtained for thermodynamic parameter calculation.

Because of the weaker interactions, phosphate buffer was selected to test the effect of pH on thermodynamics of complex formation. Surprisingly, equilibrium constant values were found to be slightly increased at acidic pH (Table 1), while ΔS increased, in the case of unmodified dendrimer practically to zero. Stoichiometry calculation (or approximation for glycodendrimers where low affinity again precluded direct derivation) showed that the increase in affinity in comparison to neutral pH is accompanied by an increase in the number of available binding sites.

3.2. Zeta potential titration

Zeta potential strongly depends on the electrolyte concentration and ionic strength (Grządka and Chibowski, 2009; Hunter, 1981), therefore we compared the dependence of dendrimer zeta potential on ATP concentration in three different environments: HEPES buffer, pH 7.4 (physiological conditions), HEPES buffer, pH 5.8 (acidic compartment) and unbuffered water as control. Titration curves (Fig. 5) at pH 7.4 showed surface potential change due to nucleotide complexation for all dendrimers even at lowest ATP concentration tested (50 μM), while at pH 5.8 the curves were sigmoidal in shape, showing a detectable complexation effect at higher ATP concentrations. The curves were also used for determination of binding stoichiometry (Table 2), again attesting to a significant increase in the number of available binding sites at acidic pH. We have confirmed again the larger number of ATP binding sites on the surface of unmodified PPI dendrimer (with regard to glycodendrimers) at both pH values, with no difference due to the type of sugar used for surface modification. Dendrimer zeta potential in water showed both similar values and similar dependence on ATP concentration as in neutral HEPES buffer (data not shown), attesting to virtually identical binding stoichiometry. While all dendrimers have positive surface potential values (indicating positive surface charge at both pH values), glycodendrimers showed consistently lower values than the unmodified dendrimer. Upon saturated complexation with ATP, zeta potential decreases to a slightly negative value of ca. −8 mV, common to all dendrimers and pH conditions.

4. Discussion

Glycodendrimers with open sugar shells have been proposed as drug

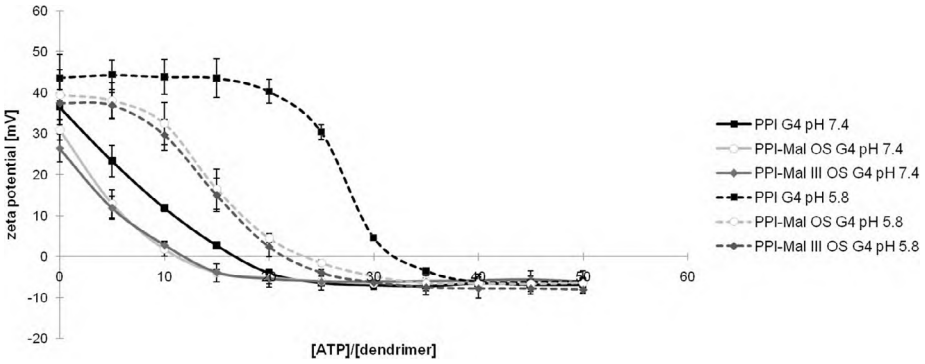

Fig. 5. Zeta potential titration curves for the experiments performed in HEPES buffer at room temperature (25 °C): effects for the calorimetric titration of 10 μM solutions of PPI G4, PPI-Mal OS G4 and PPI-Mal III OS G4 dendrimers with ATP sodium salt at pH 7.4 and 5.8 demonstrate strong dependence of binding stoichiometry and affinity on pH.

Table 2
The numbers of ATP molecules per one dendrimer macromolecule determined with zeta potential titration.

	water	HEPES pH 7.4	HEPES pH 5.8
PPI G4	20	19	32
PPI-Mal OS G4	10	10	23
PPI-Mal III OS G4	11	11	23

transporters for the targeted delivery of antileukaemic nucleoside analogues due to their reduced cytotoxicity and potential affinity for cancer cells (Szulc et al., 2016). Some types of dendrimers have been previously studied for interactions with 5-fluorouracil (Buczkowski et al., 2012, 2016) and gemcitabine (Soni et al., 2015), but it is the direct delivery of active, triphosphate forms of anticancer nucleoside analogues that these nanoparticles may be most helpful in, providing additional benefits like overcoming cellular resistance associated with decreased expression of nucleoside transporters or insufficient intracellular phosphorylation (Szulc et al., 2016). Adenosine analogues, increasingly used as frontline drugs in haematological malignancies, are excellent candidates for proof-of-concept studies with glycodendrimers. However, careful analysis of the formation and stability of complexes of dendrimers with model nucleotides is crucial to evaluate perspectives and chances of development of novel DDS.

For the present studies, we chose poly(propyleneimine) dendrimer of the 4th generation and its variants with surfaces partially modified with maltose or maltotriose and present the first results on complex formation with a model purine nucleotide (ATP). PPI G4 dendrimer possesses 64 terminal amino groups, the ionization of which strongly depends on pH and ionic strength of the environment. The 4th generation is also characterized by the semi-open structure, facilitating ligand bonding. Dendrimers of lower generations are usually more asymmetric, open and flexible compared to the higher generation macromolecules. Above the 4th generation, dendrimers adopt globular structure, in which the density increases regularly from the core region towards the surface. When the critical branched state is reached, the surface chemical groups form a closed, compact surface layer (Gorzkiewicz and Klajnert-Maculewicz, 2017). PPI G4 combines the advantages of both types of dendrimers, being characterized by moderately rigid structure with internal cavities and charged terminal groups available for the guest molecules. Moreover, the surface modification with sugar moieties may additionally increase the interaction area and stiffen the structure of the macromolecule, improving the stability of drug-dendrimer complexes. Therefore, the appropriate balance between the structure of the PPI G4 dendrimer and the degree of surface sugar modification creates perfect basis for the stable drug delivery system based on non-covalent complexes.

Comparison of the thermal effects of titration with adenosine and ATP solutions allowed to conclude that negatively charged phosphate residues of nucleotides are responsible for the interactions with cationic PPI dendrimers, most likely through the formation of ionic pairs with terminal amino moieties. The idea was further supported by the contrast between complexation efficacy of unmodified and sugar-modified PPI macromolecules with different degree of surface positive charge. Similar observations have been made for the complexes of ATP and hyperbranched PEI with various sugar shells (Appelhans et al., 2009). Moreover, the studies on the complex formation between the PPI dendrimers and 2′-/3′-O-(N′-methylanthraniloyl)-ATP (Mant-ATP) indicated the predominance of surface interactions over the encapsulation inside the dendritic scaffold (Szulc et al., 2013). Therefore, we used the model of a single type of bonding centers to describe the thermodynamic parameters of the investigated processes.

Glycodendrimers, like unmodified PPI dendrimers, bind ATP spontaneously, in an enthalpy-driven process where a low (strongly negative) enthalpy change compensates for increase in the thermodynamic order ($\Delta S < 0$), with the latter probably due to local dehydration and water structure formation. Maltose- and maltotriose-modified dendrimers bound fewer nucleotide molecules with lower equilibrium constant in comparison to unmodified PPI G4. This is an expected result of the reduction of surface positive charge of the dendrimer through the attachment of sugar residues, which limits the cytotoxicity of the dendrimer, but also prevents a fraction of terminal amino groups from interacting with ATP molecules. Similar conclusion has been drawn by Höbel et al. (2011) during the studies on interactions between glycosylated hyperbranched PEI and siRNA, where the binding capacity was significantly reduced by sugar moieties. Nevertheless, PPI G4 dendrimers with open sugar shells showed satisfactory binding capacity, which together with their reduced cytotoxic activity makes them particularly fine candidates for DDS.

Maltose-modified glycodendrimers consistently show lower enthalpy change (tighter binding due to more advantageous energetic effects) than maltotriose-modified or unmodified ones. However, they also demonstrate lowest entropy change values (weaker binding due to ordering effects), leading to slightly higher Gibbs energy change values (lower complex stability). It is interesting to note that the difference in Gibbs energy change between acidic and neutral pH is largest for maltose glycodendrimers, which makes them potentially good candidates for adenosine analogue DDS, protecting the nucleotide from hydrolysis by tighter binding in acidic compartments (e.g. lysosomes after endocytosis) and releasing it more easily in the cytoplasm, its site of action. The entropic effects probably contribute to this since higher ordering may indicate steric hindrance.

Interestingly, the defined numbers of ATP molecules attached to the studied PPI and PPI-Mal macromolecules in aqueous environment are lower than those for the analogous studies performed with the use of CTP (21 and 8 ATP molecules versus 32 and 15 CTP molecules, respectively) (Szulc et al., 2015). This discrepancy may be due to divergent concentrations of compounds and measurement parameters (since ITC is a highly precise and responsive method), but also with the structure of purine and pyrimidine rings. The imidazole ring of purines increases their molecular weight and constitutes an additional steric hindrance, preventing the attachment of more ligand molecules. It has

been often shown that increasing size of drug molecules may constitute additional limitation for complexation process (Tripp et al., 2014).

Our experiments (both ITC and zeta potential measurements) confirmed previous reports that complexation of PPI dendrimers with nucleotides is pH-dependent (Szulc et al., 2013). Acidic environment enhances electrostatic interactions and triggers changes in the conformation of dendrimer, thus facilitating dendrimer-ATP binding. Reduction of pH results in the increase of the number of active sites in PPI dendrimers, which is most likely associated with the protonation of surface amino groups acting as the ATP binding centers. This observation can be also explained by the change of conformation of the dendrimer, known as the "backfolding effect" (Boas et al., 2006; Maiti et al., 2005). At neutral pH, the hydrogen bonds between positively charged surface amino groups and uncharged amino groups inside the dendrimer may occur. As a result, terminal residues turn to the interior of the dendritic scaffold, causing further reduction of cationic surface charge and limiting the ATP binding capacity. With the decrease of pH, there is a repulsion between the positively charged surface moieties and inner amino groups of the macromolecule. As a consequence, PPI dendrimers adopt more open structure and expose multiple surface amino groups to ATP molecules in solution. Interestingly, theoretical calculations suggest that the backfolding process may also occur for maltose units of PPI glycodendrimers (McCarthy et al., 2013). PPI-Mal III OS G4 may be characterized by lower backfolding properties due to the size of the sugar residues, which may partially explain higher enthalpy and entropy values of the complexation process for this dendrimer.

When testing the effect of buffer environment on ATP complexation, we have shown that the complex is most stable in HEPES buffer (an organic buffer which is a good approximation of natural buffering properties of biological fluids) and least stable in phosphate buffer. Interestingly, complex stability in water is intermediate, although the values of thermodynamic parameters are closer to those seen in HEPES buffer. Within this comparison, we have a confirmation of prevailing influence of energetic effects (enthalpy) over ordering effects (entropy). These results are promising from the point of view of clinical application as they show that there is no simple decrease of ligand-dendrimer affinity with increasing ionic strength (which could limit the efficiency of drug delivery to distant disease sites in the organism). The strong destabilizing effect of phosphate buffer on ATP-dendrimer complex formation is another premise for the electrostatic molecular mechanism of ATP interaction with amino groups on the dendrimer via its ionized terminal phosphate moiety.

We show that full saturation with ATP decreases dendrimer zeta potential to slightly negative values. As nanoparticles with a zeta potential between −10 and +10 mV are considered approximately neutral (Clogston and Patri, 2011), we can conclude that in the final stages of titration ATP molecules completely covered the surface positive charge of the dendrimers. This may present a practical problem in future pharmaceutical applications of glycodendrimers as DDS, as neutral nanoparticles have an increased propensity to aggregation and inherently lower solubility. Since complex stoichiometry is favourable, with ca. 10 nucleotide molecules bound at saturation, it may be more advantageous in clinical applications to bind adenosine analogue drugs at sub-saturation concentrations, retaining residual positive surface charge for increased cellular uptake and decreased aggregation (Studzian et al., 2017). In that case, stoichiometric studies using a zeta potential measurements can provide help in establishing correct concentration ratio for complex formation.

5. Conclusions

For the development of an efficient drug delivery system utilizing glycodendrimers as nanocarriers for anticancer adenosine analogues, it is vital to comprehensively characterize the formation and stability of complexes between dendrimers and nucleotides at the molecular level. The application of isothermal titration calorimetry and zeta potential titration enabled the determination of nucleotide-dendrimer stoichiometry and thermal effects accompanying the interactions between the compounds under evaluation. The studied complexes have been shown to be based on ionic pairs formed by negatively charged phosphate residues and positive terminal amino moieties on the macromolecules. Such complexes are relatively stable, and the process of complexation is associated with advantageous exothermic changes in enthalpy. We show that the number of nucleotide binding sites is decreased in sugar-modified PPI dendrimers, but the resulting binding is sufficient for potential drug delivery purposes. Moreover, environmental parameters significantly affect nucleotide-dendrimer interactions, with strongest binding in organic buffer, as well as higher binding capacity at lower pH, with potentially advantageous consequences for drug delivery and release in cells.

Acknowledgements

This work was supported by National Science Centre, Poland (project UMO-2014/13/B/NZ3/04643 "Cellular and molecular mechanism of action of PPI dendrimer complexes with nucleoside analogue anticancer drugs"). Authors thank Mrs. Christiane Effenberg for synthesizing glycodendrimers and Dr. Hartmut Komber for carrying out NMR measurements.

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Article

Cite This: *Biomacromolecules* 2018, 19, 531−543

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Glycodendrimer Nanocarriers for Direct Delivery of Fludarabine Triphosphate to Leukemic Cells: Improved Pharmacokinetics and Pharmacodynamics of Fludarabine

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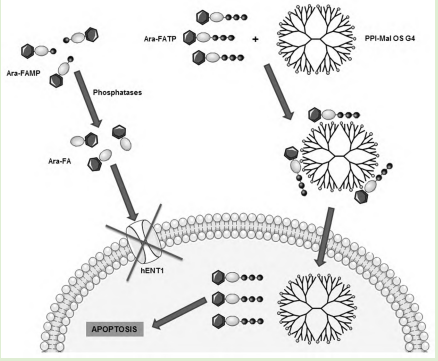
ABSTRACT: Fludarabine, a nucleoside analogue antimetabolite, has complicated pharmacokinetics requiring facilitated transmembrane transport and intracellular conversion to triphosphate nucleotide form (Ara-FATP), causing it to be susceptible to emergence of drug resistance. We are testing a promising strategy to improve its clinical efficacy by direct delivery of Ara-FATP utilizing a biocompatible glycodendrimer nanocarrier system. Here, we present results of a proof-of-concept experiment in several *in vitro*-cultured leukemic cell lines (CCRF, THP-1, U937) using noncovalent complexes of maltose-modified poly(propyleneimine) dendrimer and fludarabine triphosphate. We show that Ara-FATP has limited cytotoxic activity toward investigated cells relative to free nucleoside (Ara-FA), but complexation with the glycodendrimer (which does not otherwise influence cellular metabolism) drastically increases its toxicity. Moreover, we show that transport via hENT1 is a limiting step in Ara-FA toxicity, while complexation with dendrimer allows Ara-FATP to kill cells even in the presence of a hENT1 inhibitor. Thus, the use of glycodendrimers for drug delivery would allow us to circumvent naturally occurring drug resistance due to decreased transporter activity. Finally, we demonstrate that complex formation does not change the advantageous multifactorial intracellular pharmacodynamics of Ara-FATP, preserving its high capability to inhibit DNA and RNA synthesis and induce apoptosis via the intrinsic pathway. In comparison to other nucleoside analogue drugs, fludarabine is hereby demonstrated to be an optimal candidate for maltose glycodendrimer-mediated drug delivery in antileukemic therapy.



INTRODUCTION

Nucleoside analogues (NAs) constitute a class of pyrimidine and purine derivatives with numerous applications in chemotherapy, particularly in the medical treatment of hematological malignancies. These antimetabolites exhibit a number of cytotoxic activities, primarily based on the inhibition of DNA and RNA synthesis and disruption of metabolism of natural nucleosides and nucleotides, resulting in the induction of apoptosis. These mechanisms strongly depend on chemical structure of NAs as well as the character of their interactions with intracellular targets. In the therapy of hematological malignancies, adenosine and cytidine analogues are most commonly used.[1,2]

Because of their hydrophilic nature, nucleoside analogues cannot enter the cells by passive diffusion, and they require specialized nucleoside transporters (NTs) to pass the cellular membrane. These proteins are generally classified as equilibrative or concentrative nucleoside transporters (ENTs and CNTs, respectively), which differ in mechanisms facilitating intracellular transport of NAs.[3,4] Upon the cell entry, analogues are progressively transformed by intracellular kinases to mono-, di-, and triphosphate derivatives, which is crucial for their cytotoxic effect.

Fludarabine (Ara-FA, Figure 1A) is an adenosine analogue widely applied in the treatment of chronic lymphocytic leukemia, non-Hodgkin lymphoma, as well as acute myeloid and lymphocytic leukemias. Administrated as monophosphate prodrug (Ara-FAMP, Figure 1B) due to its higher solubility in comparison to nucleoside form, fludarabine phosphate is dephosphorylated before entering the cell.[1,2] Among human nucleoside transporters, hENT1, hENT2,[5] and hCNT3[6] have been implicated in the intracellular transport of fludarabine. Like other NAs, fludarabine requires phosphorylation by intracellular kinases to exert its cytotoxic activity. The triphosphate form of fludarabine (Ara-FATP, Figure 1C) is considered to be its only active metabolite; its presence is required for initiation of specific toxic mechanisms. These are mainly based on the direct incorporation of fludarabine into

Received: November 23, 2017
Revised: January 9, 2018
Published: January 11, 2018

DOI: 10.1021/acs.biomac.7b01650
Biomacromolecules 2018, 19, 531−543

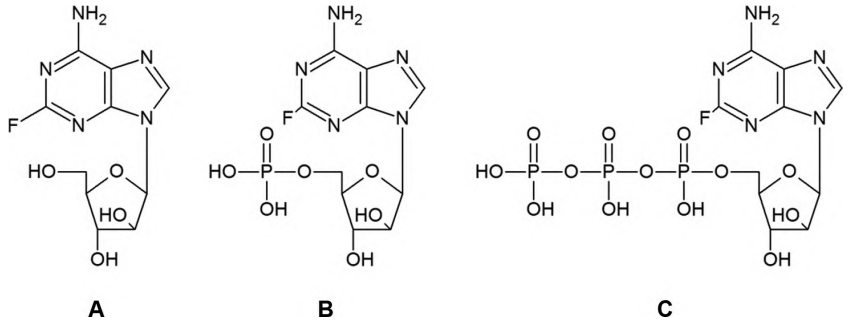


Figure 1. Chemical structures of fludarabine in (A) nucleoside, (B) monophosphate, and (C) triphosphate form.

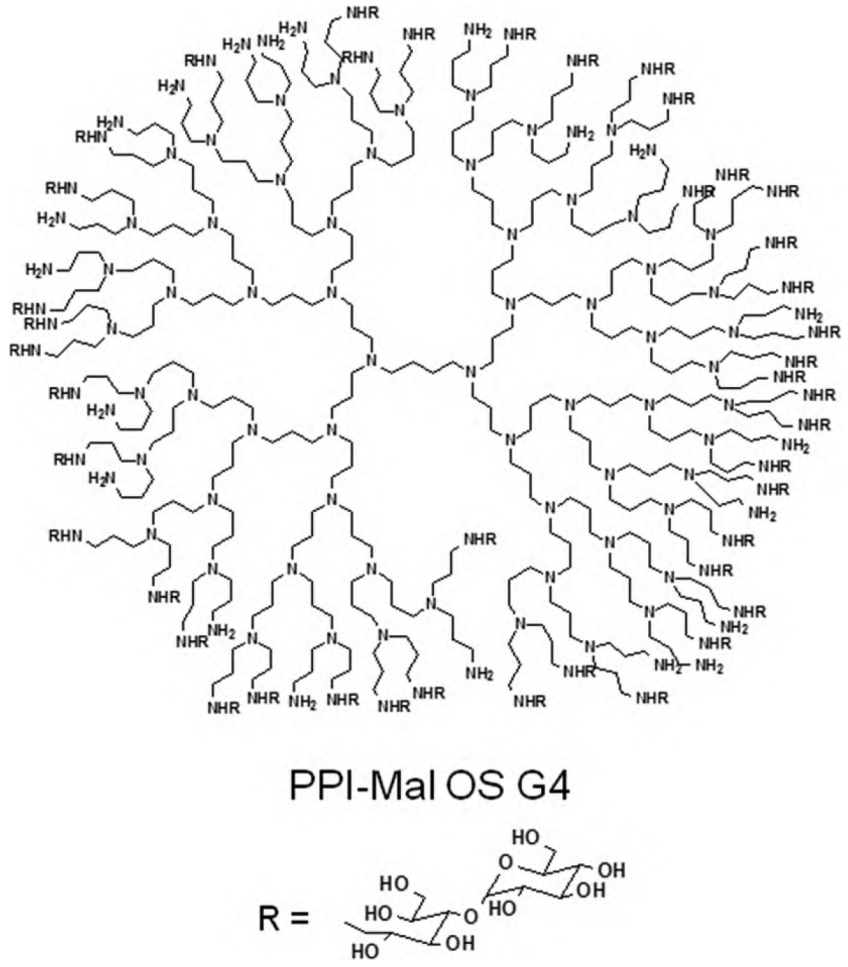


Figure 2. Chemical structure of the poly(propyleneimine) dendrimer of the 4th generation, partially modified with maltose units (PPI-Mal OS G4). The dendrimer has 43 maltose (Mal) moieties chemically coupled to 4th generation PPI scaffold.

newly synthesized chain of DNA, which results in inhibition of the DNA polymerases and chain termination, causing replication arrest and yielding an apoptotic signal. Ara-FATP is also a potent inhibitor of ribonucleotide reductase, which is responsible for maintaining the cellular pool of deoxynucleotides. Reduced activity of this enzyme favors the insertion of fludarabine into nucleic acid chains.[7]

The complicated and multistage metabolism of NAs is responsible for a number of possible resistance mechanisms, significantly limiting the therapeutic potential of these drugs. These include the decreased expression of NTs and reduced activity of intracellular kinases, leading to poor cellular accumulation and inadequate phosphorylation of NAs. The application of nucleoside analogues in anticancer therapy may be additionally hampered by their low solubility, unfavorable biodistribution, fast degradation, and systemic toxicity resulting from low specificity of interaction with target cells.[8,9]

A promising approach enabling circumvention of drug resistance involves direct delivery of therapeutic nucleoside triphosphates, using nanocarriers with autonomous modes of cellular entry. Drug delivery devices should be characterized by low toxicity, low immunogenicity, and ability to hold an adequate amount of active compound.[10,11] Of all nanoparticles proposed for the transport of nucleoside analogues, cationic dendrimers attract particular attention, mainly because of their ability to form stable, noncovalent complexes with these

DOI: 10.1021/acs.biomac.7b01650
Biomacromolecules 2018, 19, 531−543

therapeutics.[12] Dendrimers are highly branched polymers of well-defined, three-dimensional structure with numerous applications in biomedical sciences, particularly in drug delivery.[13] They have been shown to provide improved solubility of the drugs, enhanced blood circulation time, and controllable biodistribution patterns. Dendrimers with high loading capacity can sustain blood concentrations of the drugs above the minimal therapeutic dose and ensure their protection from degradation or unfavorable environmental conditions. What is more, the nanometric size and globular shape of these macromolecules are beneficial for cell entry.[11,14]

Since cationic dendrimers have high cytotoxic and hemolytic activity due to their nonspecific interactions with cellular membranes, damage to mitochondria, and generation of reactive oxygen species;[15,16] it is essential to reduce their surface positive charge to enable medical applications. This can be achieved by chemical modification of reactive terminal moieties with neutral molecules.[17] For the delivery of antileukemic drugs, glycosylation seems to be the most reasonable choice of surface modification. Leukemic cells usually overexpress surface lectin receptors, characterized by high affinity to carbohydrate ligands, enabling receptor-mediated endocytosis. Thus, sugar-coated dendrimers may actively interact with cancer cells, preventing detrimental impact on healthy tissues.[18,19] Glycosylation has been shown to enhance biocompatibility and prolong blood half-life of cationic dendrimers.[20−22]

For the development of efficient dendrimer-based nanocarriers for nucleoside analogues, it is crucial to find the right balance between the density of surface modification and the ability of macromolecules to interact with negatively charged drug molecules. So-called "open shell" (OS) glycodendrimers with surface partially modified with sugar moieties have been widely studied for interactions with both therapeutic and natural nucleosides and nucleotides, showing stable complex formation,[23] ability to shield the triphosphates from enzymatic degradation,[24] and ability to facilitate their delivery into resistant cancer cells.[25]

In the present study, we evaluated the potential of maltose-modified open shell poly(propyleneimine) dendrimer of the fourth generation (PPI-Mal OS G4) to enhance the cytotoxic activity of fludarabine triphosphate (Ara-FATP) to overcome resistance associated with decreased level of nucleoside transporters in leukemic cell lines.

MATERIALS AND METHODS

Materials. PPI dendrimer of the fourth generation with primary surface amino groups partially modified with maltose (PPI-Mal OS G4, MW 21113 g/mol) (Figure 2) was prepared as described previously.[26] The molecular weight of the PPI glycodendrimer was determined by an established ^{1}H NMR approach.[27] 2-Fluoroadenine-9-β-D-arabinofuranoside (fludarabine nucleoside, Ara-FA; Sigma-Aldrich) and fludarabine 5′-triphosphate (Ara-FATP; Jena Bioscience) were used in the course of experiments.

Cell Culture. CCRF (acute lymphoblastic T cell leukemia, subline 1301) human cell line was obtained from Sigma-Aldrich. THP-1 (acute monocytic leukemia) and U937 (histiocytic lymphoma) human cell lines were purchased from ATCC (USA). Cells were maintained under standard conditions in RPMI-1640 Medium (ThermoFisher) supplemented with 10% fetal bovine serum (Sigma-Aldrich) at 37 °C in an atmosphere of 5% CO_2. Cells were subcultured three times per week.

Complex Formation. PPI-Mal OS G4 dendrimer was dissolved in double-distilled water to a final concentration of 40 μM. Dendrimer solutions were freshly prepared and used on the same day. Fludarabine in the form of nucleoside (Ara-FA) and triphosphate (Ara-FATP) were added to the dendrimer solutions at a molar ratio of 10:1 (to a final concentration of 400 μM). This molar ratio ensures complete complexation of triphosphate molecules by maltose-modified dendrimer, as shown previously.[23] The mixtures were stirred for 0.5 h at ambient temperature. The obtained complexes were used in subsequent experiments.

Cytotoxicity Assay. To estimate the cytotoxicity of dendrimer, drugs, and their complexes, resazurin assay was performed.[28] Cells were seeded into 96-well black plates at a density of 1×10^4 cells in 90 μL of medium per well and treated with increasing concentrations of Ara-FA, Ara-FATP, and their complexes with PPI-Mal OS G4 (0.31−40 μM of the drug) or free dendrimer (0.031−4 μM) for 72 h at 37 °C in an atmosphere of 5% CO_2. Ten-times concentrated working solutions of tested compounds were prepared and added to the cells in volumes of 10 μL.

Following the incubation, resazurin was added to the culture medium to a final concentration of 10 μg/mL, and the plates were incubated at 37 °C in darkness to allow conversion of resazurin to resorufin. Fluorescence of metabolized resazurin in the cell suspension was measured after 90 min at 530 nm excitation and 590 nm emission using EnVision plate reader (PerkinElmer, Waltham, USA). Cell viability was presented as percentage of untreated control.

Gene Expression Assay. The gene expression level of fludarabine-associated nucleoside transporters (hENT1, hENT2, and hCNT3) was determined by quantitative real-time RT-PCR. Aliquots of 1×10^6 of CCRF, THP-1, and U937 cells were used for total cellular RNA isolation using TRI Reagent (Sigma-Aldrich) according to manufacturer's protocol. Complementary DNA (cDNA) was transcribed from mRNA using High Capacity cDNA Reverse Transcription Kit (ThermoFisher) and used for real-time PCR amplification with the GoTaq qPCR Master Mix (Promega) according to manufacturer's protocol. Each 16 μL reaction volume contained 1 μL of cDNA and 0.25 μM of forward and reverse intron-spanning primers. The reference genes (HPRT1 and HMBS) were selected according to the GeNorm procedure.[29] The sequences of primers are presented in Table 1.

Table 1. Primer Sequences

nucleoside transporter	gene	forward and reverse sequences (5′−3′)
	HPRT1	Fw: TGACACTGGCAAAACAATGCA
		Rv: GGTCCTTTTCACCAGCAAGCT
	HMBS	Fw: GGCAATGCGGCTGCAA
		Rv: GGGTACCCACGCGAATCAC
hENT1	SLC29A1	Fw: AGCCAGGGAAAACCGAGA
		Rv: ACCCAGCATGAAGAAGATAAGC
hENT2	SLC29A2	Fw: CAAAGCTGAGCTCCTCCAGT
		Rv: CAGGGTCAGAGCTACTTTCTGG
hCNT3	SLC28A3	Fw: GGATCATGCTAGTTACTACGGG
		Rv: TTTTCATGGCATTCTTGAGGGT

PCR reactions were performed in 96-well microplates using the CFX96 Real Time PCR Detection System (Bio-Rad). The expression level of assayed genes was expressed as number of cognate mRNA copies per 1000 copies of geometric-averaged mRNA for reference genes.

Nucleoside Transport Inhibition. To inhibit the transport of fludarabine across cell membrane, CCRF, THP-1, and U937 cells were preincubated with inhibitor of hENT1, nitrobenzylmercaptopurine (NBMPR, Sigma-Aldrich) at the final concentration 50 μM for 45 min. After preincubation with NBMPR, cells were seeded into 96-well black plates at a density of 1×10^4 cells in 90 μL of medium per well and treated with increasing concentrations of Ara-FA, Ara-FATP, and their

DOI: 10.1021/acs.biomac.7b01650
Biomacromolecules 2018, 19, 531−543

complexes with PPI-Mal OS G4 (0.31−40 μM of the drug) for 72 h at 37 °C in an atmosphere of 5% CO_2. Ten-times concentrated working solutions of tested compounds were prepared and added to the cells in volumes of 10 μL. Following the incubation, viability of the cells was evaluated using resazurin assay.

DNA/RNA Synthesis. U937 cells were incubated for 24 h with Ara-FA and Ara-FATP at final concentrations of 5, 10, and 20 μM, as well as Ara-FATP complexed with PPI-Mal OS G4 at 10:1 molar ratio in the same concentration range, and PPI-Mal OS G4 dendrimer itself only at the maximal final concentration of 2 μM. All incubations were performed at 37 °C in an atmosphere of 5% CO_2. Following the incubation, aliquots of 1×10^5 cells were withdrawn from the culture and transferred to a thin-bottom 96-well plate coated previously with poly-L-lysine. After 10 min of sedimentation at 37 °C, plates were centrifuged (5 min, 100 × g) to enhance cell adhesion to the plate. For the evaluation of DNA and RNA synthesis inhibition, Click-iT Plus EdU Imaging Kit (C10639) and Click-iT RNA Imaging Kit (C10330) from Invitrogen were used, respectively, according to manufacturer's protocols. These assays enable the detection of DNA and RNA synthesis thanks to the incorporation of EdU (5-ethynyl-2′-deoxyuridine) and EU (5-ethynyl uridine), respectively, into newly synthesized nucleic acid chains. The incorporated nucleotides are subsequently derivatized with Alexa Fluor 549 fluorophore via click chemistry. Stained cells were visualized with confocal microscopy. Images were obtained under 6300× magnification with Zeiss LSM 780 microscope equipped with 405 nm laser diode and InTune excitation laser system (Carl Zeiss Micro Imaging, USA). Quantitative measurements of EdU and EU signals were conducted with a high-content screening platform (ArrayScanVTi from ThermoFisher) using manufacturer's proprietary software. Measurements were made for >2000 cells per single well. The results were presented as mean fluorescence of stained cells.

Measurement of Phosphatidylserine Externalization−Annexin V Affinity Assay. A common signal of apoptosis involves rapid transfer of phosphatidylserine (PS) from the cytoplasmic side of plasma membrane to the cell surface. The externalized PS enables the recognition of apoptotic cells providing a binding site for annexin V, anionic lipid binding protein. Annexin V is widely applied for the detection of apoptotic cells *in vitro* and shows the potential for determination of treatment efficacy in cancer patients.[30] The technique involves incubation of cells in culture medium containing fluorescent annexin V, removal of unbound dye, and measurement of fluorescence of cell-bound dye. The value of the fluorescence intensity is a measure of apoptotic externalization of PS and indicates the involvement of cells in apoptotic processes.

U937 cells were incubated for 24 h with Ara-FA and Ara-FATP at final concentrations of 5, 10, and 20 μM, as well as Ara-FATP complexed with PPI-Mal OS G4 at 10:1 molar ratio in the same concentration range, and PPI-Mal OS G4 dendrimer itself only at the maximal final concentration of 2 μM. All incubations were performed at 37 °C in an atmosphere of 5% CO_2. Cells were subsequently washed and suspended in HBSS containing annexin-FITC dye (BD Biosciences), according to manufacturer's protocol. The cells were incubated at 37 °C in an atmosphere of 5% CO_2 for 15 min. Following the incubation, cells were washed with HBSS, resuspended in PBS, and transferred to 96-well black plate. Fluorescence measurement was performed at 485 nm excitation and 535 nm emission wavelengths using an EnVision plate reader (PerkinElmer, Waltham, USA). The results were presented as percentage of fluorescence relative to dye bound to untreated cells.

Mitochondrial Membrane Potential Assay. Depolarization of mitochondrial membrane is considered one of the central events of intrinsic apoptotic signaling pathway. The decrease of mitochondrial membrane potential leads to the release of apoptotic factors, for example, cytochrome c, triggering the assembly of apoptosome required for activation of caspase cascade.[31] To perform the measurement of changes in mitochondrial potential, the technique utilizing differences in spectral parameters of JC-1 fluorescent dye was used. JC-1 (tetraethylbenzimidazolylcarbocyanine iodide) is a cationic, membrane-permeant dye, which exhibits potential-dependent accumulation in mitochondria. Because of the physiological mitochondrial membrane potential, JC-1 can form aggregates, which cause fluorescence emission shift from green (~529 nm) to red (~590 nm). Mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio.[32] The technique involves incubation of cells with JC-1 dye, removal of extracellular dye, and measurement of fluorescence at two spectral points.

U937 cells were incubated for 24 h with Ara-FA and Ara-FATP at final concentrations of 5, 10, and 20 μM, as well as Ara-FATP complexed with PPI-Mal OS G4 at 10:1 molar ratio in the same concentration range, and PPI-Mal OS G4 dendrimer itself only at the maximal final concentration of 2 μM. All incubations were performed at 37 °C in an atmosphere of 5% CO_2. Cells were subsequently washed, suspended in HBSS containing JC-1 dye (ThermoFisher) at final concentration of 1 μM, and incubated at 37 °C in an atmosphere of 5% CO_2 for 30 min. Following the incubation, cells were washed with HBSS, resuspended in PBS, and transferred to 96-well black plate. The intensity of green (485 nm excitation and 535 nm emission wavelength) and red fluorescence (535 nm excitation and 595 nm emission wavelength) was measured using an EnVision plate reader (PerkinElmer, Waltham, USA). The results were presented as percentage of fluorescence ratio relative to untreated cells.

Caspase-3 Activity Assay. During intrinsic apoptopic pathway, mitochondrial cytochrome c is released into the cytosol and binds an adaptor protein (APAF-1), which recruits initiator caspase-9. This results in the formation of a caspase activating multiprotein complex called apoptosome. Upon activation, initiator caspases cleave and activate other executioner caspases such as caspase-3. The effector caspases are responsible for the proteolytic cleavage of a broad spectrum of cellular targets like structural and regulatory components, leading ultimately to cell death. This activity is therefore an important hallmark of initiation of apoptotic processes. Since caspase-3 serves as a convergence point for different signaling pathways, it can serve as a good marker for apoptosis assay.[33]

For the detection of apoptosis-associated caspase activity, U937 cells were incubated for 24 h with Ara-FA and Ara-FATP at final concentrations of 5, 10, and 20 μM, as well as Ara-FATP complexed with PPI-Mal OS G4 at 10:1 molar ratio in the same concentration range, and PPI-Mal OS G4 dendrimer itself only at the maximal final concentration of 2 μM. All incubations were performed at 37 °C in an atmosphere of 5% CO_2. Cells were subsequently washed with PBS and lysed by freezing and thawing in lysis buffer (containing 50 mM HEPES pH 7.4, 5 mM CHAPS, 5 mM DTT) with protease inhibitor cocktail (Sigma-Aldrich). The lysates were centrifuged at maximum speed, and 5 μL of supernatants was transferred to 96-well black polypropylene plates. For the detection of caspase-3 activity, 200 μL of assay buffer (containing 20 mM HEPES pH 7.4, 0.1% CHAPS, 5 mM DTT, 2 mM EDTA) with Ac-DEVD-AFC (Sigma-Aldrich; final concentration: 17 μM) was added to each well. Plates were gently mixed, and kinetic fluorescence measurement was performed at 380 nm excitation and 500 nm emission wavelength using an EnVision plate reader (PerkinElmer, Waltham, USA). The slopes of the fluorescence curves were considered the measure of caspase-3 activity. The results were presented as percentage of caspase-3 activity of untreated cells.

Statistics. For statistical analysis, we used one-way ANOVA for concentration series followed by posthoc Tukey's test for pairwise difference testing. For single pairwise comparisons, Student's t test was applied. In all tests, p-values < 0.05 were considered to be statistically significant. Data were presented as arithmetic mean ± SEM.

RESULTS

Cytotoxicity of Fludarabine and Fludarabine−Dendrimer Complexes in Selected Cell Line Models. In the initial step of our investigation, we verified the viability of CCRF, THP-1, and U937 cell lines exposed to nucleoside (Ara-FA) and nucleotide (Ara-FATP) forms of fludarabine, as well as their complexes with PPI-Mal OS G4 dendrimer, after 72 h of treatment.

DOI: 10.1021/acs.biomac.7b01650
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Although fludarabine is administered as monophosphate (Ara-FAMP) due to its superior solubility, it still requires dephosphorylation before entering the cell. Thus, in the course of our studies, we decided to use commercially available nucleoside form to exclude the limiting dephosphorylation stage and to investigate the maximum cytotoxic potential of the drug.

The dendrimer concentration range was selected based on the results of previous experiments on dendrimer−nucleotide complex formation, which established the optimal molar ratio for biological effects.[23,25] Within the whole concentration range used for the formation of complexes, no significant impact of free dendrimer on cell survival was detected after 72 h exposure (data not shown).

In line with our expectations, Ara-FA was found to be more toxic than its triphosphate derivative (Ara-FATP) in all cell lines under evaluation (Figure 3, Table 2). Complex of Ara-FA and maltose-modified dendrimer did not show higher toxicity compared to free nucleoside, while cytotoxicity of Ara-FATP was significantly enhanced by PPI-Mal OS G4 to a level comparable to the activity of free Ara-FA. Furthermore, there were significant differences in the extent of drug toxicity to the tested cell lines: fludarabine nucleoside was most toxic to U937 cell line, while CCRF cells exhibited the lowest susceptibility.

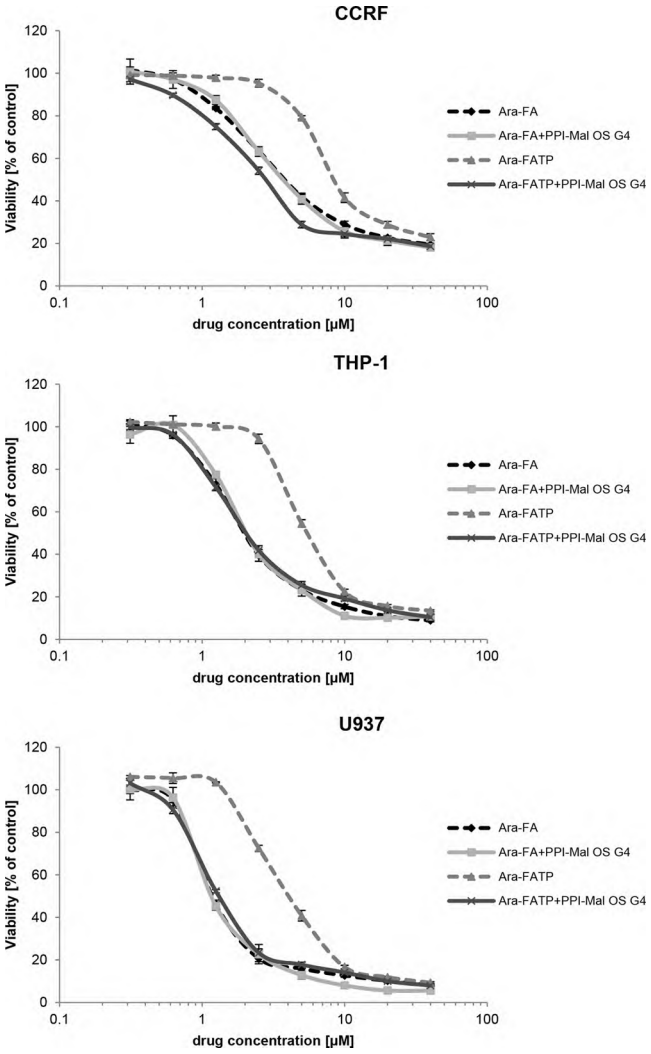

Figure 3. Cytotoxic effect of Ara-FA, Ara-FATP, and their complexes with PPI-Mal OS G4 dendrimer on CCRF, THP-1, and U937 cells. Viability was determined by the resazurin assay after 72 h of treatment. Data presented as percentage of viability of control (untreated) cells, average ± S.E.M., n = 8.

Table 2. Cytotoxic Activity (IC_{50} Values) of Ara-FA, Ara-FATP, and Their Complexes with PPI-Mal OS G4 Dendrimer on CCRF, THP-1, and U937 Cells after 72 h of Treatment

	IC_{50} ± SEM [μM]		
	CCRF	THP-1	U937
Ara-FA	2.62 ± 0.23	1.72 ± 0.15	1.11 ± 0.06
Ara-FA+PPI-Mal OS G4	2.78 ± 0.50	1.92 ± 0.20	1.13 ± 0.14
Ara-FATP	6.93 ± 0.45[a]	4.81 ± 0.21[a]	3.42 ± 0.20[a]
Ara-FATP+PPI-Mal OS G4	2.04 ± 0.18[b]	1.70 ± 0.15[b]	1.12 ± 0.08[b]

[a]Statistically significant difference compared to Ara-FA at $p < 0.05$. [b]Statistically significant difference compared to Ara-FATP at $p < 0.05$.

Nucleoside Transport Inhibition. To assess the significance of drug−dendrimer complexes in overcoming drug resistance associated with decreased activity of nucleoside transporters, we decided to determine the expression of fludarabine-specific NTs (hENT1, hENT2, and hCNT3) at mRNA level in CCRF, THP-1, and U937 cells. All cell lines showed high expression of hENT1, exceeding the expression of hENT2 by 15-fold for CCRF, 19-fold for THP-1, and 100-fold for U937 cells. Moreover, the expression of hENT1 differed significantly between these cell lines and increased in the following order: CCRF < THP-1 < U937, which correlated with susceptibility of those cells to fludarabine determined by cytotoxicity assay. The expression of hCNT3 was not observed (Table 3). Therefore, we concluded that in the tested cell lines, hENT1 is primarily responsible for intracellular accumulation of the drug.

Table 3. Relative Gene Expression Levels of Fludarabine-Specific Nucleoside Transporters in Tested Cell Lines[a]

	hENT1	hENT2	hCNT3
CCRF	1082.0 ± 31.4a,b	70.4 ± 6.9a	no expression detected
THP-1	1534.4 ± 16.9a,c	81.8 ± 2.2b	no expression detected
U937	3129.6 ± 81.4b,c	31.2 ± 2.1a,b	no expression detected

[a]Data expressed as relative mRNA copy number per 1000 copies of averaged reference mRNA, calculated by $2^{-\Delta Ct}$ transformation, presented as average ± S.E.M., n = 3. Lowercase letters denote statistically significant difference between cell lines at $p < 0.05$.

Taking the above results into consideration, we decided to further elucidate the molecular mechanism of drug−dendrimer complex mode of action and verify the differences in fludarabine toxicity during transport modulation by PPI-Mal OS G4. This step involved analysis of cytotoxic activity of Ara-FA, Ara-FATP and their complexes with PPI-Mal OS G4 dendrimer in the presence of long-established inhibitor of hENT1, nitrobenzylmercaptopurine (NBMPR).[34] The preincubation of cells with NBMPR resulted in significant decrease of the cytotoxic activity of both Ara-FA and Ara-FATP to the same extent, which indicates that the triphosphate form requires extracellular conversion to nucleoside to enter the cell via hENT1. The cytotoxicity of Ara-FA−dendrimer complex was comparable to those observed for Ara-FA and Ara-FATP. On the other hand, in all three cell lines, the

DOI: 10.1021/acs.biomac.7b01650
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complex of Ara-FATP and PPI-Mal OS G4 retained its cytotoxic activity, although the IC_{50} values for these compounds were slightly higher compared to the experiments without NBMPR (Figure 4, Table 4). This indicates that the

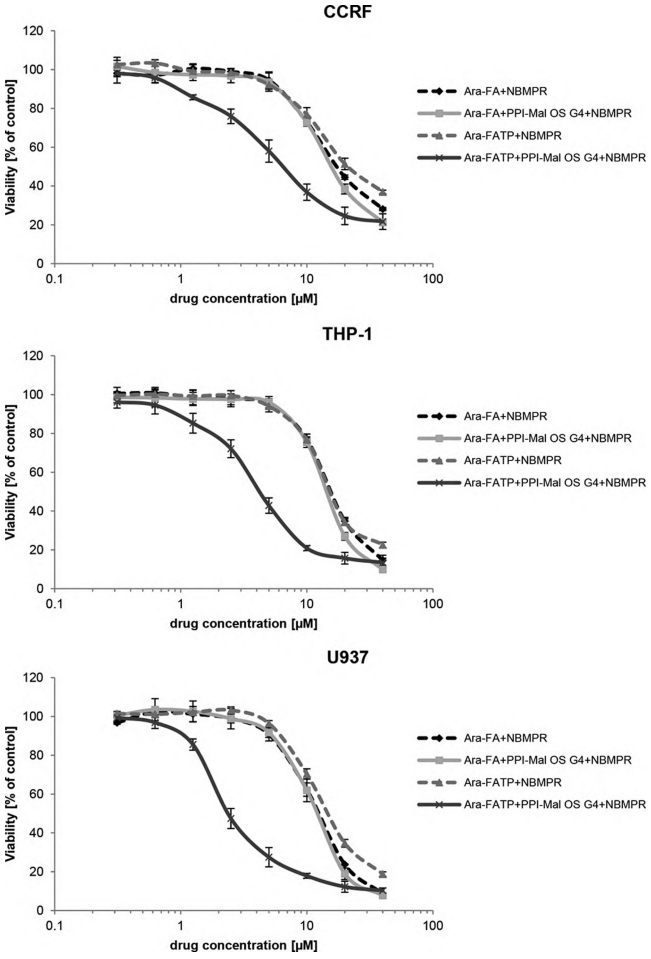

Figure 4. Cytotoxic effect of Ara-FA, Ara-FATP, and their complexes with PPI-Mal OS G4 dendrimer on CCRF, THP-1, and U937 cells in the presence of hENT1 inhibitor NBMPR. Viability was determined by the resazurin assay after 72 h of treatment. Data presented as percentage of viability of control (untreated) cells, average ± S.E.M., $n = 6$.

triphosphate−dendrimer formulation must possess a separate, specific mode of transport into the cell, independent of hENT1, although Ara-FATP cannot entirely bypass the hENT1 entry pathway using the nanocarrier. Interestingly, at the highest concentration of fludarabine drugs, NBMPR seemed to lose its inhibitory properties, which may be associated with the fact that NBMPR (as a nucleoside analogue) is a competitive inhibitor.[35]

Fludarabine and Its Complexes with Maltose-Modified PPI Dendrimer: Mechanisms of Toxicity. The final stage of our investigation was meant to elucidate the intracellular mechanisms associated with the toxic effects of fludarabine in nucleoside and triphosphate form as well as Ara-FATP−dendrimer complex. Since the main cytotoxic activity of fludarabine is based on the inhibition of DNA and RNA elongation leading to the induction of apoptosis,[7,36] we evaluated the impact of Ara-FA, Ara-FATP, and Ara-FATP−PPI-Mal OS G4 complex on nucleic acid synthesis and parameters directly related to apoptotic processes (phosphatidylserine externalization, reduction of mitochondrial membrane potential, and increase in caspase-3 activity).

Table 4. Cytotoxic Activity (IC_{50} Values) of Ara-FA, Ara-FATP, and Their Complexes with PPI-Mal OS G4 Dendrimer on CCRF, THP-1, and U937 Cells in the Presence of hENT1 Inhibitor NBMPR after 72 h of Treatment

	IC_{50} ± SEM [μM]		
	CCRF	THP-1	U937
Ara-FA+NBMPR	13.14 ± 2.22	14.34 ± 2.33	11.69 ± 1.24
Ara-FA+PPI-Mal OS G4+NBMPR	13.79 ± 2.74	13.71 ± 1.45	11.20 ± 1.72
Ara-FATP+NBMPR	13.75 ± 3.68	12.99 ± 1.41	12.30 ± 0.99
Ara-FATP+PPI-Mal OS G4+NBMPR	4.75 ± 1.41[ab]	3.78 ± 0.67[ab]	2.21 ± 0.32[ab]
(Ara-FATP +NBMPR)/(Ara-FATP+PPI-Mal OS G4+NBMPR)	2.89	3.44	5.57

[a]Statistically significant difference compared to Ara-FA at $p < 0.05$. [b]Statistically significant difference compared to Ara-FATP at $p < 0.05$.

Because the cellular apoptotic mechanisms are rapid, they are usually tested after shorter incubation times than in case of traditional cytotoxicity assays which determine only the percentage of dying cells. Therefore, all experiments in this part were performed after 24 h of incubation.

To comprehensively illustrate the potential differences in action of different forms of the drug, the tests were performed on U937 cell line due to its highest sensitivity to fludarabine (Figure 3, Table 2) and the highest cytotoxic activity of Ara-FATP−dendrimer complex in cells with inhibited hENT1 (Figure 4, Table 4).

In all experiments, free PPI-Mal OS G4 at the highest concentration used (2 μM) did not cause significant changes compared to untreated control (data not shown), which indicates once again that this type of dendrimer may serve as a safe, inert drug carrier.

All forms of fludarabine have been shown to inhibit the synthesis of nucleic acids in a concentration-dependent manner (Figures 5 and 6). The drug was a more potent inhibitor of RNA synthesis, which may be associated with the rapidity of this process and faster incorporation of the nucleoside analogue to RNA than DNA. In both cases, the activity of Ara-FATP was lower than Ara-FA, although this effect was more noticeable for inhibition of RNA synthesis. As expected, the triphosphate−dendrimer complex exhibited stronger inhibition effect than free Ara-FATP, as seen both in microscope images (Figures 5A and 6A) and in quantitative measurements (Figures 5B and 6B). In the case of DNA synthesis, this inhibitory activity appeared at even lower concentrations compared to Ara-FA (Figure 5B).

Ara-FA, Ara-FATP, and Ara-FATP−PPI-Mal OS G4 complex triggered concentration-dependent increase in the amount of cell-bound annexin V, indicating apoptosis-linked phosphatidylserine exposure (Figure 7). Drug−dendrimer complex showed the strongest effect, with the activity of Ara-FA being only slightly lower. Free Ara-FATP exhibited the lowest activity.

Ara-FATP in complex with PPI-Mal OS G4 dendrimer caused a rapid, concentration-dependent depolarization of mitochondrial membrane (Figure 8). Ara-FA exhibited this activity at 10 and 20 μM concentrations, while free Ara-FATP

DOI: 10.1021/acs.biomac.7b01650
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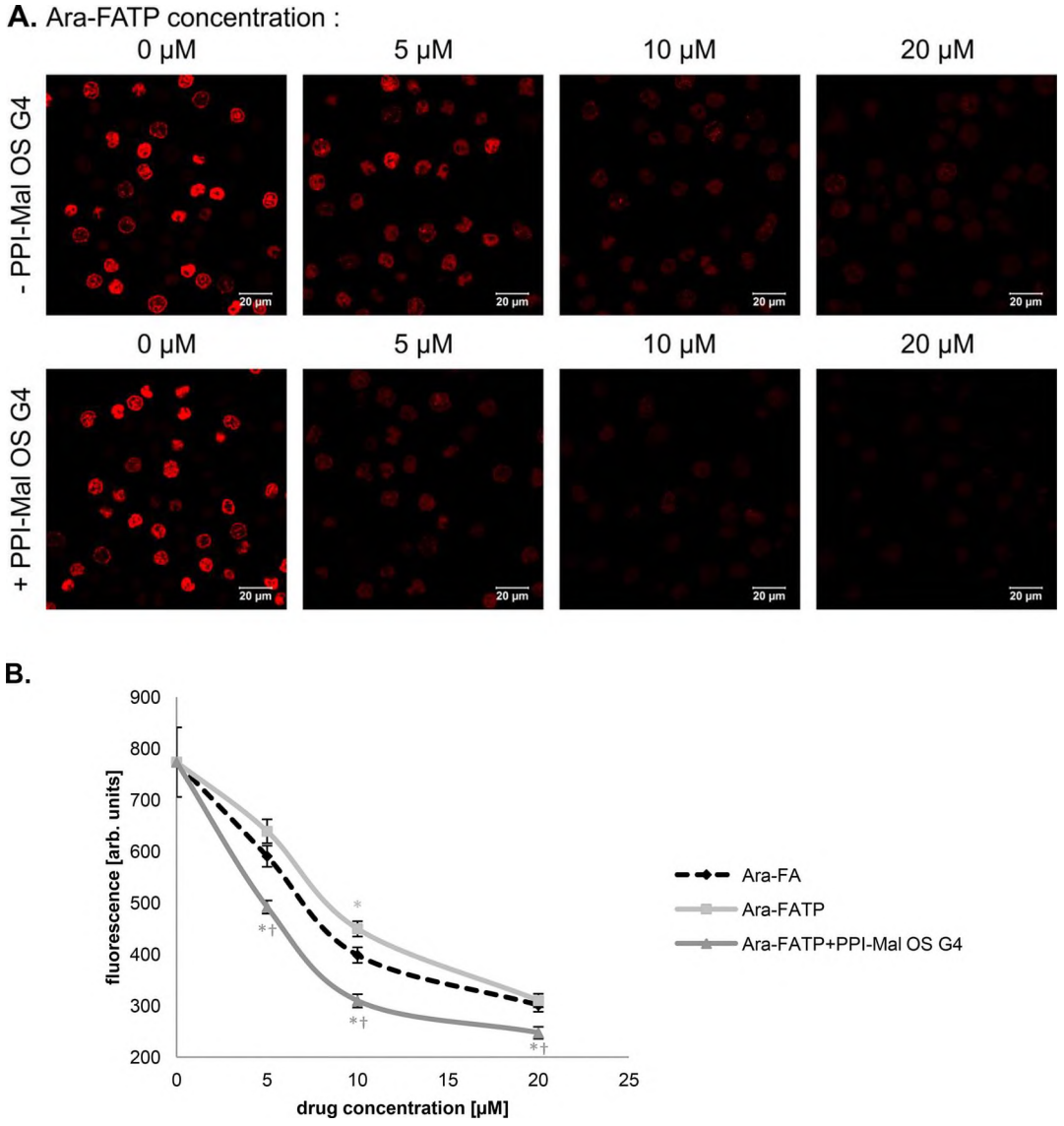

Figure 5. DNA synthesis inhibition by Ara-FA, Ara-FATP, and Ara-FATP−PPI-Mal OS G4 complex in U937 cells after 24 h of treatment. (A) Representative confocal images for each treatment with Ara-FATP and Ara-FATP−PPI-Mal OS G4 complex. (B) Quantitative evaluation of DNA synthesis inhibition by high-content screening. Data presented as average incorporated EdU fluorescence ± S.E.M., $n = 4$. ∗, Statistically significant difference compared to Ara-FA at $p < 0.05$. †, Statistically significant difference compared to Ara-FATP at $p < 0.05$.

reduced mitochondrial potential only at the highest concentration tested (20 μM).

The complex of Ara-FATP and PPI-Mal OS G4 dendrimer significantly boosted the activity of caspase-3, exceeding almost twice the effect of Ara-FA, and about five-times the effect of free Ara-FATP over the entire concentration range (Figure 9).

In summary, the results of apoptotic assays corroborate the preliminary conclusions drawn from viability assays. The applied methods confirmed higher toxicity of Ara-FA and Ara-FATP−dendrimer complex compared to Ara-FATP. In some cases, the activity of triphosphate−dendrimer complex exceeded the effects of Ara-FA. What is more, the apoptotic mode of cell death induced by fludarabine remained unchanged in the additional presence of PPI-Mal OS G4 dendrimer. The conserved mechanism of toxicity and increased activity of Ara-FATP−dendrimer complex suggests that the phenomenon of dendrimer enhancement of fludarabine toxicity is probably based exclusively on enhanced cellular transport of the triphosphate form of fludarabine.

DISCUSSION

Since the late 1980s, successful application of cytarabine in the therapy of leukemias and lymphomas has generated interest in the development of other anticancer nucleoside analogues. Fludarabine, a deamination-resistant analogue of deoxyadenosine synthesized by Montgomery and Hewson,[37] displays remarkable activity in many types of hematological malignancies, particularly in chronic lymphocytic leukemia (CLL), but also in acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myelogenous leukemia (CML), or non-Hodgkin lymphoma.[38] Unlike other NAs, fludarabine is administered as monophosphate prodrug due to the higher solubility and ionization at physiologic pH. Before entering a blood cell, fludarabine monophosphate must undergo dephosphorylation to the nucleoside form (Ara-FA), catalyzed by phosphatases present in blood serum or more efficiently by the membrane-bound ectoenzyme 5′-nucleotidase (CD73), which is frequently expressed in acute leukemias. Ara-FA is delivered

DOI: 10.1021/acs.biomac.7b01650
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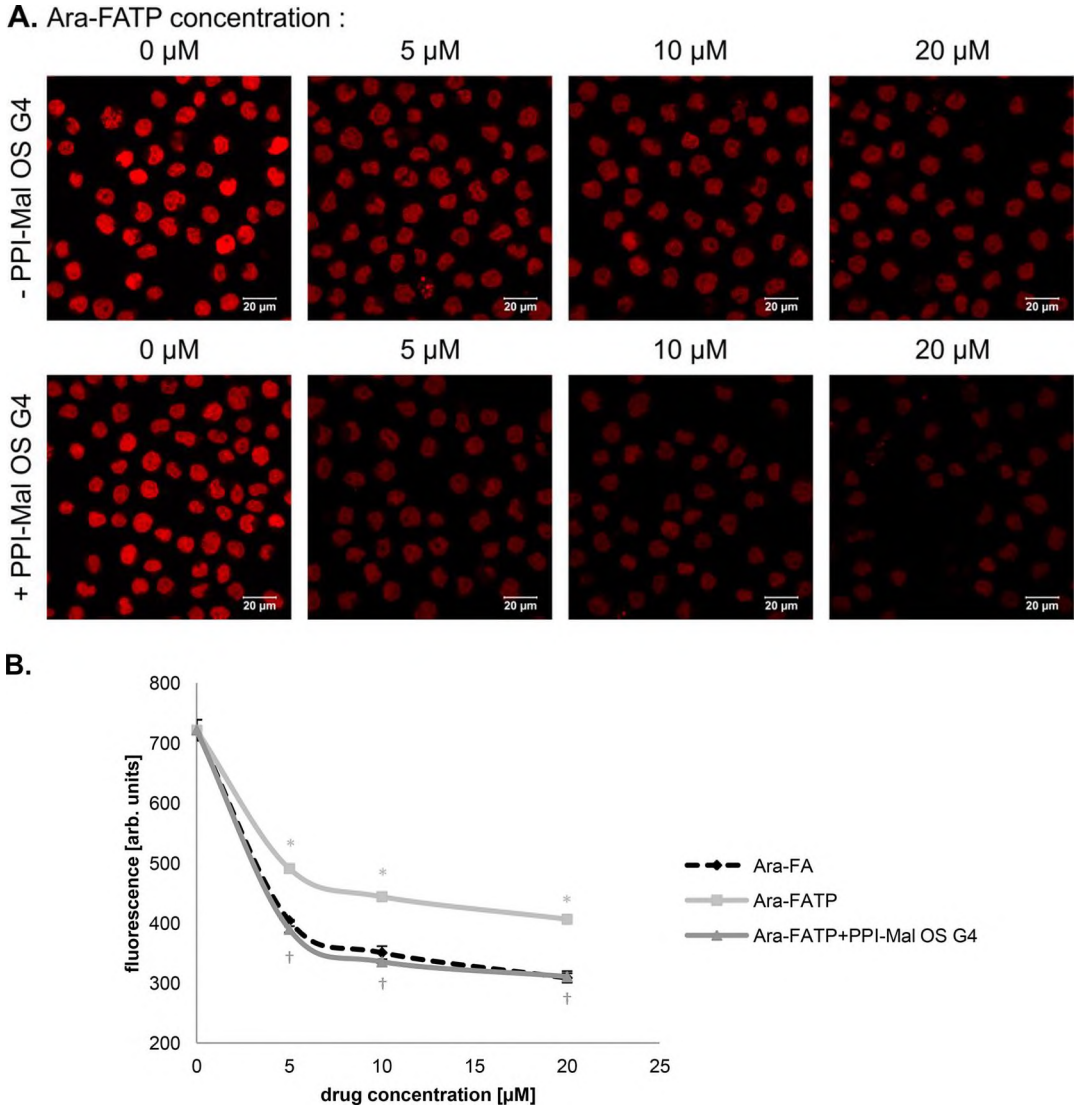

Figure 6. RNA synthesis inhibition by Ara-FA, Ara-FATP, and Ara-FATP−PPI-Mal OS G4 complex in U937 cells after 24 h of treatment. (A) Representative confocal images for each treatment with Ara-FATP and Ara-FATP−PPI-Mal OS G4 complex. (B) Quantitative evaluation of DNA synthesis inhibition by high-content screening. Data presented as average incorporated EU fluorescence ± S.E.M., $n = 4$. ∗, Statistically significant difference compared to Ara-FA at $p < 0.05$. †, Statistically significant difference compared to Ara-FATP at $p < 0.05$.

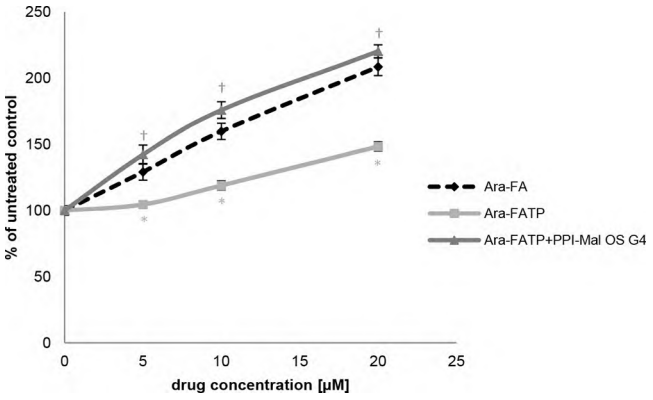

Figure 7. Annexin V binding by U937 cells treated with Ara-FA, Ara-FATP, and Ara-FATP−PPI-Mal OS G4 complex for 24 h. Data presented as percentage of annexin V bound to control (untreated) cells, average ± S.E.M., $n = 4$. ∗, Statistically significant difference compared to Ara-FA at $p < 0.05$. †, Statistically significant difference compared to Ara-FATP at $p < 0.05$.

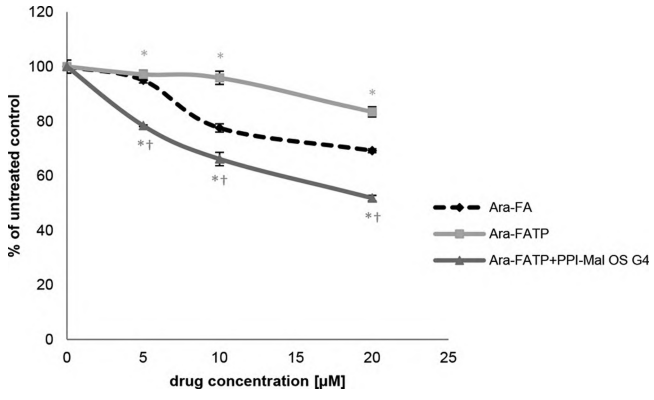

Figure 8. Mitochondrial membrane depolarization in U937 cells treated with Ara-FA, Ara-FATP, and Ara-FATP−PPI-Mal OS G4 complex for 24 h. Data presented as percentage of JC-1 fluorescence ratio of control (untreated) cells, average ± S.E.M., $n = 4$. ∗, Statistically significant difference compared to Ara-FA at $p < 0.05$. †, Statistically significant difference compared to Ara-FATP at $p < 0.05$.

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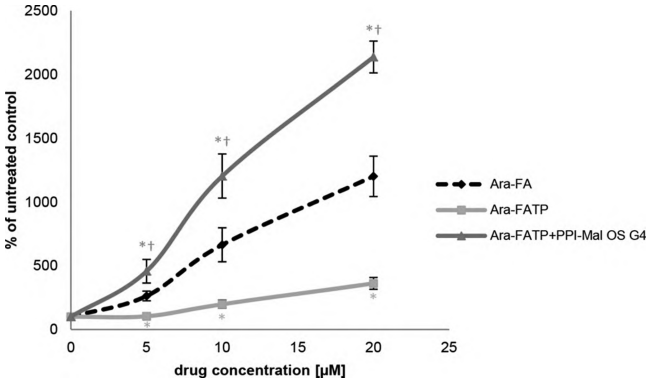

Figure 9. Caspase 3 activity in U937 cells treated with Ara-FA, Ara-FATP, and Ara-FATP−PPI-Mal OS G4 complex for 24 h. Data presented as percentage of caspase 3 substrate fluorescence in control (untreated) cells, average ± S.E.M., $n = 5$. ∗, Statistically significant difference compared to Ara-FA at $p < 0.05$. †, Statistically significant difference compared to Ara-FATP at $p < 0.05$.

by facilitated transport into cytoplasm, where it is rephosphorylated by deoxycytidine kinase (dCK) and subsequently converted to the diphosphate and triphosphate form. Ara-FATP is thought to be the only metabolite of fludarabine directly interacting with cellular targets. The relatively low intracellular concentrations of fludarabine mono- and diphosphate suggest that the activity of dCK is the rate-limiting step for triphosphate formation.[7] In our cellular models, these assumptions were fully validated, with Ara-FA exhibiting much stronger overall cytostatic effect when added extracelularly than Ara-FATP.

Fludarabine-based treatment in hematological cancers achieves superior remission rates compared to traditional therapies, such as chlorambucil- and anthracycline-based regimens.[38] Several *in vivo* studies focused on the relationship between the dose rate of Ara-FAMP, Ara-FA concentrations in plasma, and intracellular accumulation of Ara-FATP. The standard Ara-FAMP infusion of 25−30 mg/m^2 over 30 min gives C_{max} values of Ara-FA reaching 3−5 μM. The intracellular peak concentration of Ara-FATP is proportional to the dose of Ara-FAMP[39] and reaches maximum within 3−4 h after termination of fludarabine infusion, declining monophasically with a median half-life of 23 h.[40] This suggests that Ara-FATP is a relatively long-lived active metabolite. This body of data influenced our choice of working concentrations of fludarabine drugs, so that our conclusions on potential drug delivery improvement could be clinically relevant.

Ara-FATP has multiple molecular targets in the cell (see below), which reduces the possibility of selection of drug resistant cells,[41] so the major obstacle in the development of efficient fludarabine-based therapy arises from an insufficient intracellular accumulation of Ara-FATP. This is mainly associated with membrane impermeability of this active drug form, leading to the necessity of using precursor forms (Ara-FAMP, Ara-FA) with complex pharmacokinetics. Thus, decreased therapeutic effect may be due to reduced transmembrane transport, insufficient activity of intracellular kinases, or increased degradation.[1]

Intracellular fludarabine accumulation is primarily mediated by human equilibrative nucleoside transporters 1 (hENT1) and 2 (hENT2), which transport exclusively dephosphorylated substrates. Human concentrative nucleoside transporter 3 (hCNT3) has also been reported for the transport of Ara-FA.[5,6] hCNTs actively transport nucleosides and nucleoside analogues depending on the inwardly directed sodium gradient, while hENTs facilitate their passive diffusion. hENTs differ in their sensitivity to inhibition by nitrobenzylmercaptopurine (NBMPR): hENT1 is characterized by "es" activity (equilibrative and NBMPR-sensitive), while hENT2 possesses "ei" (equilibrative and NBMPR-insensitive) characteristic.[34]

Scientific data indicate significant differences in the expression of hENT1 depending on the type of leukemia.[42] *In vitro* studies have demonstrated that NT-deficient cells are highly resistant to NAs. In isolated leukemic blasts, sensitivity to fludarabine correlated with the cellular abundance of hENT1. In addition, high cellular proliferation rates are associated with high levels of hENT1 activity, which enhance nucleoside drug uptake.[1] Therefore, the decreased expression of hENT1 seems to be the most severe cellular resistance mechanism against fludarabine. In our study, one of the reasons for initially applying three different cellular models was to verify the potential differences in hENT1 expression and their impact on fludarabine toxicity. Our results conformed to the expectations, with hENT1 demonstratively responsible for the bulk of Ara-FA cellular entry and its inhibition mimicking a drug-resistant phenotype.

To overcome above-mentioned drug resistance and to increase intracellular concentration of fludarabine triphosphate, nanocarrier systems may be applied. Various nanostructures, such as liposomes or nanogels, were evaluated for the potential of delivering fludarabine both in a prodrug[43] and triphosphate form.[44] The idea of direct transport of active triphosphate seems to be a promising strategy, enabling not only the circumvention of drug resistance associated with insufficient cellular uptake of the drug, but also with inadequate intracellular phosphorylation. However, the use of nanocarriers in most cases is concentrated on nucleosides, while the available scientific data on the delivery of triphosphates are limited.[12] We decided to focus our efforts on leukemic cells (which had not hitherto been a target of any study on fludarabine drug delivery by nanocarrier) to ensure clinical relevance of our results. In contrast to other studies, we also employed a pharmacological model (hENT1 inhibition) to mimic the drug-resistant phenotype that nanocarrier application is designed to circumvent. Our study focused on maltose-modified open shell poly(propyleneimine) dendrimers of the fourth generation (PPI-Mal OS G4) with the potential to form stable, noncovalent complexes with nucleoside triphosphates and to facilitate their intracellular delivery. The aim of our investigation was to evaluate the ability of drug−dendrimer complexes to enhance the cytotoxicity of Ara-FATP and to bypass drug resistance based on the decreased activity of hENT1.

PPI dendrimers partially coated with maltose units are of particular interest for medical application, especially for drug delivery, due to their greatly reduced toxicity with regard to naked PPI dendrimers[21] while maintaining positive surface charge enabling electrostatic interaction with negatively charged phosphate residues.[23,45] This makes them particularly promising vehicles for nucleotides. Compared to chemical conjugation, incorporation of therapeutic molecules based on physical interactions gives the advantage of relatively facile preparation without affecting pharmacological activity of the drug.[46] Drug−dendrimer complexation was proved to enhance drug solubility and bioavailability as well as to enable controlled release and drug targeting. Such complexes may deliver active

DOI: 10.1021/acs.biomac.7b01650
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form of the therapeutic directly to cancer cells, potentially overcoming limitations of classical chemotherapy such as multidrug resistance, disadvantageous biodistribution, or fast metabolism of the drug.[47,48] Application of drug delivery system may also eliminate harmful side effects, which in case of fludarabine treatment include myelosuppression (neutropenia, thrombocytopenia, and anemia), lymphocytopenia, and respiratory tract infections.[7]

PPI G4 dendrimer with 64 surface amino groups is characterized by semiopen and moderately rigid structure, which facilitates binding of ligand molecules.[12] Terminal amino groups, although responsible for high toxicity, play an important role in many medical applications.[49] Partial modification with sugar moieties reduces surface positive charge of PPI dendrimer, which increases its biocompatibility, at the same time leaving the possibility of forming ion pairs with negative molecules. Glycosylation has been found not only to significantly lower the innate cytotoxicity of PPI dendrimers, but also to improve specificity of their interactions with selected cell types.[50,51] Therefore, appropriate balance between the structure of PPI G4 dendrimer and the degree of surface sugar coating gives the possibility to create stable drug delivery system based on noncovalent complexes. Previous studies have shown that maltose-modified PPI dendrimers form stable complexes with nucleotides and are able to protect them from enzymatic degradation.[23,24] Furthermore, PPI-Mal OS G4 dendrimer was actively taken up by the leukemic cell, most probably along the receptor-mediated endocytosis pathway, reaching high intracellular concentrations,[48] and was able to deliver the triphosphate form of cytarabine (Ara-CTP) into CCRF cells.[25] When comparing our results to those previously obtained with Ara-CTP−dendrimer complexes, we see a much stronger impact of dendrimer on toxicity of Ara-FATP, especially in the presence of the hENT1 inhibitor. This is perhaps partially due to the fact that Ara-C can more readily than fludarabine circumvent facilitated transport and enter the cell passively (its polar surface area is only 129 A^2, whereas it is 140 A^2 for fludarabine−data from PubChem, pubchem.ncbi.nlm.nih.gov) and partially to the potentially better biophysical parameters of association of Ara-FATP with the dendrimer. These observations give hope for potential development of the maltose glycodendrimer as a highly specific carriers for fludarabine and potentially other nucleotide active forms of anticancer nucleoside analogue drugs.

For our research concerning fludarabine, we chose three human cell lines of different origin, representing various types of hematological malignancies: CCRF (acute lymphoblastic T cell leukemia), THP-1 (acute monocytic leukemia), and U937 (histiocytic lymphoma). Since previous studies involving cytarabine showed significant differences in drug activity and mode of nucleotide−dendrimer action,[25] we started with evaluation of cytotoxic activity of fludarabine in nucleoside and nucleotide form in selected cell lines. The obtained results were consistent with the scientific data on this subject−fludarabine triphosphate was significantly less toxic in comparison to nucleoside form, which is most likely associated with the necessity of Ara-FATP dephosphorylation before entering the cell. Further, the tested cell lines differed in susceptibility to fludarabine, which turned out to correlate with the level of hENT1 expression. On the other hand, the expression of hENT2 was significantly lower, and hCNT3 expression was not observed, which indicates the major role of hENT1 in the transport of fludarabine across cell membrane. Thus, the next part of our research involved the application of hENT1 inhibitor (NBMPR), which equally inhibited the activity of both forms of fludarabine, suggesting that the observed differences in cytotoxicity are related to the rate of intracellular transport.

Most importantly, the results of further experiments provided evidence for the facilitation of Ara-FATP cellular entry by PPI-Mal OS G4 dendrimer. The cytotoxicity of nucleotide−dendrimer complex, detected at the level of cell viability, was comparable to this of free nucleoside, while the addition of PPI-Mal OS G4 to the solution of Ara-FA did not enhance its activity. Subsequent use of hENT1 inhibitor enabled the final confirmation of our hypothesis that sugar-modified PPI dendrimers can be successfully applied as nanocarriers for phosphate forms of NAs bypassing their complicated pharmacokinetics, enabling the direct and specific delivery of the active drug form into cancer cells. Upon pretreatment with NBMPR, maltose-modified PPI dendrimer was able to deliver nucleotide through the cell membrane independently of the nucleoside transporter. This is especially important in view of above-mentioned resistance mechanisms associated with hENT1-dependent transport of fludarabine. On the other hand, after inhibition of hENT1, the cytotoxic activity of Ara-FA−dendrimer complex was similar to those observed for Ara-FA and Ara-FATP. This is not unexpected since previous data show that PPI-Mal OS G4 does not form complexes with adenosine.[24] Further, interactions between dendrimers and nucleoside form of cytarabine were proved to be very weak and physiologically irrelevant,[25] corroborating a similar effect for fludarabine nucleoside.

The final stage of our investigation involved determination of mechanism of action of Ara-FATP−dendrimer complex on subcellular level and direct comparison with the activity of fludarabine itself. The aim was to prove that the Ara-FATP action in complex with PPI-Mal OS G4 is not altered by the dendrimer macromolecule. Therefore, we evaluated an impact of free dendrimer, Ara-FA, Ara-FATP, and nucleotide−dendrimer complex on parameters directly associated with fludarabine pharmacodynamics, that is, DNA and RNA synthesis arrest, phosphatidylserine externalization, decrease in mitochondrial membrane potential, and activity of caspase-3. As expected, all tested forms of fludarabine influenced the same facets of cellular biochemistry, inhibiting both DNA and RNA synthesis and inducing the appearance of apoptosis hallmarks.

Fludarabine was the first anticancer nucleoside analogue proved to exhibit multiple mechanisms of cytotoxicity. The principal action of Ara-FATP is thought to involve inhibition of DNA synthesis. In particular, Ara-FATP competes as an alternative substrate with natural deoxyadenosine 5′-triphosphate (dATP), directly inhibiting the DNA polymerases and primase. Other enzyme targets of Ara-FATP in dividing cells include DNA ligase and topoisomerase II.[1] Fludarabine triphosphate is also a potent inhibitor of ribonucleotide reductase (RR), leading to decrease in cellular deoxynucleotide pools, decreasing the amount of dATP available for incorporation in newly synthesized DNA chains and consequently potentiating the competitive inhibitory potential of fludarabine toward DNA synthesis enzymes. This results in replication arrest and induction of DNA damage. Fludarabine-associated DNA breaks trigger mitochondrial permeability transition via p53 activation. Together, these actions result in complete inactivation of DNA synthesis followed by an initiation of programmed cell death. In dividing cells,

DOI: 10.1021/acs.biomac.7b01650
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incorporation of purine analogues into DNA, resulting in impaired replicative DNA synthesis and induction of apoptosis, appears to be crucial for cytotoxicity.[52] Furthermore, purine analogues have been known to inhibit RNA synthesis. A correlation between fludarabine-induced inhibition of RNA elongation and subsequent cell death in CLL cells was demonstrated, which indicated that global repression of gene transcription may contribute to the cytotoxic action of purine analogues by reducing the expression of proteins that are important for cell survival.[36] In fludarabine-treated cell lines, inhibition of RNA synthesis appears to result primarily from direct incorporation into RNA.[53,54] Finally, fludarabine has been reported as the most potent nucleotide activator of APAF-1, with the subsequent activation of caspase-9 and caspase-3 pathways.[55]

The design of our experiments, with multiple measurements reflecting both molecular mechanism and overall phenomenology of cell death, enabled us to draw interesting conclusions about the respective importance of intracellular targets of Ara-FATP action in our cellular model. Since our results demonstrate clearly that the glycodendrimer itself has no direct action on any targets related to cell death and it appears to function exclusively as a drug delivery vehicle for Ara-FATP (which otherwise would have to undergo dephosphorylation outside the cell and rephosphorylation inside it), the increased activity of the Ara-FATP−dendrimer complex with regard to DNA synthesis and apoptotic end points (in comparison to Ara-FA) must stem from issues related to different intracellular concentrations of Ara-FATP (the active metabolite), which can be reached via direct delivery versus phosphorylation of Ara-FA. On the other hand, Ara-FA and the Ara-FATP−dendrimer complex had identical activities toward RNA synthesis as well as long-term cell proliferation/cell death. It is a strong suggestion that the two molecular modes of action of fludarabine known from literature function simultaneously, with different affinities of relevant cellular targets. While DNA synthesis is inhibited by relatively high intracellular Ara-FATP concentrations, leading to fast-track apoptosis by intrinsic pathway activation (i.e., this mechanism will be more susceptible to direct Ara-FATP delivery as dendrimer complex), RNA synthesis is efficiently inhibited already at lower intracellular Ara-FATP concentrations, easily reachable also by Ara-FA delivery and intracellular phosphorylation. This view is supported also by our finding that the extent of RNA synthesis inhibition at the lowest drug concentrations tested (5 μM) is much deeper than for DNA synthesis. Moreover, we found that U937 is the cell line most vulnerable to fludarabine toxicity; among the cell lines studied by us, it is also the one with highest basal overall transcriptional rate[56] and our data. Therefore, we suggest that the major mechanism of long-term cell death/growth inhibition in leukemic cells at clinically relevant drug concentrations is actually inhibition of RNA synthesis, which has a less immediate effect on apoptotic pathways, but leads to an efficient cytostatic effect. Therefore, in our studies, the overall cytotoxicity of Ara-FA and Ara-FATP−dendrimer complex is virtually indistinguishable, and the crucial advantage of using dendrimer-mediated delivery rests on circumvention of potential drug resistance caused by impairment of nucleoside entry and phosphorylation rather than on any synergistic effects of the dendrimer on intracellular targets. The dendrimer behaves thus in this system as a perfectly bioorthogonal drug delivery agent.

CONCLUSIONS

To sum up, we were able to prove that PPI-Mal OS G4 dendrimer may serve as an efficient nanocarrier for active, triphosphate metabolite of fludarabine by enhancing its intracellular cytotoxic activity and bypassing hENT1-associated resistance, most probably due to the autonomous mode of cell entry. All evidence indicates mechanistic explanation of this phenomenon, where inherently nontoxic PPI-Mal OS G4 dendrimer does not alter specific activity of fludarabine, at the same time enhancing its cellular entry and enabling the direct and phosphorylation-independent toxicity due to noncovalent interaction with the nucleotide. These observations are promising prognoses for application of this type of dendrimer as nanocarrier, which may provide supreme alternative for classical chemotherapy, reducing its detrimental side effects and overcoming drug resistance mechanisms.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by National Science Centre, Poland (Project No. UMO-2014/13/B/NZ3/04643 "Cellular and molecular mechanism of action of PPI dendrimer complexes with nucleoside analogue anticancer drugs"). Graphical abstract was prepared in part using image vectors from Servier Medical Art (www.servier.com) licensed under the Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/). The authors thank Robin Zinke for synthesizing the glycodendrimer and Dr. Hartmut Komber for NMR measurement.

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on January 30, 2018, with errors in Figures 5 and 6. The corrected version was reposted on January 31, 2018.

DOI: 10.1021/acs.biomac.7b01650
Biomacromolecules 2018, 19, 531−543

Article

Cite This: *Biomacromolecules* 2019, 20, 1429−1442

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Fludarabine-Specific Molecular Interactions with Maltose-Modified Poly(propyleneimine) Dendrimer Enable Effective Cell Entry of the Active Drug Form: Comparison with Clofarabine

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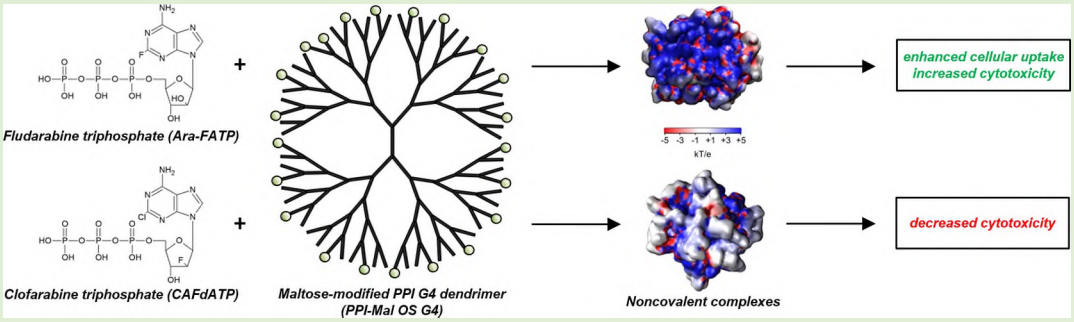
Supporting Information



ABSTRACT: Fludarabine is an anticancer antimetabolite essential for modern chemotherapy, but its efficacy is limited due to the complex pharmacokinetics. We demonstrated the potential use of maltose-modified poly(propyleneimine) dendrimer as drug delivery agent to improve the efficiency of therapy with fludarabine. In this study, we elaborated a novel synthesis technique for radioactively labeled fludarabine triphosphate to prove for the first time the direct ability of nucleotide−glycodendrimer complex to enter and kill leukemic cells, without the involvement of membrane nucleoside transporters and intracellular kinases. This will potentially allow to bypass the most common drug resistance mechanisms observed in the clinical setting. Further, we applied surface plasmon resonance and molecular modeling to elucidate the properties of the drug−dendrimer complexes. We showed that clofarabine, a more toxic nucleoside analogue drug, is characterized by significantly different molecular interactions with poly(propyleneimine) dendrimers than fludarabine, leading to different cellular outcomes (decreased rather than increased treatment efficiency). The most probable mechanistic explanation of uniquely dendrimer-enhanced fludarabine toxicity points to a crucial role of both an alternative cellular uptake pathway and the avoidance of intracellular phosphorylation of nucleoside drug form.

INTRODUCTION

Nucleoside analogues (NAs), first used clinically as antiviral agents over 50 years ago, quickly became the basis of modern anticancer chemotherapy due to the numerous antimetabolic activities. They are commonly used in the treatment of various types of cancer, including leukemias, lymphomas, and solid tumors. Their mechanism of action involves inhibition of enzymes engaged in the metabolism of natural nucleosides and nucleotides, as well as the direct incorporation into newly synthesized nucleic acid chains, resulting in inhibition of DNA and RNA synthesis. All these processes ultimately lead to cell death via apoptosis.[1,2] However, the possibility of application of NAs in cancer therapy is limited due to complicated and multistage metabolism. Because of their hydrophilic nature, these compounds cannot enter the cell by passive diffusion and require facilitated transport through cell membrane, mediated by nucleoside transporters (NTs). These proteins are commonly divided into two subclasses: equilibrative and concentrative NTs (ENTs and CNTs, respectively), with different affinities for various NAs. Inside the cell, nucleosides

Received: January 3, 2019
Revised: January 28, 2019
Published: February 1, 2019

DOI: 10.1021/acs.biomac.9b00010
Biomacromolecules 2019, 20, 1429−1442

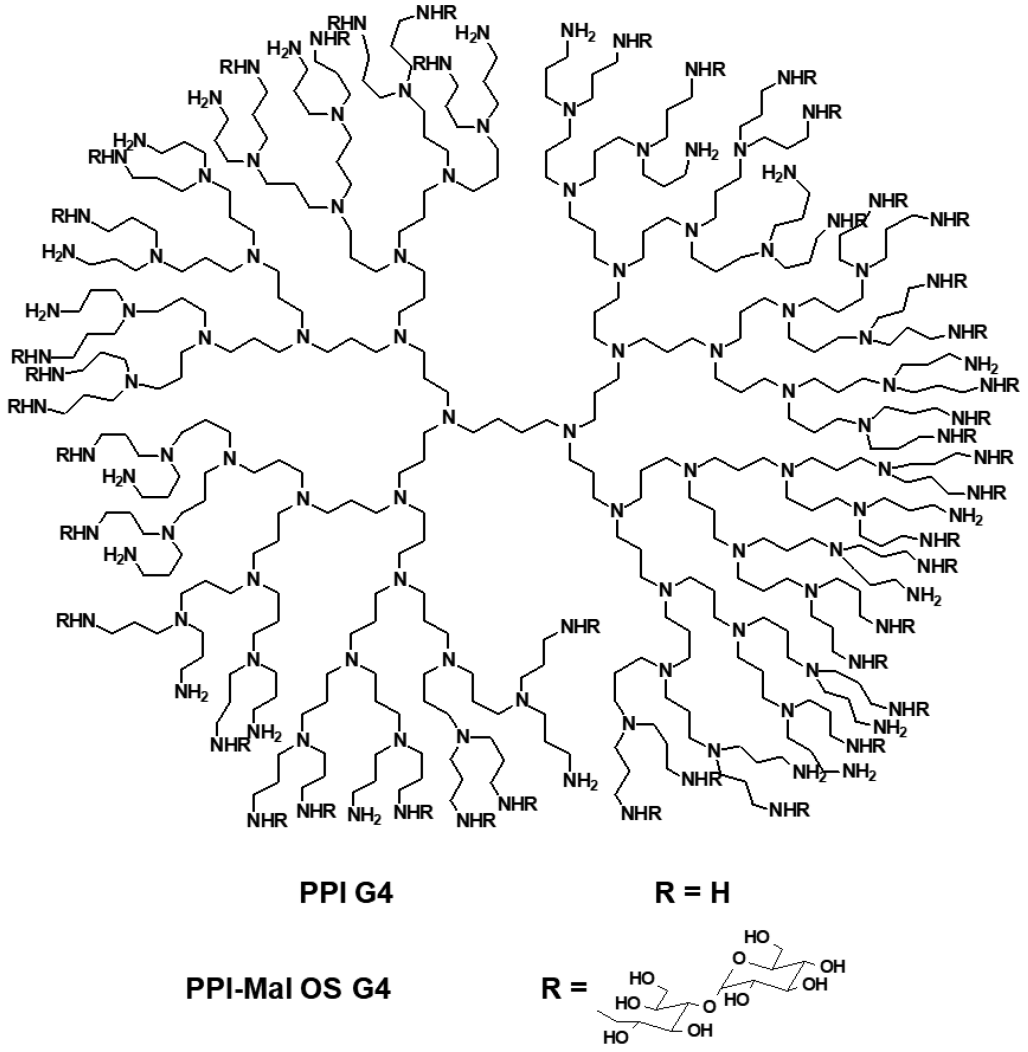


Figure 1. Chemical structure of unmodified (PPI G4) and partially modified with maltose units (PPI-Mal OS G4) poly(propyleneimine) dendrimers of the 4th generation. PPI-Mal OS G4 dendrimer has 43 maltose (Mal) moieties chemically coupled to 4th generation PPI scaffold.

are progressively phosphorylated to their triphosphate forms, which is essential for cytotoxic activity of this class of therapeutics.[1,3] Since NTs can transport only dephosphorylated substrates,[4,5] the administration of NAs is limited to prodrug forms instead of active triphosphates. This generates a number of possible resistance mechanisms, of which the most severe involve reduced surface expression of nucleoside transporters and decreased activity of cellular kinases, resulting in inefficient intracellular accumulation of cytotoxic nucleoside triphosphates.[1,2,6]

For the purpose of conquering resistance mechanisms and improvement of anticancer activity of NAs, several drug delivery systems, primarily based on the application of nanoparticles, have been proposed. Nanosystems with their autonomous ways of transmembrane passage and cargo release may overcome the limitations associated with NT-dependent cellular uptake of nucleoside analogues, at the same time protecting them from degradation or catabolism. Further, this approach may enable the delivery of active, triphosphate forms of NAs, thus preventing their inadequate phosphorylation inside the target cells. The use of nanocarriers could additionally diminish undesirable interactions of the drugs with normal cells due to the targeted delivery, thus reducing detrimental side effects of classical chemotherapy.[1,7−9]

The variety of nanoparticles proposed as drug carriers for NAs provides a number of possibilities to improve the efficiency of nucleoside analogue-based therapies. Of these, highly branched cationic dendrimers with well-defined, three-dimensional structure are of particular interest, primarily due to their ability to form stable, noncovalent complexes with nucleoside triphosphates.[10] Numerous studies on the drug delivery potential of these macromolecules indicate that dendrimers provide high loading capacity, enhanced solubility, prolonged blood half-life, and controllable biodistribution of therapeutics. Moreover, the nanometric size and globular shape of dendrimers are beneficial for cell uptake.[11−14] All these features make them particularly fine candidates for drug delivery devices.

Since cationic dendrimers (such as poly(amidoamine) (PAMAM) and poly(propyleneimine) (PPI) macromolecules) are characterized by high cytotoxic activity associated with nonspecific interactions with cellular membranes, damage to mitochondria, and generation of reactive oxygen species,[15,16] it is crucial to reduce their surface positive charge in order to enable clinical applications. This can be achieved by chemical conjugation of surface amino groups with neutral molecules,[17] such as sugar moieties. Glycosylation may bring additional benefits for the delivery of antileukemic drugs, as leukemic cells

DOI: 10.1021/acs.biomac.9b00010
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are usually overexpressing surface lectin receptors with high affinity for carbohydrate ligands. This may enable specific, receptor-mediated cellular uptake of sugar-coated dendrimers, further enhancing drug delivery efficiency.[18,19]

PPI dendrimers with the surface partially modified with maltose moieties are particularly promising vehicles for nucleotides due to their significantly reduced cytotoxic activity compared to unmodified macromolecules and maintained surface positive charge allowing for electrostatic interactions with negatively charged phosphate groups.[20−22] In comparison to chemical conjugation, noncovalent physical incorporation of drug molecules into the dendrimer's structure enables relatively facile preparation of carrier system without changing the pharmacological activity of the therapeutic.[23] Drug−dendrimer complexation has been shown to improve drug solubility and bioavailability, as well as to provide targeted delivery and controlled release. Noncovalent complexes may transport active forms of therapeutics directly to cancer cells, potentially overcoming limitations of standard chemotherapy such as multidrug resistance, unfavorable biodistribution, or rapid metabolism of the drug.[24] Further, glycosylation not only decreases the innate cytotoxicity of PPI dendrimers, but also provides specific interactions with selected cell types.[25,26] Thus, an appropriate degree of surface sugar coating gives the possibility to create a stable, biocompatible drug delivery system based on noncovalent complexes between cationic PPI dendrimers and nucleotides.

Our research interests focused on the possibility of using so-called "open-shell" (OS) poly(propyleneimine) dendrimers with surface partially modified with maltose moieties for the delivery of active, triphosphate forms of NAs to tumor cells. Such PPI glycodendrimers have been previously shown to possess high biocompatibility, form stable complexes with nucleoside triphosphates,[20,22] and shield nucleotides from enzymatic degradation.[27] We were able to prove that maltose-coated PPI dendrimer of the fourth generation (PPI-Mal OS G4) may serve as an efficient nanocarrier for fludarabine triphosphate (Ara-FATP), enhancing its cytotoxic activity and bypassing NT-associated resistance, most probably due to the autonomous mode of cell entry.[28] In this work, we have followed up on the mechanistic explanation of this phenomenon; a novel enzymatic method for the synthesis of fludarabine triphosphate from radiolabeled nucleoside allowed us to ultimately confirm that cellular uptake of nucleotide−dendrimer complexes occurs via an alternative, NT-independent mode. In the further course of our studies, we decided to determine whether PPI-Mal OS G4 dendrimer may serve as a universal carrier for adenosine analogues. Comparison of impact of dendrimer on clofarabine and fludarabine toxicity together with surface plasmon resonance (SPR) and molecular modeling studies allowed us to highlight the phenomenological and mechanistic differences in pharmacological drug−dendrimer interactions.

MATERIALS AND METHODS

Materials. Poly(propyleneimine) dendrimer of the fifth generation (MW 7167.97 g/mol) with 64 terminal amino groups (Figure 1) was obtained from SyMO-Chem (Eindhoven, The Netherlands) and specified as fourth generation (PPI G4) following the uniform nomenclature description of polyamine dendrimers for poly(propyleneimine) and poly(amidoamine) macromolecules.[29]

PPI dendrimer of the fourth generation with primary surface amino groups partially modified with maltose (PPI-Mal OS G4, MW 21113 g/mol; so-called "open shell" glycodendrimer, Figure 1) was prepared as described previously.[30,31] Validation of PPI glycodendrimer molecular properties (structure confirmation by ^{13}C NMR,[30] molecular weight verification by a ratiometric ^{1}H NMR approach[30] and retention of positive surface charge by zeta potential measurement[22]) was also performed as described previously.

In the course of experiments, the following nucleoside analogues were used:

- 2-fluoroadenine-9-β-D-arabinofuranoside (fludarabine, Ara-FA) and 2-chloro-2′-arabino-fluoro-2′-deoxyadenosine (clofarabine, CAFdA; Figure 2A and C, respectively) from Sigma-Aldrich,

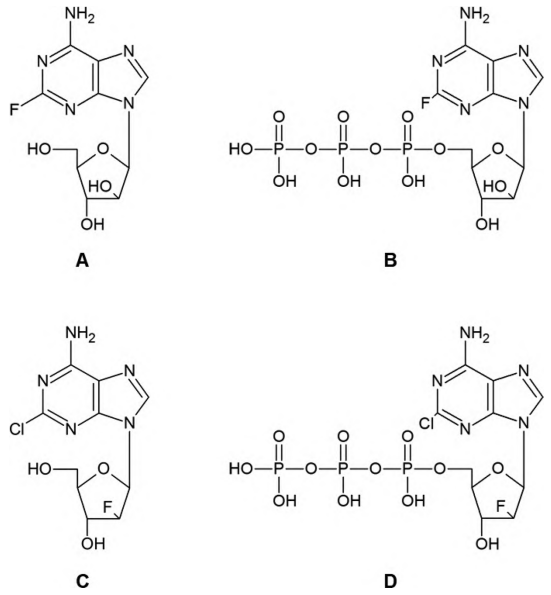

Figure 2. Chemical structures of fludarabine and clofarabine in nucleoside (A and C, respectively) and triphosphate forms (B and D, respectively).

- fludarabine-5′-triphosphate (Ara-FATP) and clofarabine-5′-triphosphate (CAFdATP; Figure 2B and D, respectively) from Jena Bioscience,
- radiolabeled 2-fluoroadenine-9-β-D-arabinofuranoside (^{3}H-Ara-FA, [8-^{3}H]-) from Moravek Biochemicals Inc. packaged in ethanol/water (1:1) solution at a concentration of 24.04 μg/mL, 1.0 mCi/mL; specific activity 11.9 Ci/mmol.

Synthesis and Purification of Tritium-Labeled Fludarabine Triphosphate (^{3}H-Ara-FATP). A total of 7 nmol of nucleoside (^{3}H-Ara-FA) was used as a substrate in the enzymatic phosphorylation to form fludarabine triphosphate (^{3}H-Ara-FATP). Initially, ethanol was removed from the sample of radiolabeled nucleoside by centrifugation in low temperature (20 °C). Then the nucleoside was dissolved in low volume of 2.5 mM formic acid. Phosphorylation of fludarabine nucleoside to the triphosphate form was performed by multistep, single-tube enzymatic reaction. The reaction mixture (10 μL) contained: buffer (10 mM Tris-HCl, pH 8.0; 20 mM KCl; 2 mM $MgCl_2$), 7 nmol ^{3}H-Ara-FA, 5 nmol adenosine-5′-triphosphate (ATP, Sigma-Aldrich), 200 nmol phosphocreatine (PC, Sigma-Aldrich), 2 mU deoxycytidine kinase (dCK, NovoCIB), 1.4 U adenylate kinase (AK, Sigma-Aldrich), and 2 U creatine kinase (CK, Sigma-Aldrich). Fludarabine triphosphate was formed in following reactions:

catalyzed by dCK:

$$\text{Ara-FA} + \text{ATP} \rightarrow \text{Ara-FAMP} + \text{ADP} \quad (1)$$

catalyzed by AK:

$$\text{Ara-FAMP} + \text{ATP} \leftrightarrow \text{Ara-FADP} + \text{ADP} \quad (2a)$$

$$\text{Ara-FAMP} + \text{Ara-FATP} \leftrightarrow 2\text{Ara-FADP} \quad (2b)$$

DOI: 10.1021/acs.biomac.9b00010
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catalyzed by CK:

$$\text{ADP} + \text{PC(excess)} \rightarrow \text{ATP} + \text{creatine} \quad (3a)$$

$$\text{Ara-FADP} + \text{PC(excess)} \rightarrow \textbf{Ara-FATP} + \text{creatine} \quad (3b)$$

Incubation was performed at 37 °C for 2 h, then the reaction was stopped by denaturation of enzymes with 1 μL of concentrated formic acid (ca. 20 M), and the reaction tube was centrifuged (14000 × g, 5 min) to remove denatured proteins. Supernatant was then separated by thin-layer chromatography on the TLC plastic sheets covered with PEI-cellulose (Merck KGaA). A total of 1 μL of mixture was used for analytical separation (visualized by autoradiography, Figure S1 in Supporting Information) and 10 μL was used for preparative chromatography. Chromatography was run with 3 M ammonium formate (pH 3.5) containing 0.35 M NaCl. In these conditions, nucleotide products and potentially unreacted radiolabeled nucleoside substrate are well separated: R_f (Ara-FATP) = 0.13, R_f (Ara-FA) = 0.71, R_f (ATP) = 0.33. Preparative TLC plate was visualized under UV light at 254 nm. Plate fragment containing ^{3}H-Ara-FATP was cut and nucleotide was extracted with 40 μL of 1.5 M ammonia solution. Next, extracted radiolabeled nucleotide was mixed with equal volume of 100% ethanol and dried by centrifugation at 8 °C. Finally, purified ^{3}H-Ara-FATP was dissolved in 50 μL of deionized water and stored at −20 °C.

Cell Culture. U937 (histiocytic lymphoma) human cell line was purchased from ATCC (U.S.A.). Cells were maintained under standard conditions in RPMI-1640 Medium (ThermoFisher) supplemented with 10% fetal bovine serum (Sigma-Aldrich) at 37 °C in an atmosphere of 5% CO_2. Cells were subcultured three times per week.

Internalization of ^{3}H-Ara-FA, ^{3}H-Ara-FATP and Their Complexes with PPI-Mal OS G4 in U937 Cells. PPI-Mal OS G4 dendrimer was dissolved in double-distilled water to a final concentration of 100 μM. Dendrimer solutions were freshly prepared and used on the same day. Stock solutions of the drugs were prepared by mixing 99 parts of nonradioactive Ara-FA or Ara-FATP and 1 part of ^{3}H-Ara-FA or ^{3}H-Ara-FATP, respectively. Compounds were then mixed with the dendrimer at a drug/dendrimer molar ratio of 10:1 (to a final drug concentration of 100 μM). This molar ratio ensures complete complexation of triphosphate molecules by maltose-modified dendrimer, as shown previously.[20,22] The mixtures were stirred for 0.5 h at room temperature, and obtained complexes were used in subsequent experiments.

U937 cells were seeded into 96-well transparent plates at a density of 2.8 × 10^5 cells in 130 μL of medium per well. To inhibit the transport of fludarabine across cell membrane, cells were preincubated with inhibitor of hENT1, nitrobenzylmercaptopurine (NBMPR, Sigma-Aldrich) at the final concentration of 50 μM for 45 min. The cells (with or without NBMPR) were subsequently treated with free drugs or drug-dendrimer complexes (final drug concentration: 10 μM) and incubated at 37 °C in an atmosphere of 5% CO_2 for 5 min, 1, 3, 6, and 9 h. Following the incubation, the cells were transferred to MultiScreen HTS BV Filter Plates (Merck KGaA) and medium was removed by filtration. Cells were washed twice with fresh medium, and then Optiphase Supermix scintillation cocktail (PerkinElmer, Waltham, U.S.A.) was added to each well. For total radioactivity measurements, the same amount of radiolabeled drug or drug−dendrimer complex was added directly to the scintillation cocktail. Intracellular and total radioactivity was determined by counting (1 min) in Wallac 1410 scintillation counter. Intracellular drug content was calculated relative to total amount of added drug and presented as percentage.

Surface Plasmon Resonance (SPR) Analysis. SPR experiments were performed using Biacore X100 (GE Healthcare). 150−170 resonance units (RUs) of PPI G4 or 500−600 RUs of PPI-Mal OS G4 were immobilized on the surface of CM5 chip (GE Healthcare) using amine coupling chemistry in 10 mM HEPES buffer pH 5.5. Higher level of immobilization in case of PPI-Mal OS G4 was dictated by surface modification with maltose units and almost 3× higher molar mass compared to PPI G4. Binding kinetics assays were performed in 10 mM HEPES buffer pH 7.4, using at least five different concentrations of the analyte (fludarabine or clofarabine in nucleoside or triphosphate forms). Analytes were injected at a flow rate of 30 μL/min with an association time of 120 s and a dissociation time of 120 s. A 10 mM NaOH was used for chip regeneration. The binding data were fitted to the Langmuir 1:1 binding model using Biacore X100 Evaluation software and analyzed using the affinity approach, allowing to calculate dissociation constant (K_D) by determining the sensor response in equilibrium (steady state) as a function of analyte (drug) concentration (Figure S2 in Supporting Information). Determinations of association and dissociation rate constants (k_a and k_d, respectively) were performed, but yielded values outside the measurement limits of the instrument.

Cytotoxicity Assay. To estimate the cytotoxicity of the dendrimers, drugs and their complexes, resazurin assay was performed.[32] PPI G4 and PPI-Mal OS G4 dendrimers were dissolved in double-distilled water to a final concentration of 1.2 μM. Dendrimer solutions were freshly prepared and used on the same day. Clofarabine in the form of nucleoside (CAFdA) or triphosphate (CAFdATP) was added to the dendrimer solutions at a molar ratio of 10:1 (to a final drug concentration of 12 μM). The mixtures were stirred for 0.5 h at room temperature.

U937 cells were seeded into 96-well black plates at a density of 1 × 10^4 cells in 90 μL of medium per well, and treated with increasing concentrations of CAFdA, CAFdATP, and their complexes with PPI G4 or PPI-Mal OS G4 (10−1200 nM drug concentration) or free dendrimers (1−120 nM) for 72 h at 37 °C in an atmosphere of 5% CO_2. The 10× concentrated working solutions of tested compounds were prepared and added to the cells in volumes of 10 μL.

Following the incubation, resazurin was added to the culture medium to a final concentration of 10 μg/mL and the plates were incubated at 37 °C in darkness to allow conversion of resazurin to resorufin. Fluorescence of metabolized resazurin in the cell suspension was measured after 90 min at 530 nm excitation and 590 nm emission using EnVision plate reader (PerkinElmer, Waltham, U.S.A.). Data were presented as percentage of viability of control (untreated) cells.

In Silico Studies. The 3D models of negatively charged nucleoside triphosphates (Ara-FATP, CAFdATP) and cationic dendrimers (PPI G4, PPI-Mal OS G4) were developed using Avogadro chemical editor[33] and Linux bash scripts made in house.

The General Amber Force Field (GAFF)[34] was used to describe the molecule topologies. Partial charges were calculated considering separate residues by the RESP fitting method at the HF/6-31G level of theory using AM1-BCC charges as in a number of previous works.[35−40] A proper distribution of partial charges was provided, which correctly takes into account the conformation of the dendrimers and related bonds. Then, the topology and parametrization were developed using antechamber and GROMACS associated tools[41−45] and an in house code.[40]

Four molecular systems were created by combining nucleoside triphosphates and dendrimers. Each system consisted of a single triphosphate and a single dendrimer (ratio 1:1). More in detail, the triphosphate and the dendrimer were positioned at a starting distance of about 1.5 nm and a random orientation in a dodecahedron box filled with TIP3P water molecules[46] and ions to neutralize the system charge. The resulting molecular systems (e.g., CAFdATP-PPI G4) were composed of roughly 50000 interacting particles. Each system was energy-minimized by 1000 steps of steepest descent energy minimization algorithm. A 100 ps position restrained MD was performed at 300 K[47] and 1 atm.[48] Finally, a 100 ns long MD was performed in the NVT ensemble (N = number of particles, V = volume, and T = temperature) at 300 K. Atom velocities were randomly initialized following a Maxwell−Boltzmann distribution.

For each system, a combination of three independent repetitions of the above-explained procedure was carried out. In total, 300 ns MD was performed to sample the conformational space of each considered system. The last 50 ns of 3 MD repetitions were considered as a single ensemble trajectory. Hence, the total ensemble trajectory considered

DOI: 10.1021/acs.biomac.9b00010
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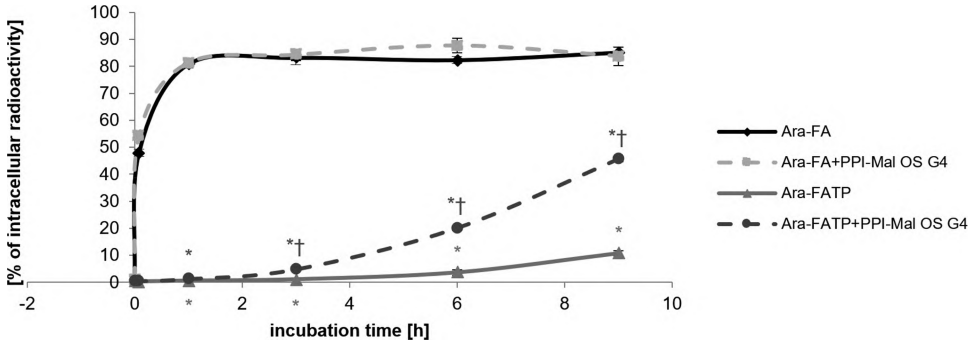

Figure 3. Cellular uptake of radiolabeled Ara-FA and Ara-FATP complexed with PPI-Mal OS G4 into U937 cells. Data presented as ratio of intracellular radioactivity to total radioactivity (percentage), average ± S.E.M., $n = 3$. *Statistically significant difference compared to Ara-FA at $p < 0.05$. †Statistically significant difference compared to Ara-FATP at $p < 0.05$.

for statistical analysis was 150 ns long and composed of 75 million molecular configurations.

Moreover, the impact of the nucleoside triphosphate on the nanoparticle surface properties was investigated. Further set of MD simulations has been carried out considering the ratio 10:1 between the nucleotide and the dendrimer. Following above-described approach, each dendrimer was simulated in the presence of ten triphosphate molecules randomly oriented in an explicitly solvated box. In particular, for each combination (CAFdATP-PPI G4, CAFdATP-PPI-Mal OS G4, Ara-FATP-PPI G4, Ara-FATP-PPI-Mal OS G4) five repetitions of a 100 ns long MD simulation were performed. The last 50 ns of each repetition were considered as an ensemble trajectory (250 ns in total and 125 million of molecular configurations analyzed). Nanoparticles were further investigated in terms of surface properties by quantifying the surface electrostatic potential. Simulation frames were extracted from the ensemble trajectory of nucleotide-dendrimer complexes. Electrostatic potentials in the presence of bound triphosphates were compared with electrostatic potential calculated for single dendrimers in water. The electrostatic potentials were computed by the APBS package.[49] In detail, nonlinear Poisson−Boltzmann equation was applied using single Debye−Huckel sphere boundary conditions on a 200 × 200 × 200 grid with a spacing of 1 Å centered at the COM of the molecular system. The relative dielectric constants of the solute and the solvent were set to 4 and 78.4,[50,51] respectively. The ionic strength was set to 150 mM and the temperature was fixed at 300 K.[50,51]

GROMACS 5 package was used for all MD simulations and data analysis.[43] Long-ranged electrostatic interactions were calculated at every step with the Particle-Mesh Ewald method with a cutoff of 1 nm. The same cutoff was also applied to Lennard-Jones interactions. LINCS algorithm[52] approach enabled an integration time step of 2 fs. Visual Inspection of Simulations was performed by The Visual Molecular Dynamics (VMD)[53] package and used for the visual inspection of the simulated systems.

Statistics. For statistical analysis we used one-way ANOVA for concentration series followed by posthoc Tukey's test for pairwise difference testing. For single pairwise comparisons, Student's t test was applied. In all tests, p values <0.05 were considered to be statistically significant.

RESULTS

Fludarabine Uptake into U937 Cells. In order to comprehensively illustrate the drug delivery potential of PPI-Mal OS G4 dendrimer, cellular uptake tests were performed on U937 cell line. We have previously demonstrated high sensitivity of this cell line to fludarabine, as well as high cytotoxic activity of Ara-FATP-dendrimer complex in U937 cells with inhibited hENT1, the main nucleoside transporter responsible for Ara-FA cellular uptake.[28]

For direct measurements of cellular uptake of nucleoside and nucleotide drug forms, the development of a novel efficient synthesis method for Ara-FATP from radiolabeled Ara-FA was needed. Application of a chain of enzymatic reactions with equilibrium shift allowed us to reach near-quantitative yields, which facilitated purification. Thus, we were able to directly monitor the intracellular drug concentration and we showed that, in line with previous reports,[54,55] the internalization of Ara-FA was very rapid, reaching the maximum intracellular concentration (about 80% of the initial amount of Ara-FA) after 1 h of incubation. The addition of maltose-modified PPI dendrimer did not affect cellular uptake of fludarabine nucleoside (Figure 3).

On the other hand, internalization of free Ara-FATP began after 6 h, which was most probably associated with its hydrolysis to Ara-FA, since only dephosphorylated (nucleoside) forms can undergo NT-mediated transmembrane transport.[1,2,4,5] In this case, an intracellular concentration of the drug reached about 10% after 9 h of incubation (Figure 3).

Interestingly, PPI-Mal OS G4 dendrimer was able to efficiently deliver Ara-FATP into the cells (with intracellular content reaching about 5% after 3 h, 20% after 6 h, and 45% after 9 h of incubation; Figure 3). These results indicate a large fraction of dendrimer-complexed fludarabine triphosphate entering the cell directly, in comparison to a much smaller uptake of Ara-FA generated by Ara-FATP dephosphorylation. Although the drug−dendrimer ratio of 10:1 provides complete complexation of triphosphate molecules by the dendrimer macromolecule, previous reports show that about 26% of triphosphate in the complex may detach and hydrolyze.[27] Therefore, the impact of dendrimer on nucleotide hydrolysis rate is most probably negative and the fraction of drug entering as Ara-FATP−dendrimer complex is even greater than can be calculated from difference with spontaneously hydrolyzing uncomplexed Ara-FATP.

To further validate the hypothesis that the majority of drug enters cells as nucleotide−dendrimer complex, we tested the cellular uptake of drugs and drug−dendrimer formulations in the presence of a well-known inhibitor of hENT1, nitrobenzylmercaptopurine (NBMPR).[56] The preincubation of cells with NBMPR resulted in complete inhibition of Ara-FA uptake into U937 cells both in absence and presence of the dendrimer. The residual uptake of (hydrolysis-derived) Ara-FA after incubation with free Ara-FATP was also totally inhibited. By contrast, maltose-modified PPI dendrimer was able to deliver Ara-FATP into the cells in a virtually uninhibited manner. The decrease in internalized drug amount compared to tests

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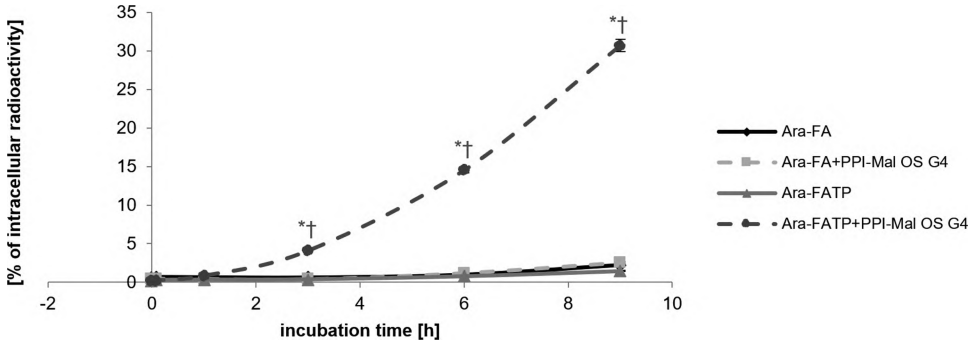

Figure 4. Cellular uptake of radiolabeled Ara-FA and Ara-FATP complexed with PPI-Mal OS G4 into U937 cells in the presence of hENT1 inhibitor NBMPR. Data presented as ratio of intracellular radioactivity to total radioactivity (percentage), average ± S.E.M., $n = 3$. *Statistically significant difference compared to Ara-FA at $p < 0.05$. †Statistically significant difference compared to Ara-FATP at $p < 0.05$.

without hENT1 inhibitor was slight and attributable, as mentioned above, to a fraction of Ara-FATP complexed with dendrimer subjected to detachment/dephosphorylation (Figure 4). These observations provide an ultimate proof for the hypothesis previously formulated on the basis of drug and drug−dendrimer activity assays:[28] the triphosphate−dendrimer complex possesses an alternative mode of cellular entry, independent of hENT1.

Confirmation of Drug−Dendrimer Complexation by SPR. Since drug transport measurements are not enough to prove the direct physicochemical interactions between drug and carrier molecules, we applied label-free biophysical method of surface plasmon resonance to characterize the properties of such binding. According to our expectations, no changes in SPR signal from chip-immobilized PPI-Mal OS G4 dendrimer were detected upon incubation with nucleoside form of the drug (Ara-FA), indicating lack of complex formation. Since there is abundant literature data on nucleoside/nucleotide interactions with unmodified (amino-terminated) PPI dendrimers, we confirmed that also for this type of macromolecule (PPI G4) no interactions with Ara-FA occurred. This lack of nucleoside-dendrimer interaction is not specific to Ara-FA: the nucleoside form of another commonly used anticancer drug, clofarabine (CAFdA), was also tested and showed no detectable binding. By contrast, triphosphate forms of both drugs showed binding to both PPI-Mal OS G4 and unmodified PPI G4 with quantifiable affinities (Table 1). The strength of the interactions was slightly increasing in the following order: Ara-FATP + PPI-Mal OS G4 < Ara-FATP + PPI G4 < CAFdATP + PPI-Mal OS G4 < CAFdATP + PPI G4 (with the decrease of K_D values).

Table 1. K_D Values Determined by SPR (Affinity Analysis), Presented as Average ± S.E.M.[a]

	K_D ± S.E.M. (μM)
Ara-FATP + PPI-Mal OS G4	157.65 ± 5.85
Ara-FATP + PPI G4	100.20 ± 0.21
CAFdATP + PPI-Mal OS G4	87.60 ± 1.74
CAFdATP + PPI G4	54.95 ± 4.37

[a]Statistically significant differences between all values have been observed at $p < 0.05$.

Cytotoxicity of Clofarabine and Clofarabine−Dendrimer Complexes in U937 Cell Line Model. Since clofarabine is known to be more toxic than fludarabine, we verified whether application of dendrimer carrier would improve the cellular pharmacokinetics of clofarabine triphosphate analogously to previous observations for fludarabine. For this purpose, we verified the viability of U937 cells exposed to nucleoside (CAFdA) and nucleotide (CAFdATP) forms of clofarabine, as well as their complexes with PPI-Mal OS G4 dendrimer, after 72 h of treatment.

In line with our expectations, CAFdA was found to be more toxic than its triphosphate derivative (CAFdATP). Remarkably, upon complexation with PPI-Mal OS G4 dendrimer, the activity of CAFdATP was decreased compared to free nucleotide. Thus, we also applied unmodified PPI G4 as a potential carrier of clofarabine triphosphate, since we previously demonstrated the formation of CAFdATP-PPI G4 complex. In this case, the CAFdATP activity was inhibited to an even greater extent by PPI G4 than PPI-Mal OS G4 complexation. CAFdA showed no altered cytotoxic activity in formulations with both dendrimers compared to the nucleoside form alone (Figure 5, Table 2).

In control experiments, no significant impact of the dendrimer alone on cell survival was detected after 72 h exposure within the whole concentration range used for the formation of complexes, for both dendrimers (data not shown).

Molecular Modeling. In order to explain observed differences in the nucleotide delivery potential of both PPI macromolecules, we set out to characterize properties of drug−dendrimer complexes using molecular modeling.

In every case, the nucleotide−dendrimer binding was detected in the first 50 ns of the MD simulation, whereas unbinding events were not detected throughout the entire simulation. This evidence suggests that the binding free energy was significantly higher than thermal energy for all investigated systems.

The interaction mode has been investigated by determining nucleotide orientation and distance with respect to the dendrimer over all conformational states of nucleotide−dendrimer systems (75 million of configuration states were considered for each system).

The nucleotide orientation was quantified by analyzing the variation of the amplitude of a specific angle (ϑ) during the molecular dynamics (Figure 6A,B). The angle was defined by 3 regions identified as follows: the dendrimer center of mass, the center of mass of the atoms of phosphate "tail" (brown particles in Figure 6A) and nitrogen atoms of the nucleotide "head" (blue particles in Figure 6A). The calculated angle directly correlated with the nucleotide orientation with respect to the dendrimer surface. The continuous variation of ϑ during

DOI: 10.1021/acs.biomac.9b00010
Biomacromolecules 2019, 20, 1429−1442

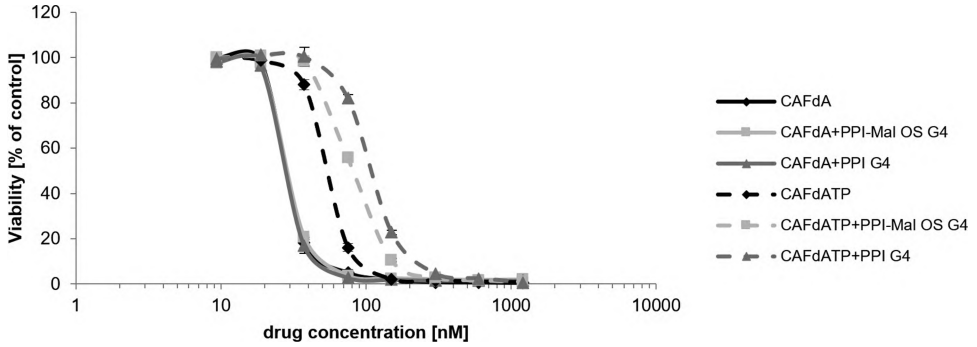

Figure 5. Cytotoxic effect of CAFdA, CAFdATP and their complexes with PPI G4 and PPI-Mal OS G4 dendrimer on U937 cells. Viability was determined by resazurin assay after 72 h of treatment. Data presented as percentage of viability of control (untreated) cells, average ± S.E.M., $n = 3$.

Table 2. Cytotoxic Activity (IC_{50} Values) of CAFdA, CAFdATP, and Their Complexes with PPI G4 and PPI-Mal OS G4 Dendrimer on U937 Cells after 72 h of Treatment

	IC_{50} ± S.E.M. (nM)
CAFdA	31.05 ± 2.66
CAFdA + PPI-Mal OS G4	32.81 ± 2.50
CAFdA + PPI G4	30.69 ± 1.67
CAFdATP	54.45 ± 1.48[a]
CAFdATP + PPI-Mal OS G4	78.70 ± 1.39[a,b]
CAFdATP + PPI G4	107.40 ± 4.74[a,b,c]

[a]Statistically significant difference compared to CAFdA at $p < 0.05$. [b]Statistically significant difference compared to CAFdATP at $p < 0.05$. [c]Statistically significant difference compared to CAFdATP+PPI-Mal OS G4 at $p < 0.05$.

the simulation was discretized in three main orientations and the probability to adopt particular orientation was quantified in histograms reported in Figure 6B.

It has been shown that CAFdATP orientation changes significantly when bound to PPI G4 or PPI-Mal OS G4 dendrimer. In CAFdATP-PPI G4 complex the nucleotide was mainly oriented with the head toward the dendrimer interior, whereas CAFdATP-PPI-Mal OS G4 was characterized by the nucleotide tail directed to the center of macromolecule (Figure 6B). On the other hand, Ara-FATP had a tendency to lie flat on the surface of PPI G4 dendrimer, whereas for PPI-Mal OS G4 no clear trend was observed (Figure 6B).

The nucleotide penetration data (Figure 6C,D) highlighted higher tendency of CAFdATP to reach inner layers of the PPI G4 in comparison to Ara-FATP (Figure 6D), most probably due to the higher probability of clofarabine nucleotide to bind the dendrimer with the head oriented toward the macromolecule center. Interestingly, in case of complexes with PPI-Mal OS G4, CAFdATP distance from the dendrimer center of mass was greater, while Ara-FATP adopted a flat orientation on the dendrimer surface (Figure 6D). Combining information from penetration data (Figure 6D) together with data from orientation analysis (Figure 6B), it can be concluded that CAFdATP was stably bound to the PPI-Mal OS G4 dendrimer due to the interactions between dendrimer surface amino groups and the nucleotide charged phosphate tail. In this case, CAFdATP head was more exposed to the solvent.

It is worth mentioning that the thickness of calculated PPI-Mal OS G4 layers (Figure 6D) differed for Ara-FATP and CAFdATP. However, the differences were always in the same order of layer size standard deviation (roughly 0.05 nm in all cases). Hence, those differences were negligible and mostly related to the dendrimer orientation and structure fluctuation in the sampled ensemble.

The nucleotide Radial Distribution Function (RDF) versus dendrimer distance quantification (Figure 7) enabled more comprehensive understanding of molecular phenomena driving the nucleotide−dendrimer complexation. For each system, RDF was calculated for two well-defined nucleotide groups of atoms (electronegative atoms of head and tail identified in Figure 7, top) with respect to the dendrimer outer layer. Hence, RDF plots provided a clear indication of short-range electrostatic interactions that stabilize the bonding.

RDF corroborated the orientation and penetration analysis (Figure 6). More in detail, CAFdATP head did not show significant primary interactions with the dendrimer outer layer when bound to both dendrimers (black line in Figure 7A,C). The nucleotide head was either positioned in the inner layer (when bound to PPI G4) or exposed to the solvent (when bound to PPI-Mal OS G4). Only the CAFdATP tail was stabilized by primary interactions with amino groups of the dendrimer outer layer.

By contrast, both the head (in approximately 25%) and the tail (in approximately 75%) of Ara-FATP contributed to primary interactions with the dendrimer amine layer (red line in Figure 7A,C).

Data from orientation, penetration (Figure 6), and RDF analysis (Figure 7) indicated different interaction modes characterizing CAFdATP and Ara-FATP when bound to PPI G4 and PPI-Mal OS G4 dendrimers. Figure 8 shows representative trajectory snapshots, providing a clear picture of the interaction mode. Again, CAFdATP-PPI G4 interaction was characterized by the nucleotide head completely embedded into the inner layer of the dendrimer, whereas for the CAFdATP-PPI-Mal OS G4 complex, the nucleotide head was exposed to the solvent. This may result in a different nanoparticle ability to release the nucleotide and eventually affect its efficiency. In particular, one might expect the CAFdATP-PPI-Mal OS G4 complex (nucleotide head exposed to the solvent) to release the drug more efficiently than the CAFdATP-PPI G4 (nucleotide head buried in the inner layer of the dendrimer).

Ara-FATP complexed either with PPI-G4 or PPI-Mal OS G4 did not show significant differences in the interaction mode. In both cases, a flat orientation of the nucleotide with respect to the dendrimer surface has been observed. Moreover, also RDF plots showed a noteworthy contribution of nucleotide head in primary interactions with the dendrimer surface amino groups.

Molecular interaction mode may also affect overall nanoparticle surface properties. To investigate this aspect, another

DOI: 10.1021/acs.biomac.9b00010
Biomacromolecules 2019, 20, 1429−1442

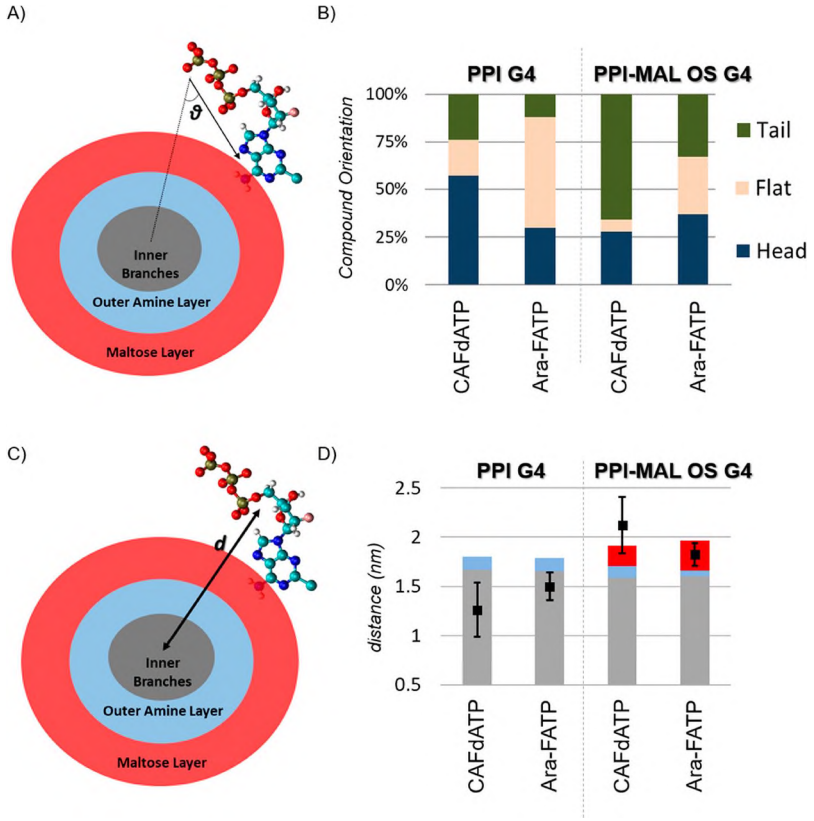

Figure 6. (A) Scheme of the nucleotide−dendrimer interaction mode with a focus on nucleotide orientation. The dendrimer structure is simplified in inner branches (gray area), and the outer surface is made by an amine layer (cyan area). For the PPI-Mal OS G4 dendrimer, there is an additional layer composed of maltose units (red area). To calculate the orientation of the ligand over the dendrimer, the angle (ϑ) has been defined. The angle ϑ connects three regions: the dendrimer center of mass, the center of mass of the phosphate tail atoms (brown particles), and nitrogen atoms of the nucleotide head (blue particles). (B) Binding orientation probability. Blue histogram indicates the probability of binding with the nucleotide head turned toward the center of the dendrimer ($0° \leq \vartheta \leq 75°$). Creamy-white histogram shows the probability of binding with the nucleotide lying flat over the dendrimer surface ($75° \leq \vartheta \leq 105°$). Green histogram represents the probability of binding with the nucleotide tail turned toward the center of the dendrimer ($105° \leq \vartheta \leq 180°$). (C) Scheme of the nucleotide−dendrimer interaction mode, with a focus on the nucleotide distance from the dendrimer core. The dendrimer structure is simplified as inner branches (gray area) and an outer surface composed of an amine layer (cyan area). For PPI-Mal OS G4 dendrimer, there is an additional layer composed of maltose units (red area). The distance is calculated between the center of mass of the nucleotide and the dendrimer center of mass. (D) Nucleotide−dendrimer distance (black squares with standard deviation) combined with dendrimer radius of gyration histograms. Histograms provide a quantification of size of each dendrimer layer drawn in panel C. The combination of two plots provides a clear view of nucleotide penetration into the dendrimer structure.

set of simulations considering a 10:1 drug−dendrimer ratio was performed. Following the same approach (described in Materials and Methods), each dendrimer was simulated in the presence of ten nucleotides randomly oriented in an explicitly solvated box. In all considered trajectories, 10 nucleotide molecules bound to the dendrimer and never detached throughout the simulation. Figure 9 provides a representative snapshots of obtained complexes in which the entire dendrimer surface is completely covered by bound nucleotides. Data analyzed in terms of orientation, penetration, and RDF did not show statistically significant differences compared to the results obtained for 1:1 nucleotide−dendrimer ratio (Figures 6 and 7).

Complexes were further investigated in terms of surface properties by quantifying the surface electrostatic potential (Figure 10). Simulation frames were extracted from the ensemble trajectory of nucleotide−dendrimer systems. Electrostatic potentials in the presence of bound nucleotides were compared, with electrostatic potentials calculated for single dendrimer macromolecules in water.

The most obvious observation was the overall positive electrostatic potential of the single dendrimer. Further, quantitative examination of electrostatic data showed interesting differences in the nucleotide binding regions. Noteworthy, CAFdATP showed much better ability to neutralize the dendrimer electrostatic potential with respect to Ara-FATP.

DISCUSSION

Our studies concentrated on the use of PPI-Mal OS G4 for the delivery of an active form of fludarabine, an adenosine analogue displaying exceptional cytotoxic activity in many types of hematological malignancies.[57] We were previously able to show that fludarabine triphosphate (Ara-FATP) has limited toxicity toward several cancer cell lines compared to free nucleoside (Ara-FA), while complexation with maltose-coated PPI dendrimer (which does not otherwise influence cellular metabolism) significantly increases its activity. Further, we proved that cellular uptake of Ara-FA mediated by equilibrative nucleoside transporter 1 (hENT1) is a limiting step in the cytotoxicity of fludarabine. However, the complexation with PPI-Mal OS G4 dendrimer allowed Ara-FATP to

DOI: 10.1021/acs.biomac.9b00010
Biomacromolecules 2019, 20, 1429−1442

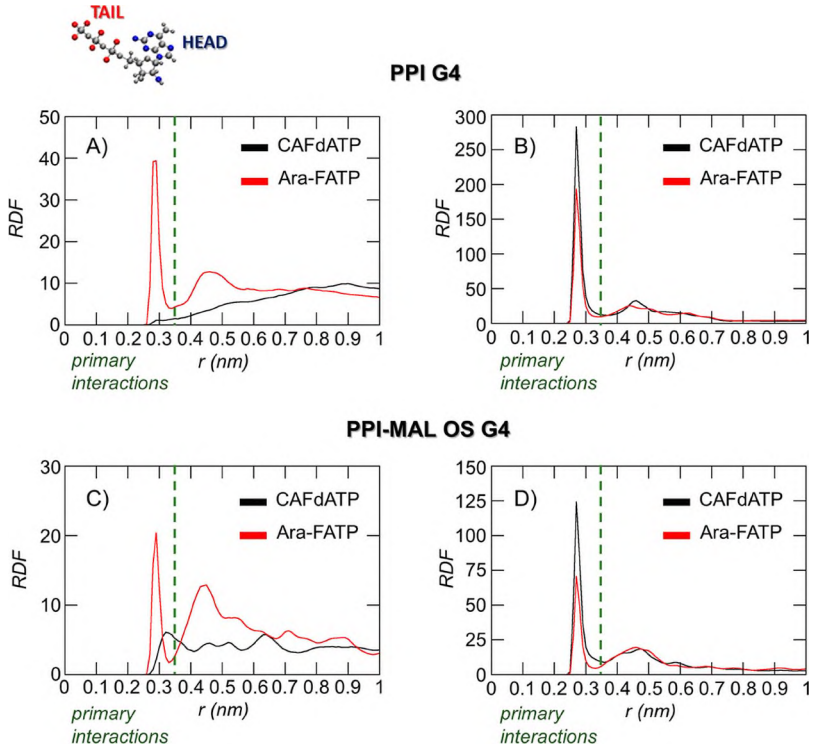

Figure 7. Radial Distribution Functions of CAFdATP (black curve) and Ara-FATP (red curve) head interacting with PPI G4 and PPI-Mal OS G4 outer layer (A, C). Radial Distribution Functions of CAFdATP (black curve) and Ara-FATP (red curve) tail interacting with PPI G4 and PPI-Mal OS G4 outer layer (B, D). Dendrimer outer layer is composed of primary amino groups in case of PPI G4 and primary and secondary (maltose-conjugated) amino groups in case of PPI-Mal OS G4.

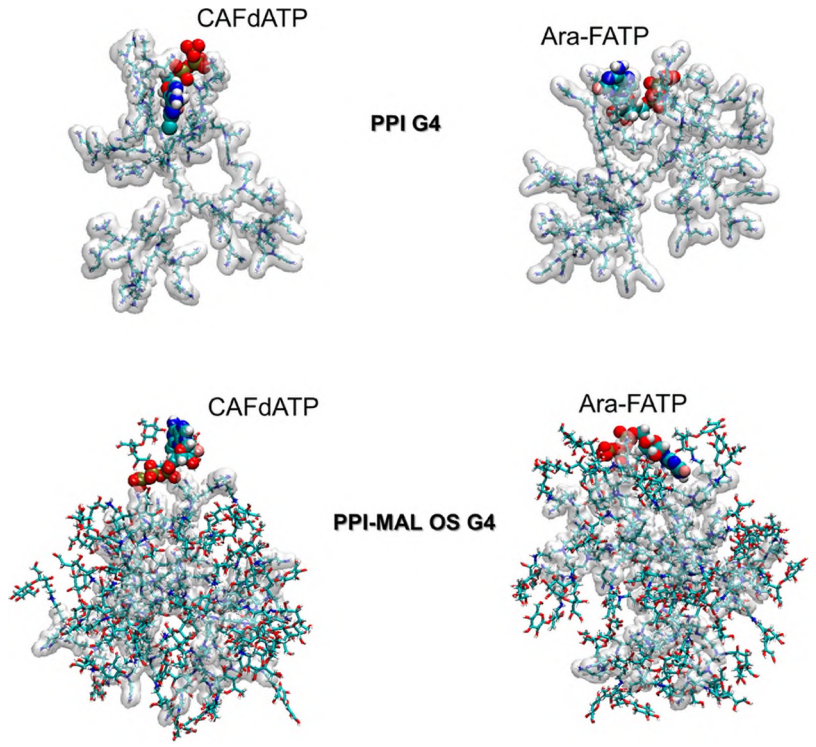

Figure 8. Representative snapshots of nucleotide−dendrimer (1:1 ratio) configurations for each investigated system.

kill cells even in the presence of nitrobenzylmercaptopurine (NBMPR), an hENT1 inhibitor. Finally, using a set of assays for the evaluation of phosphatidylserine externalization, mitochondrial membrane potential, caspase-3 activity and DNA/RNA synthesis, we showed that complex formation does not change intracellular pharmacodynamics of Ara-FATP, preserving its capability to inhibit the elongation of nucleic acid chains and to induce apoptosis via intrinsic pathway.

DOI: 10.1021/acs.biomac.9b00010
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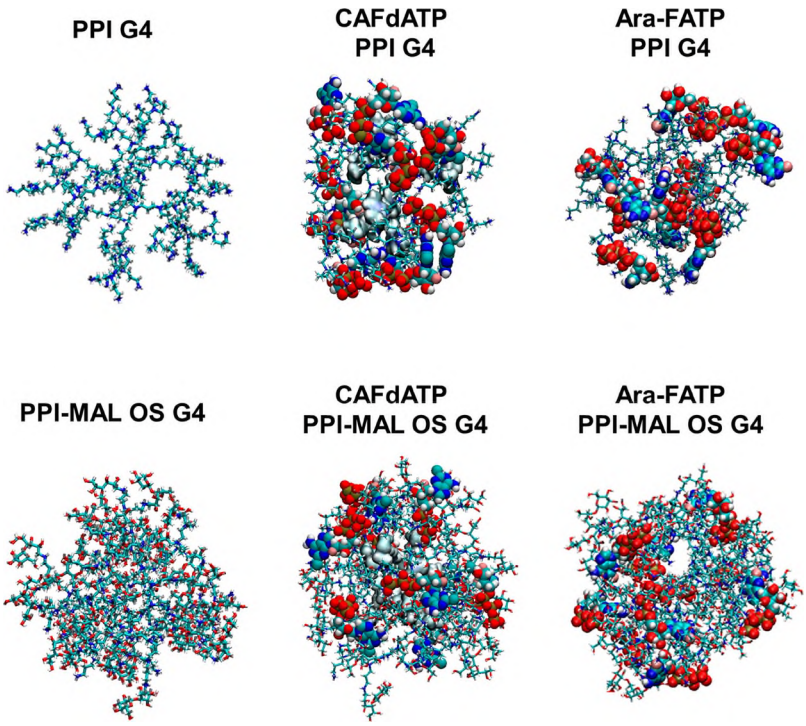

Figure 9. Representative snapshots of single dendrimer macromolecules and nucleotide−dendrimer (10:1 ratio) configurations for each investigated system.

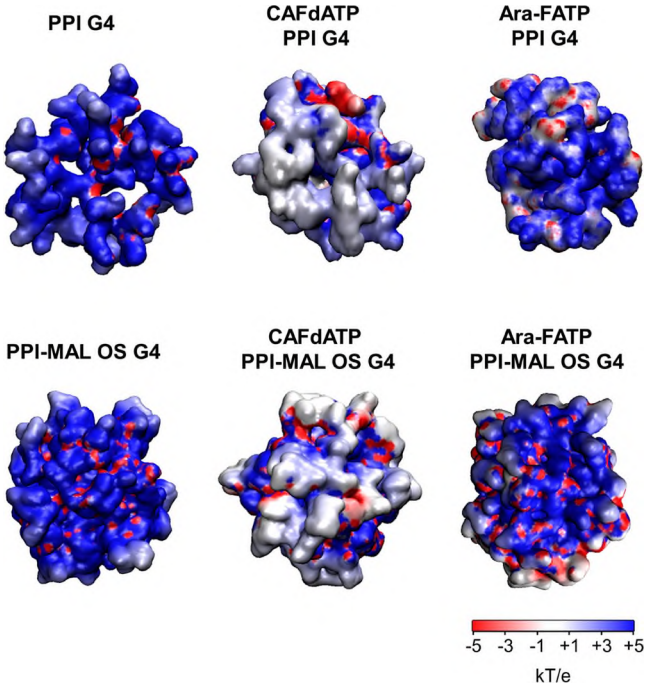

Figure 10. Electrostatic map presented for single dendrimer macromolecules and nucleotide−dendrimer complexes (10:1 ratio). Potential isocontours (obtained by solution of the NLPBE at 150 mM ionic strength with solute dielectric of 4 and solvent dielectric of 78.4) are shown in the range from +5 kT/e (blue) to −5 kT/e (red).

Thus, we concluded that maltose-coated PPI dendrimer may serve as an efficient nanocarrier for active metabolite of fludarabine, enhancing its cytotoxic activity and bypassing hENT1-associated resistance, most probably due to the autonomous mode of cell entry.[28]

One of the main goals of the present work was to conclusively prove the inference that NT-independent cellular uptake of nucleotide−dendrimer complexes is the main cause of increased activity of fludarabine triphosphate. Therefore, we designed and optimized the method for enzymatic synthesis and subsequent purification of tritium-labeled fludarabine triphosphate (^{3}H-Ara-FATP), starting from commercially available nucleoside (^{3}H-Ara-FA). Such method of Ara-FATP synthesis has never been reported before. The reaction sequence (based on the use of deoxycytidine, adenylate and creatine kinases) proved to be very efficient, with the yield close to 100%. In cellular uptake studies using radiolabeled Ara-FA and Ara-FATP, we were able to show a strong difference in the internalization rate of free nucleoside and triphosphate, which confirms that the nucleotide form must undergo dephosphorylation before entering the cell.[1,2] We have been able to demonstrate for the first time directly that PPI-Mal OS G4 dendrimer significantly enhances cellular uptake of Ara-FATP. It has been shown previously that "open-shell" maltose-modified PPI (PPI-Mal OS) dendrimers form stable complexes with nucleoside triphosphates and have the ability to shield them from enzymatic degradation.[20,27] Furthermore, PPI-Mal OS G4 is actively taken up by the leukemic cells, most probably via the receptor-mediated endocytosis pathway, reaching high intracellular concentrations,[58] and can deliver cytarabine triphosphate (Ara-CTP) into CCRF cells.[21] Since in our study, dendrimer-facilitated Ara-FATP uptake occurs also in the presence of hENT1 inhibitor (NBMPR), it finally proves the existence of an alternative, NT-independent internalization pathway for the drug-dendrimer complex. On the other hand, glycodendrimer did not influence the rate of internalization of Ara-FA, which is expected since PPI dendrimers do not form stable complexes with nucleosides.[22,27]

DOI: 10.1021/acs.biomac.9b00010
Biomacromolecules 2019, 20, 1429−1442

Another direct piece of evidence that was hitherto lacking in this model of dendrimer action as drug delivery vehicle was a direct mechanistic determination of drug−dendrimer binding parameters. In previous work, we used analogous natural nucleotides to study these interactions by isothermal titration calorimetry and electrophoretic light scattering.[20,22] These techniques are indirect (rely on binding-associated physical phenomena) and require large amounts of analyte. In the present study, we used surface plasmon resonance (SPR) for the first time to study nucleotide drug−dendrimer interactions. By this method, we were able to explicitly confirm the formation of a noncovalent, equilibrium-driven complex between PPI-Mal OS G4 and Ara-FATP, as well as lack of the interactions between the dendrimer and nucleoside form of the drug. Taken together, these results confirm the applicability of maltose-modified PPI dendrimers as delivery agents for the active form of fludarabine and invite further pharmacokinetic studies in vivo.

At this point, the outstanding question concerned the uniqueness of fludarabine as a potential glycodendrimer-delivered anticancer payload. We set out to compare its properties with clofarabine, a superior second generation nucleoside analogue drug.[59,60] Since the suggested intracellular metabolism and mechanism of action of the two compounds are highly alike, we decided to test the differential impact of maltose-modified glycodendrimer on the cellular pharmacokinetics of these drugs. The SPR study showed that clofarabine triphosphate (CAFdATP) was able to bind to the dendrimer with slightly higher affinity than Ara-FATP. Similarly to Ara-FA, the nucleoside form of clofarabine (CAFdA) did not bind to the dendrimer. Viability assays showed that cytotoxic activity of CAFdA in our cellular system was higher compared to its triphosphate form. The toxicity of clofarabine nucleoside (IC_{50} = 31.05 ± 2.66 nM) significantly exceeded that previously observed for fludarabine nucleoside in the same cell line (IC_{50} = 1.11 ± 0.06 μM[28]). While the higher potency of clofarabine was expected and in line with data from other cell types,[55] its extent (more than 35-fold) is unprecedented in the literature. Even more interestingly, while for both drugs the triphosphate form was significantly less potent than the nucleoside form, this gap in toxicity was more pronounced for fludarabine (3.1-fold; IC_{50} = 3.42 ± 0.20 μM for Ara-FATP[28]) than for clofarabine (1.7-fold in the present study). Since both literature consensus and our direct uptake data indicate that the nucleotide form can be taken up exclusively after hydrolysis to free nucleoside, and there are no indications of any differences in extracellular hydrolysis rate (occurring either spontaneously by acid−base catalysis or by cellular ectoenzymes), these differences can reflect divergent drug−cell interactions at subsequent steps. It is known that CAFdA has greater affinity for nucleoside transporters, leading to higher cellular concentration of CAFdA than Ara-FA.[55] Furthermore, clofarabine is characterized by higher affinity for dCK compared to fludarabine,[57,61] which results in faster intracellular phosphorylation and accumulation of cytotoxic CAFdATP.[61] It is worth noting that, despite the differences in the intracellular accumulation of both drugs, the rate of transmembrane transport of their nucleoside forms remains very high, the intracellular concentration reaches a maximum after little more than 5 min (present study and[55]); therefore, this step is unlikely to form the bottleneck for cellular toxicity.

Surprisingly and in contrast to Ara-FATP, complexation with maltose-modified dendrimer did not increase the toxicity of CAFdATP, unexpectedly further weakening its toxic activity. This effect was even stronger upon the application of unmodified PPI G4. This result must be related to nucleotide−dendrimer interactions, since we show that nucleoside forms (Ara-FA and CAFdA) do not interact directly with dendrimers and their toxicity is unaffected by the presence of PPI macromolecules. At this stage it can be hypothesized that the qualitative difference in dendrimer impact on toxicity must stem from divergent influence on the intracellular fate of the drug. We postulate that, for Ara-FATP, the presence of a dendrimer has a crucial positive impact on the therapeutic efficiency at two stages: net drug flux through the cell membrane and direct delivery of pharmacodynamically active triphosphate form to its intracellular site of action. By contrast, since CAFdA is known to be converted to CAFdATP more efficiently than Ara-FA to Ara-FATP,[61] the contribution of potential direct nucleotide form delivery to clofarabine toxicity is negligible. Moreover, the potential ability of the CAFdATP−dendrimer complex to bypass NT-mediated uptake may be rendered moot either by hindered intracellular release due to tighter binding or by weakening of the internalization process itself.

Since these results point to significant differences in practical effects of nucleotide−dendrimer complexation between the two drugs, we undertook a molecular modeling study to explain these phenomena. Obtained models indeed revealed divergences in nucleotide−dendrimer interaction patterns. As expected, for both drugs, the nucleotide's phosphate residues (referred to as phosphate "tail") were responsible for primary interactions with surface amino groups of PPI dendrimers, explaining similar phenomenology of binding. In the case of Ara-FATP, the nucleoside part (referred to as the "head" of the nucleotide) also interacted with the amino surface layer of both dendrimers, although to a much lesser extent than phosphate moieties. Overall, Ara-FATP remained on the surface of the dendrimer and did not penetrate into its interior, which may contribute to an easier release of the nucleotide from the complex. As for CAFdATP, its "head" did not interact with the outer layer of PPI macromolecules. Interestingly, the nucleoside part of clofarabine triphosphate had a tendency to penetrate into deeper layers of PPI G4. This could further stabilize the interaction by trapping the drug within the dendrimer structure, hampering the release of clofarabine triphosphate from the complex. On the other hand, the nucleoside part of CAFdATP did not penetrate inside the PPI-Mal OS G4 dendrimer, most probably because of the steric hindrance posed by maltose units. CAFdATP-PPI-Mal OS G4 interaction mode was featured with the nucleotide "head" exposed to the solvent and the "tail" interacting mainly with surface primary amino groups, making the release of clofarabine from this complex potentially easier than in the case of PPI G4.

The observed differences in interaction patterns may be partially due to the fact that Ara-FA is more polar (its polar surface area is 140 A^2, whereas it is only 119 A^2 for CAFdA; data from PubChem, pubchem.ncbi.nlm.nih.gov) and exhibits higher hydrophilicity,[62] which can cause fludarabine to interact with the charged surface of the dendrimer, while clofarabine may enter its interior more easily. Moreover, the presence of a fluorine atom in the sugar ring of clofarabine may additionally stiffen the structure of its triphosphate, preventing simultaneous interactions of the "tail" and the "head" with the dendrimer's surface.

DOI: 10.1021/acs.biomac.9b00010
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Interestingly, surface electrostatic potential modeling revealed significant differences between the impact of Ara-FATP and CAFdATP complexation, independent of the binding pose. CAFdATP has a substantially higher capability of masking the inherently positive surface potential of the PPI dendrimer (both unmodified and sugar-modified) than Ara-FATP. This unexpected CAFdATP feature may limit the ability of the drug−dendrimer complex to enter the cell, since positively charged nanoparticles have a much greater tendency to penetrate cell membranes than neutral or negatively charged ones.[63−65] In this case, the drug delivery potential of PPI macromolecules may be significantly decreased.

It is important to note that structural differences between fludarabine and clofarabine are restricted to two halide atoms, which were introduced in clofarabine for purposes related to simple extracellular pharmacokinetics. The introduction of a fluorine atom in the 2′-position of the arabinofuranosyl ring significantly enhanced the stability of the glycosidic bond in acidic pH and decreased its sensitivity to nucleoside phosphorylases, thus enabling both intravenous and oral administration of CAFdA. Clofarabine is also distinguished from fludarabine by the substitution of fluorine with a chlorine atom in the 2-position of the adenine base, which was meant to prevent deamination.[59,60] Still, these restricted changes led to a broader difference in biological activity: clofarabine is more potent than fludarabine in ribonucleotide reductase (RR) inhibition and incorporates more efficiently into DNA and RNA chains, leading to inhibition of their synthesis and subsequent induction of apoptosis via intrinsic pathway.[55,59,60] In addition, CAFdA has been shown to directly trigger the depolarization of mitochondria, resulting in fast release of cytochrome c and apoptosis inducing factor (AIF).[66] Thus, the differences in drug−dendrimer interactions leading to positive effect of dendrimer only on fludarabine toxicity are not entirely unexpected and can be explained by distinctive effects on cellular pharmacokinetics. In our model, direct delivery of Ara-FATP (active drug form) by dendrimer is of utmost importance for the toxic effect, while clofarabine is already highly efficiently internalized and phosphorylated by endogenous mechanisms and CAFdATP−dendrimer complexation can only hinder this streamlined process. Of course, our model tests toxicity toward isolated leukemic cells, so we can only model the minimal therapeutic concentration and not the maximal one (full therapeutic range). Since fludarabine is inherently less toxic, the decrease of effective concentration by glycodendrimer certainly extends the therapeutic range, making glycodendrimers a viable drug delivery option in this case. On the other hand, the applied nucleotide−dendrimer ratio of 10:1 ensures full saturation of the PPI-Mal OS G4.[20,22] While this seems optimal for fludarabine delivery, it is possible that a change of CAFdATP−dendrimer ratio to suboptimal (lower) values (by increasing dendrimer concentration) could remove the negative impact of surface charge neutralization, allowing the complex to efficiently deliver also CAFdATP into the cell.[22,58]

CONCLUSIONS

In conclusion, we proved for the first time that substantial increase in Ara-FATP toxicity toward leukemic cells in the presence of PPI-Mal OS G4 glycodendrimer is due to formation of a noncovalent complex through the surface amino groups of the dendrimer and nucleotide's phosphate moieties. This complex is then efficiently taken up by the cell, bypassing the need to utilize both nucleoside transporters in the plasma membrane and intracellular kinases, to deliver active (triphosphate) drug form directly to the site of action. This demonstration was made possible by a novel synthesis method for radioactively labeled Ara-FATP. Comparison with clofarabine showed significant differences both in molecular features of complex formation and in resulting biological activity. SPR and molecular modeling proved to be important tools for explaining phenomenological features of binding, for example, why the nucleotide "tail" is crucial for complex formation. In summary, our results suggest that, for fludarabine, the intracellular metabolism is as important or maybe even more important than transport as a limiting factor for toxicity; thus, the dendrimer is an efficient fludarabine delivery vehicle both because it helps to bypass the transporters, but also (more importantly) because it delivers a ready-made triphosphate. The potential practical advantages of glycodendrimer application in fludarabine delivery are further underscored by the potential to overcome drug resistance associated with mutations or downregulation of NTs. For clofarabine, where delivering the triphosphate is not crucial due to efficient phosphorylation, this advantage of dendrimer is nullified. Combined with better pharmacodynamics (activity toward intracellular targets) and perhaps more efficient NT-mediated intake, this makes clofarabine a drug for which the dendrimer may not confer a significant beneficial effect. Moreover, potentially stronger interactions of CAFdATP with PPI dendrimers and its ability to mask surface positive charge of these macromolecules may decrease the ability of complexes to cross the cell membrane and release clofarabine triphosphate, which requires further studies.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.9b00010.

Figure S1: Autoradiography of analytical TLC; Figure S2: Sample analysis of the affinity of Ara-FATP for PPI-Mal OS G4 dendrimer immobilized on the CM5 sensor (PDF).

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by National Science Centre, Poland (Project UMO-2014/13/B/NZ3/04643 "Cellular and molecular mechanism of action of PPI dendrimer complexes with nucleoside analogue anticancer drugs") and Swiss National Supercomputing Centre (CSCS). Authors would like to thank Dr. Tomasz Wójcik and Dr. Alicja Sochaj-Gregorczyk from

DOI: 10.1021/acs.biomac.9b00010
Biomacromolecules 2019, 20, 1429−1442

Jagiellonian Center of Innovation (Cracow, Poland) for performing the SPR measurements and Dr. Krzysztof Skowronek from International Institute of Molecular and Cell Biology (Warsaw, Poland) for his great help in the interpretation of SPR analysis results.

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DOI: 10.1021/acs.biomac.9b00010
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Supporting Information

Fludarabine-specific molecular interactions with maltose-modified poly(propyleneimine) dendrimer enable effective cell entry of the active drug form: comparison with clofarabine

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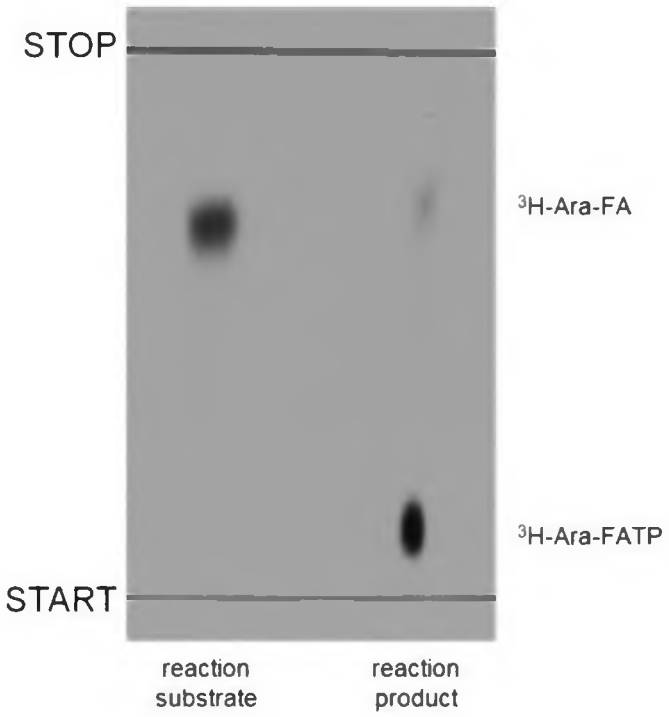

Figure S1. Autoradiography of analytical TLC. The reaction efficiency was close to 100% (almost all ^{3}H-Ara-FA was phosphorylated into ^{3}H-Ara FATP).

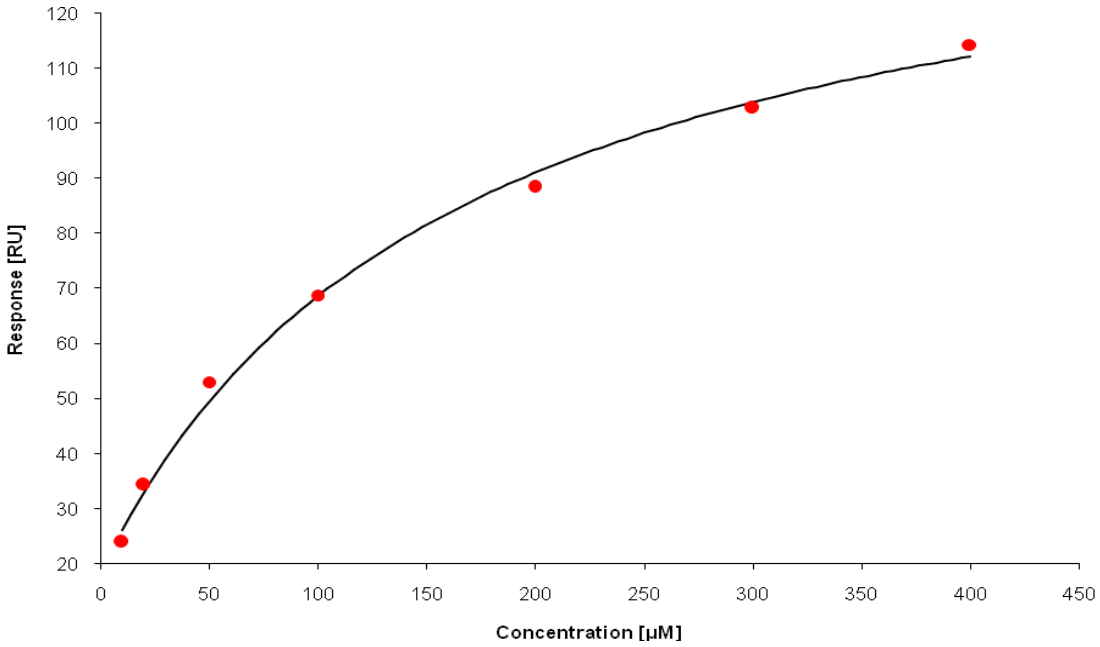

Figure S2. Sample analysis of the affinity of Ara-FATP for PPI-Mal OS G4 dendrimer immobilized on the CM5 sensor. The model calculates the equilibrium dissociation constant K_D for a 1:1 interaction from a plot of steady state binding levels (RU) against analyte concentration (C). The equation includes a term for the bulk refractive index contribution RI, which is assumed to be the same for all samples: $RU = (CR_{max}/K_D+C)+RI$

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Effect of the Structure of Therapeutic Adenosine Analogues on Stability and Surface Electrostatic Potential of their Complexes with Poly(propyleneimine) Dendrimers

Michał Gorzkiewicz, Dietmar Appelhans, Susanne Boye, Albena Lederer, Brigitte Voit, and Barbara Klajnert-Maculewicz*

Poly(propyleneimine) glycodendrimers are proposed as nanocarriers for triphosphate forms of anticancer adenosine analogues to improve the efficiency of chemotherapy and to overcome drug resistance mechanisms. This approach has proven successful for fludarabine administration—an autonomous way of cellular entry of a nucleotide–dendrimer noncovalent complex enables an increase in the intracellular accumulation and cytotoxic activity of the active metabolite of the drug. However, the attempt to apply an analogous strategy for clofarabine results in the inhibition of drug activity. To better understand this phenomenon, characterization and comparison of drug-dendrimer complexes were needed to indicate the differences in their surface properties and the strengths of fludarabine-dendrimer and clofarabine-dendrimer interactions. Here, zeta potential measurements, ultrafiltration, and asymmetric flow field-flow fractionation are applied to determine the surface electrostatic potential and stability of nucleotide–dendrimer formulations. This approach significantly extends the authors' research on the complexation potential of perfectly branched macromolecules, ultimately explaining previously observed differences and their consequences.

Nucleoside analogues (NAs) belong to a class of anticancer therapeutics with a number of cytotoxic activities based on the inhibition of enzymes specific for nucleoside metabolism, disruption of DNA and RNA synthesis processes, and induction of apoptosis. The mechanism of action of therapeutic NAs strongly depends on their chemical structure and character of interactions with intracellular targets.[1] Most anticancer NAs share similar metabolic pathways. Being administered as prodrugs in the form of nucleosides, they are transported through the cell membrane by specialized proteins (nucleoside transporters, NTs) and subjected to intracellular phosphorylation resulting in the formation of triphosphates,[2] which is a crucial step for their cytotoxic activity. This phenomenon generates several potential resistance mechanisms, significantly limiting the efficacy of chemotherapy based on these type of drugs. The decreased expression of surface proteins facilitating cell entry and reduced activity of intracellular kinases lead to insufficient cellular uptake and inadequate formation of the active forms of NAs. These events are most commonly observed in drug-resistant cells.[3,4]

One of the most promising strategies to overcome drug resistance and enhance the effectiveness of treatment involves a direct delivery of therapeutic nucleotides to target cells using a dendrimer-based nanocarrier system with an autonomous mode of cellular uptake.[5] Our scientific interests have concentrated on the possibility of using so-called open-shell (OS) poly(propyleneimine) (PPI) dendrimers with their surfaces partially coated with maltose units for the intracellular delivery of active triphosphate forms of adenosine analogues. Such glycodendrimers exhibit high biocompatibility as well as the capacity to form stable complexes with nucleoside triphosphates[6,7] and to protect them from enzymatic degradation.[8] We proved that the fourth generation maltose-modified PPI dendrimer (PPI-Mal OS G4) can serve as an efficient nanocarrier for an active metabolite of fludarabine (Ara-FATP), increasing its cytotoxic activity and overcoming NT-associated resistance due to the independent mechanism of cell entry.[9,10]

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DOI: 10.1002/marc.201900181

The comparison of the impact of the dendrimer on fludarabine and clofarabine toxicity together with the characterization of drug-dendrimer complexes using surface plasmon resonance (SPR) and molecular modeling indicated significant differences in nucleotide–dendrimer interaction patterns, which affected the possibility of using PPI glycodendrimers as universal carriers for different therapeutic adenosine analogues. In the case of clofarabine triphosphate (CAFdATP), the decrease in its cytotoxic activity upon complexation with PPI macromolecules is thought to be related to stronger drug-dendrimer interactions and the ability of CAFdATP to mask the surface charge of cationic dendrimers, resulting in reduced cellular uptake and drug release.[10]

In the present study, we applied 1) zeta potential analysis to evaluate the surface electrostatic potential of drug-dendrimer formulations and 2) two separation techniques, ultrafiltration and asymmetric flow field-flow fractionation (AF4), which differ in terms of flow rate and shear force impact on the complexes, in order to characterize their stability in different environmental conditions. The aim was to enhance the understanding of decisive factors that affect the properties of nucleotide-PPI dendrimer complexes.

Zeta potential measurements have enabled the determination of the surface electrostatic properties of dendrimers and nucleotide–dendrimer complexes. In this case, two different drug-dendrimer ratios have been evaluated. The drug-dendrimer ratio of 10:1 had been applied previously for both unmodified and maltose-modified macromolecules in molecular modeling[10] and constitutes the maximum saturation of PPI-Mal OS G4 by adenosine-5′-triphosphate (ATP) molecules in pH 7.4.[7] Additionally, since we showed that PPI G4 has the ability to bind approximately 20 ATP molecules under the same pH conditions,[7] we have tested a ratio of 20:1 for this dendrimer. These experiments have confirmed the outcome of in silico simulations:[10] in all cases, CAFdATP showed a significantly higher tendency to mask the positive surface charge of PPI dendrimers than fludarabine triphosphate (**Table 1**). Since cationic nanoparticles have much greater capacities to penetrate cellular membranes than neutral or anionic nanoparticles,[11] this property of CAFdATP may hamper its intracellular delivery by PPI macromolecules, explaining the decreased cytotoxicity of this drug upon complexation with the dendrimers.[10]

Table 1. Zeta potential of PPI dendrimers and nucleotide–dendrimer complexes, presented as average ± SD, $n = 9$.

	zeta potential [mV] ± SD
PPI G4	35.7 ± 5.7
Ara-FATP-PPI G4 (10:1)	26.8 ± 1.8[a)]
Ara-FATP-PPI G4 (20:1)	13.0 ± 0.3[b)]
CAFdATP-PPI G4 (10:1)	10.5 ± 0.9[a)]
CAFdATP-PPI G4 (20:1)	−1.4 ± 0.8[b)]
PPI-Mal OS G4	28.3 ± 3.3
Ara-FATP-PPI-Mal OS G4 (10:1)	13.2 ± 1.6[c)]
CAFdATP-PPI-Mal OS G4 (10:1)	4.0 ± 0.3[c)]

[a–c)]Statistically significant difference between samples at $p < 0.05$.

To determine the stability of complexes and examine the differences in complexation potential of PPI macromolecules under different pH conditions, we applied ultrafiltration and AF4 analysis. First, a combination of ultrafiltration and UV–vis measurements for the evaluation of the amount of noncomplexed nucleosides and nucleotides was used. The principle of this technique is to retain the dendritic polymers with complexed analytes in a stirring cell under shear force conditions, while the free analyte molecules can pass through the separation membrane and be detected in the filtrate by UV-Vis spectroscopy. The shear forces applied during ultrafiltration constitute additional factors for determining the stability of complexes.

In line with our previous results,[7,10] ultrafiltration experiments proved that nucleotides form stable complexes with cationic PPI dendrimers (**Figure 1**) due to the electrostatic interactions between the negatively charged phosphate tails of nucleotides and the positive amino groups of dendrimer macromolecules. This conclusion is further supported by the fact that PPI dendrimers have not bound nucleoside forms of the drugs under evaluation (Ara-FA and CAFdA, data not shown).

Both unmodified and maltose-modified PPI dendrimers have shown similar complexation profiles. Nucleotide–dendrimer complexation is pH-dependent, being most efficient in acidic environment. Under such conditions, the degree of protonation of the primary and tertiary amino groups of the dendrimer increases, and so does the number of potential nucleotide binding sites. At acidic pH values, PPI dendrimers adopt a more open conformation due to the repulsion between the positively charged surface moieties and the inner amino groups of the macromolecule,[12,13] enhancing electrostatic interactions and facilitating nucleotide–dendrimer binding. Upon an increase in pH, PPI dendrimers bind less nucleotide molecules, most likely due to deprotonation of the amino groups of the macromolecule (Figure 1).

Further, we decided to supplement our previous research with the evaluation of the influence of inorganic ions on the drug-dendrimer complexes. The increase of ionic strength (by the addition of NaCl at the physiological concentration) has reduced the stability of complexes (in 10 mM HEPES buffer pH 7.4 with 150 mM NaCl, the nucleotide–dendrimer interactions were the weakest compared to other tested buffers) (Figure 1). This may affect future application of such noncovalent formulations in vivo upon intravenous administration.

The stability of the complexes depends significantly on the drug-dendrimer complexation ratios. The nucleotide–dendrimer molar ratios of 20:1 for PPI G4 and 10:1 for PPI-Mal OS G4 (due to the partial maltose coating) allow for the complete saturation of polymers with triphosphate molecules.[7] Thus, it is not unexpected that for these and lower ratios (10:1 and 5:1 for PPI G4 and PPI-Mal OS G4, respectively), approximately 100% complexation has been observed. However, for the same ratios at pH 8.5 and 7.4 with the addition of NaCl, a lower complexation degree was determined, which again indicates the importance of positive charge on the surface of the macromolecule and the impact of the presence/absence of competing ions in solution on the stability of complexes. The percentage of complexed drug molecules decreases with increasing drug-dendrimer ratio (Figure 1).

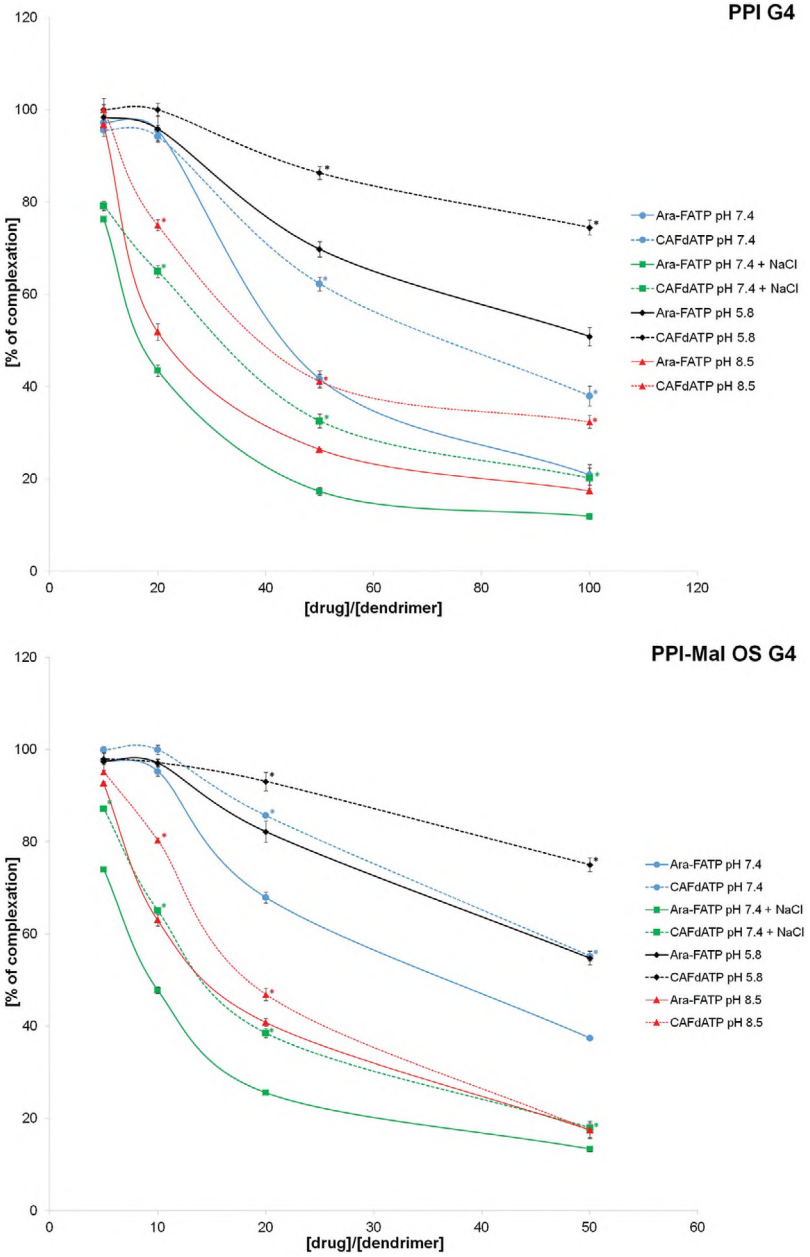

Figure 1. Results of ultrafiltration experiments for PPI G4 (upper panel) and PPI-Mal OS G4 (lower panel), presented as percentage of drug complexation, average ± SD, $n = 8$. *Statistically significant difference compared to Ara-FATP at $p < 0.05$.

Most importantly, CAFdATP interacts more strongly with PPI dendrimers than fludarabine triphosphate (more CAFdATP molecules are bound to the dendrimer under the same conditions, as shown in Figure 1 and Table S3, Supporting Information). Such a trend has been observed consistently for all buffers and for the majority of drug-dendrimer ratios. This outcome corroborates our previous SPR measurements,[10] which indicated that PPI dendrimers were able to differently interact with nucleotides under continuous flow conditions. It also supports the hypothesis of the reduced release of CAFdATP from the complex with the dendrimer, which may decrease the delivery potential of PPI macromolecules in the case of this drug. The differences in the strength of the interactions (and thus the stability of complexes) can be explained by the diverse modifications introduced in the structure of tested adenosine analogues, leading to different drug-dendrimer interaction patterns.[10] Structural differences between fludarabine and clofarabine are restricted to two halide atoms; fludarabine possesses a fluorine atom in the 2-position of the adenine base, while in the case of clofarabine, it has been substituted by chlorine. Moreover, an additional fluorine atom has been introduced in the 2′-position of the arabinofuranosyl ring of CAFdA (Figure S2, Supporting Information). Ara-FA has been shown to be more hydrophilic, which may cause the nucleoside part of fludarabine triphosphate to compete with the phosphate tail for binding to the surface amino groups of the PPI dendrimer.[10] Indeed, computer simulations showed that both the nucleoside part (in approx. 25%) and the phosphate tail (in approx. 75%) contribute to interactions with the dendrimer amine layer.[10] Since the nucleotide's phosphate residues are responsible for the primary interactions with surface amino groups of PPI dendrimers, this may facilitate drug release from the complex.

The results of ultrafiltration experiments were confirmed by AF4, an elution-based chromatography-like separation method commonly applied for the characterization of nanoparticles, polymers, and proteins. The advantages of AF4 include a broad separation range accessible by a single channel and significantly reduced risk of shear degradation.[14–16] The application of this method constitutes a significant extension of our previous studies on the complexation patterns of glycosylated branched polymers.[17,18] The experiments have been carried out for PPI-Mal OS G4 at pH 7.4 (physiological conditions) and 5.8 (acidic cellular compartment) (**Figure 2**). Here, the observed complexation efficiency has been slightly higher in comparison to ultrafiltration, which may be attributed to the lower flow rate and the absence of shear force conditions in the case of AF4. Nevertheless, the dendrimer has again shown greater capacity of stable binding of CAFdATP compared to Ara-FATP.

Interestingly, the application of higher than optimal drug-dendrimer ratios has shown that PPI macromolecules have the ability to form stable complexes with greater amounts of nucleotide molecules (Figure 1 and Table S3, Supporting Information) than those determined previously by isothermal titration

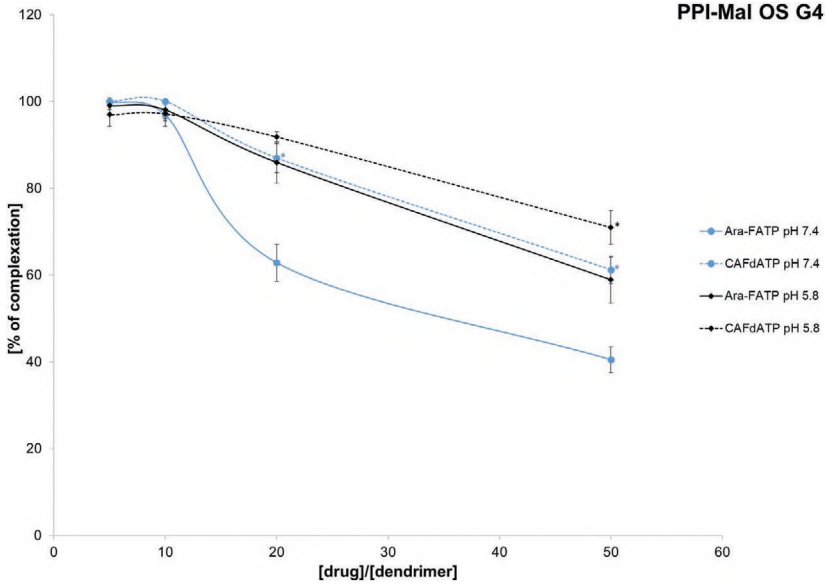

Figure 2. Results of AF4 experiments, presented as percentage of drug complexation, average ± SD, $n = 8$. *Statistically significant difference compared to Ara-FATP at $p < 0.05$.

calorimetry (ITC) and zeta potential titration.[7] This can be explained by the different characters of the applied techniques. The ITC measurements are more dynamic due to the relatively short time between consecutive injections of a titrant solution, favoring the observation of ligand binding with high affinity to primary active sites located on or just below the surface of the dendritic scaffolds. Thus, the detection of potential active sites with lower affinity and/or located deeper within the structure is hindered due to the limited diffusion of the drug to the interior of the PPI macromolecules. As a result, the tested system does not fully reach thermodynamic equilibrium, and the number of determined binding sites may be lower compared to the results obtained with more static techniques (such as dialysis or ultrafiltration) that allow for the detection of active sites with lower affinity.[19,20] Moreover, assuming the predominance of surface interactions over encapsulation inside the dendritic scaffold, the model of a single type of bonding centers to describe the thermodynamic parameters of complexation processes is commonly applied in ITC measurements.[7] This may also contribute to the determination of lower numbers of nucleotide molecules complexed by the dendrimer.

Another possible explanation for the observed complexation efficiencies involves the formation of aggregates by nucleotide–dendrimer formulations with an excess of the drug. This may be relevant especially in the case of the complexes with CAFdATP, since particles with a high surface potential have lower aggregation tendency compared with particles with a low surface potential under the same environmental conditions.[17,21]

In conclusion, the obtained results highlight the differences in the complexation properties of PPI dendrimers toward Ara-FATP and CAFdATP. The comparison of surface electrostatic potential and stability of drug-dendrimer noncovalent complexes has allowed us to indicate the most probable cause of previously observed differences in the cytotoxic activity of such formulations. This in turn points to an urgent need for comprehensive physicochemical characterization of such drug delivery systems before further biological tests in order to fully exploit their therapeutic potential.

Experimental Section

A fifth generation poly(propyleneimine) dendrimer (MW 7167.97 g mol^{-1}; Figure S1, Supporting Information) with 64 terminal amino groups was obtained from SyMO-Chem (Eindhoven, The Netherlands) and specified as fourth generation (PPI G4) following the uniform nomenclature of polyamine dendrimers for poly(propyleneimine) and poly(amidoamine) macromolecules.[22]

A fourth generation PPI dendrimer with primary surface amino groups partially modified with maltose (PPI-Mal OS G4, MW 21113 g mol^{-1}) (a so-called open shell glycodendrimer; Figure S1, Supporting Information) was prepared and characterized as described previously.[7,23,24]

Fludarabine (2-fluoroadenine-9-β-D-arabinofuranoside, Ara-FA) and clofarabine (2-chloro-2′-arabino-fluoro-2′-deoxyadenosine, CAFdA) were purchased from Sigma-Aldrich. Fludarabine-5′-triphosphate and clofarabine-5′-triphosphate (Ara-FATP and CAFdATP, respectively; Figure S2, Supporting Information) were purchased from Jena Bioscience.

The surface electrostatic potential and stability of drug-dendrimer complexes were evaluated using zeta potential measurements, ultrafiltration, and AF4. A detailed description of the methods applied in this study is included in the Supporting Information.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This work was supported by the National Science Centre, Poland (project UMO-2014/13/B/NZ3/04643 "Cellular and molecular mechanism of action of PPI dendrimer complexes with nucleoside analogue anticancer drugs") and is based upon work from COST Action "Nano2Clinic. Cancer Nanomedicine – from the bench to the bedside" CA17140 supported by COST (European Cooperation in Science and Technology).

Conflict of Interest

The authors declare no conflict of interest.

Keywords

clofarabine, complexation, drug delivery systems, fludarabine, PPI glycodendrimers

Received: April 11, 2019
Revised: May 13, 2019
Published online: May 28, 2019

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Effect of the structure of therapeutic adenosine analogues on stability and surface electrostatic potential of their complexes with poly(propyleneimine) dendrimers

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SUPPORTING INFORMATION

Materials and Methods

- Zeta potential analysis
- Ultrafiltration
  - **Table S1.** The composition of mixtures for the preparation of drug-dendrimer complexes for ultrafiltration experiments.
- Asymmetric flow field-flow fractionation (AF4)
  - **Table S2.** The composition of mixtures for the preparation of drug-dendrimer complexes for AF4 experiments.
- Statistics

Figure S1. Chemical structure of unmodified (PPI G4) and partially modified with maltose units (PPI-Mal OS G4) poly(propyleneimine) dendrimers of the 4th generation. PPI-Mal OS G4 dendrimer has 43 maltose (Mal) moieties chemically coupled to 4th generation PPI scaffold.

Figure S2. Chemical structures of fludarabine (A) and clofarabine (B) in the triphosphate forms.

Table S3. The results of ultrafiltration experiments, presented as the number of complexed nucleotide molecules.

MATERIALS AND METHODS

Zeta potential analysis

Measurements of zeta potential were performed with the use of Zetasizer Nano ZS (Malvern Instruments Ltd., UK). All samples were placed in the folded capillary cell (DTS 1070, Malvern) and measured at 25 °C. The data were analyzed using the Malvern software.

PPI G4 and PPI-Mal OS G4 dendrimers were dissolved in double-distilled water to a final concentration of 100 μM. Dendrimer solutions were freshly prepared and used on the same day. Fludarabine and clofarabine triphosphates (Ara-FATP and CAFdATP, respectively) were added to the dendrimer solutions at a molar ratio of 10:1 (to a concentration of 1 mM for both dendrimers) or 20:1 (to a concentration of 2 mM for PPI G4 dendrimer).

The mixtures were stirred for 0.5 h at ambient temperature. Prior the measurements, complexes and dendrimer solutions were diluted 10 times in the testing buffer (HEPES pH 7.4) to the final dendrimer concentration of 10 μM.

Ultrafiltration

For the ultrafiltration procedures, solvent-resistant stirred cell XFUF07601 (Millipore Corp., USA) with ultrafiltration membrane of poly(ethylene sulfone) (PES) with the nominal molecular weight limit (NMWL) of 5 kDa (PBCC07610, Millipore Corp., USA) at a rotation speed of 200 rpm under 5 atm pressure of nitrogen was used.

PPI G4 and PPI-Mal OS G4 dendrimer solutions in double-distilled water were freshly prepared and used on the same day. Fludarabine and clofarabine in the form of nucleosides (Ara-FA and CAFdA, respectively) or triphosphates were mixed with dendrimer solutions in the required complexation ratios (Table S1) in 100 μl of double-distilled water and stirred for 0.5 h at ambient temperature. Prior the experiment, the prepared mixtures were diluted 100 times by moving to 10 ml of appropriate buffer (HEPES pH 7.4, 5.8, 8.5 or HEPES pH 7.4 with 150 mM NaCl) to the final nucleoside/nucleotide concentration of 50 μM. The complexes were separated by ultrafiltration via a 5 kDa PES membrane, followed by the determination of non-complexed nucleoside/nucleotide molecules in the filtrate by UV-Vis spectroscopy.

The ultrafiltration was carried out with the solution of pure nucleoside/nucleotide until the collection of the first 4 ml of the filtrate, and then the samples of 500 μl were collected in the range from 4 to 6 ml. The remaining solution was subsequently removed, the membrane was rinsed with water and drug-dendrimer complexes have been subjected to analogous ultrafiltration procedure.

UV-Vis measurements were carried out using UV Micro-cuvettes (z = 8.5 mm, Carl Roth GmbH + Co. KG, Germany) in a Specord 210 Plus Spectrophotometer (Analytik Jena, Germany) at a wavelength of 254 nm.

Table S1. *The composition of mixtures for the preparation of drug-dendrimer complexes for ultrafiltration experiments.*

Ratio		Preparation		
nucleoside/nucleotide	PPI G4	nucleoside/nucleotide [µl from 50 mM stock]	PPI G4 [µl from 5 mM stock]	water [µl]
		10		90
10	1	10	10	80
20	1	10	5	85
50	1	10	2	88
100	1	10	1	89

Ratio		Preparation		
nucleoside/nucleotide	PPI-Mal OS G4	nucleoside/nucleotide [µl from 50 mM stock]	PPI-Mal OS G4 [µl from 5 mM stock]	water [µl]
		10		90
5	1	10	20	70
10	1	10	10	80
20	1	10	5	85
50	1	10	2	88

Asymmetric flow field-flow fractionation (AF4)

AF4 measurements were performed with an Eclipse Dualtec system (Wyatt Technology Europe, Germany). The separation device was poly(ethylene sulfone) (PES) hollow fiber (HF, Superon GmbH, DE) with a molecular weight cut-off of 10 kDa. Flows were controlled with an Agilent Technologies 1200 series isocratic pump equipped with vacuum degasser. The detection system comprised of a variable wavelength detector (Agilent Technologies, UK) set to 254 nm. The UV detector was arranged at the cross flow outlet for the determination of free drug amount transported out of the HF. All injections were performed with an autosampler (1200 series, Agilent Technologies, UK). The channel flow rate (Fc) was maintained at 0.35 ml/min for all AF4 operations. Samples were injected during the focusing/relaxation step within 40 min. The focus flow (Ff) was set to 0.70 ml/min. The cross flow rate (Fx) during the elution step was optimized by an isocratic step of 0 ml/min for 10 min. At least two series of samples were prepared and measured in quadruplicates. After every two samples a blank run was performed, which was necessary for the baseline subtraction of the UV signal.

For the quantification of free drug, a calibration curve for each drug and buffer was established. For this purpose, different concentration series were prepared, injected and the resulting UV peak area related to the injected sample mass was used as basis for the quantification. During the experiment a constant sample recovery has been observed, indicating the lack of drug adsorption. The determination of complexation efficiency was performed by measurements of defined mixtures of the drug and the dendrimer with known amounts of both compounds.

For the preparation of complexes, PPI-Mal OS G4 dendrimer solutions in double-distilled water were freshly prepared and used on the same day. Fludarabine and clofarabine in the form of triphosphates were mixed with dendrimer solutions in the required complexation ratios (Table S2) in 100 µl of double-distilled water and stirred for 0.5 h at ambient temperature. Prior the experiment, the

prepared mixtures were diluted 10 times by moving to 900 µl of appropriate buffer (HEPES pH 7.4 or 5.8) to the final nucleotide concentration of 120 µM.

HEPES buffer pH 7.4 or 5.8 was used as eluent for all measurements. Collecting and processing of detector data were made by the Astra software, version 6.1 (Wyatt Technology, USA).

Table S2. *The composition of mixtures for the preparation of drug-dendrimer complexes for AF4 experiments.*

Ratio		Preparation		
nucleotide	PPI-Mal OS G4	nucleotide [µl from 5 mM stock]	PPI-Mal OS G4 [µl from 0.5 mM stock]	water [µl]
		24		76
5	1	24	48	28
10	1	24	24	52
20	1	24	12	64
50	1	24	4.8	71.2

Statistics

For single pairwise comparisons, Student's t test was applied. In all tests, p values < 0.05 were considered to be statistically significant. Data were presented as average ± SD.

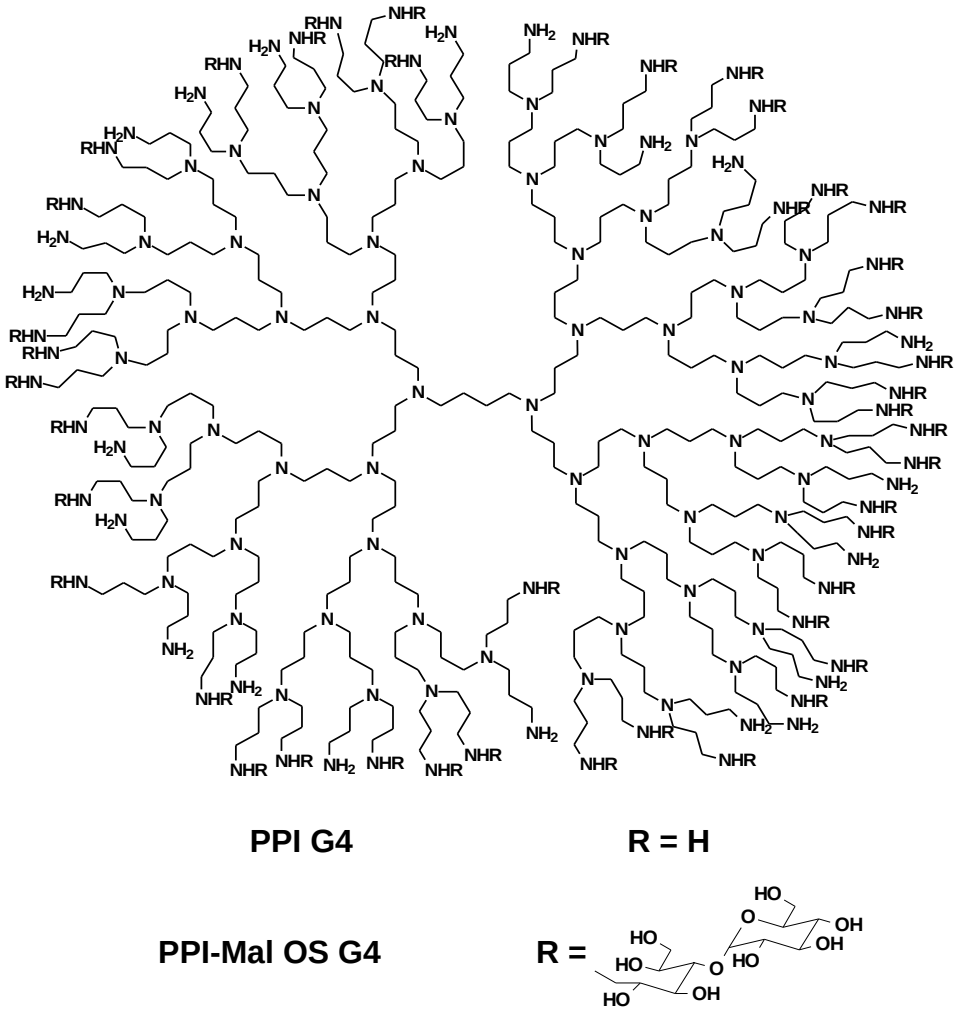

Figure S1. *Chemical structure of unmodified (PPI G4) and partially modified with maltose units (PPI-Mal OS G4) poly(propyleneimine) dendrimers of the 4th generation. PPI-Mal OS G4 dendrimer has 43 maltose (Mal) moieties chemically coupled to 4th generation PPI scaffold.*

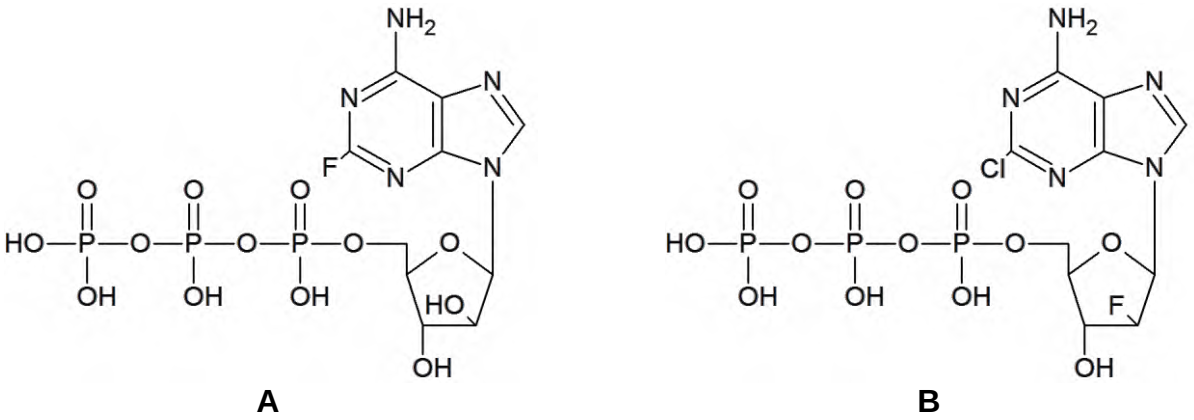

Figure S2. *Chemical structures of fludarabine (A) and clofarabine (B) in the triphosphate forms.*

Table S3. *The results of ultrafiltration experiments, presented as the number of complexed nucleotide molecules.*

		PPI G4	
		Ara-FATP	CAFdATP
buffer	drug-dendrimer complexation ratio	[number of complexed molecules]	
HEPES pH 7.4	100:1	21	38
	50:1	21	31
	20:1	19	19
	10:1	10	10
HEPES pH 7.4 + NaCl	100:1	12	20
	50:1	9	16
	20:1	9	13
	10:1	8	8
HEPES pH 5.8	100:1	51	75
	50:1	35	43
	20:1	19	20
	10:1	10	11
HEPES pH 8.5	100:1	17	32
	50:1	13	21
	20:1	10	15
	10:1	10	10

		PPI-Mal OS G4	
		Ara-FATP	CAFdATP
buffer	drug-dendrimer complexation ratio	[number of complexed molecules]	
HEPES pH 7.4	50:1	19	28
	20:1	14	17
	10:1	10	10
	5:1	5	5
HEPES pH 7.4 + NaCl	50:1	7	9
	20:1	5	8
	10:1	5	7
	5:1	4	4
HEPES pH 5.8	50:1	27	38
	20:1	16	19
	10:1	10	10
	5:1	5	5
HEPES pH 8.5	50:1	9	9
	20:1	8	9
	10:1	6	8
	5:1	5	5

Oświadczenia współautorów publikacji wchodzących w skład rozprawy doktorskiej

Łódź 9.09.2019

(miejsce, data)

Mgr inż. Michał Gorzkiewicz

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ul. Pomorska 141/143, 90-236 Łódź

Oświadczenie

Oświadczam, że w pracy:

Gorzkiewicz M., Klajnert-Maculewicz B. (2017), Dendrimers as nanocarriers for nucleoside analogues, European Journal of Pharmaceutics and Biopharmaceutics, 114:43-56 mój udział polegał na zebraniu materiałów oraz przygotowaniu manuskryptu (90%),

Gorzkiewicz M., Buczkowski A., Appelhans D., Voit B., Pułaski Ł., Pałecz B., Klajnert-Maculewicz B. (2018), Poly(propyleneimine) glycodendrimers non-covalently bind ATP in a pH-and salt-dependent manner-model studies for adenosine analogue drug delivery, International Journal of Pharmaceutics, 544(1):83-90 mój udział polegał na planowaniu i wykonaniu większości doświadczeń, analizie i interpretacji wyników, przygotowaniu manuskryptu (60%),

Gorzkiewicz M., Jatczak-Pawlik I., Studzian M., Pułaski Ł., Appelhans D., Voit B., Klajnert-Maculewicz B. (2018), Glycodendrimer nanocarriers for direct delivery of fludarabine triphosphate to leukemic cells: improved pharmacokinetics and pharmacodynamics of fludarabine, Biomacromolecules, 19(2):531-543 mój udział polegał na planowaniu i wykonaniu większości doświadczeń, analizie i interpretacji wyników, przygotowaniu manuskryptu (65%),

Gorzkiewicz M., Deriu M.A., Studzian M., Janaszewska A., Grasso G., Pułaski Ł., Appelhans D., Danani A., Klajnert-Maculewicz B. (2019), Fludarabine-Specific Molecular Interactions with Maltose-Modified Poly(propyleneimine) Dendrimer Enable Effective Cell Entry of the Active Drug Form: Comparison with Clofarabine, Biomacromolecules, 20(3):1429-1442 mój udział polegał na planowaniu i wykonaniu większości doświadczeń, analizie i interpretacji wyników, przygotowaniu manuskryptu (55%),

Gorzkiewicz M., Appelhans D., Boye S., Lederer A., Voit B., Klajnert-Maculewicz B. (2019), Effect of the structure of therapeutic adenosine analogues on stability and surface electrostatic potential of their complexes with poly(propyleneimine) dendrimers, Macromolecular Rapid Communications, 40(15):1900181 mój udział polegał na planowaniu i wykonaniu większości doświadczeń, analizie i interpretacji wyników, przygotowaniu manuskryptu (70%).

Gorzkiewicz

(podpis)

Łódź 10.09.2019

(miejsce, data)

Prof. dr hab. Barbara Klajnert-Maculewicz

Katedra Biofizyki Ogólnej

Wydział Biologii i Ochrony Środowiska

Uniwersytet Łódzki

ul. Pomorska 141/143, 90-236 Łódź

Oświadczenie

Oświadczam, że w pracy:

Gorzkiewicz M., Klajnert-Maculewicz B. (2017), Dendrimers as nanocarriers for nucleoside analogues, European Journal of Pharmaceutics and Biopharmaceutics, 114:43-56 mój udział polegał na korekcie merytorycznej oraz zatwierdzeniu ostatecznej wersji artykułu (10%),

Gorzkiewicz M., Buczkowski A., Appelhans D., Voit B., Pułaski Ł., Pałecz B., Klajnert-Maculewicz B. (2018), Poly(propyleneimine) glycodendrimers non-covalently bind ATP in a pH-and salt-dependent manner-model studies for adenosine analogue drug delivery, International Journal of Pharmaceutics, 544(1):83-90 mój udział polegał na planowaniu badań, kierowaniu projektem obejmującym badania, konsultacji merytorycznej oraz zatwierdzeniu ostatecznej wersji artykułu (5%),

Gorzkiewicz M., Jatczak-Pawlik I., Studzian M., Pułaski Ł., Appelhans D., Voit B., Klajnert-Maculewicz B. (2018), Glycodendrimer nanocarriers for direct delivery of fludarabine triphosphate to leukemic cells: improved pharmacokinetics and pharmacodynamics of fludarabine, Biomacromolecules, 19(2):531-543 mój udział polegał na planowaniu badań, kierowaniu projektem obejmującym badania, konsultacji merytorycznej oraz zatwierdzeniu ostatecznej wersji artykułu (5%),

Gorzkiewicz M., Deriu M.A., Studzian M., Janaszewska A., Grasso G., Pułaski Ł., Appelhans D., Danani A., Klajnert-Maculewicz B. (2019), Fludarabine-Specific Molecular Interactions with Maltose-Modified Poly(propyleneimine) Dendrimer Enable Effective Cell Entry of the Active Drug Form: Comparison with Clofarabine, Biomacromolecules, 20(3):1429-1442 mój udział polegał na planowaniu badań, kierowaniu projektem obejmującym badania, konsultacji merytorycznej oraz zatwierdzeniu ostatecznej wersji artykułu (5%),

Gorzkiewicz M., Appelhans D., Boye S., Lederer A., Voit B., Klajnert-Maculewicz B. (2019), Effect of the structure of therapeutic adenosine analogues on stability and surface electrostatic potential of their complexes with poly(propyleneimine) dendrimers, Macromolecular Rapid Communications, 40(15):1900181 mój udział polegał na planowaniu badań, kierowaniu projektem obejmującym badania, konsultacji merytorycznej oraz zatwierdzeniu ostatecznej wersji artykułu (5%).

B. Klajnert-Maculewicz

(podpis)

Łódź, 2019 05 31

(miejsce, data)

Dr hab. Łukasz Pułaski

Katedra Biofizyki Molekularnej

Wydział Biologii i Ochrony Środowiska

Uniwersytet Łódzki

ul. Pomorska 141/143, 90-236 Łódź

Oświadczenie

Oświadczam, że w pracy:

Gorzkiewicz M., Buczkowski A., Appelhans D., Voit B., Pułaski Ł., Pałecz B., Klajnert-Maculewicz B. (2018), Poly(propyleneimine) glycodendrimers non-covalently bind ATP in a pH-and salt-dependent manner-model studies for adenosine analogue drug delivery, International Journal of Pharmaceutics, 544(1):83-90 mój udział polegał na częściowym przygotowaniu manuskryptu (5%),

Gorzkiewicz M., Jatczak-Pawlik I., Studzian M., Pułaski Ł., Appelhans D., Voit B., Klajnert-Maculewicz B. (2018), Glycodendrimer nanocarriers for direct delivery of fludarabine triphosphate to leukemic cells: improved pharmacokinetics and pharmacodynamics of fludarabine, Biomacromolecules, 19(2):531-543 mój udział polegał na planowaniu i wykonaniu części doświadczeń, analizie i interpretacji wyników, częściowym przygotowaniu manuskryptu (5%),

Gorzkiewicz M., Deriu M.A., Studzian M., Janaszewska A., Grasso G., Pułaski Ł., Appelhans D., Danani A., Klajnert-Maculewicz B. (2019), Fludarabine-Specific Molecular Interactions with Maltose-Modified Poly(propyleneimine) Dendrimer Enable Effective Cell Entry of the Active Drug Form: Comparison with Clofarabine, Biomacromolecules, 20(3):1429-1442 mój udział polegał na planowaniu i wykonaniu części doświadczeń, analizie i interpretacji wyników, częściowym przygotowaniu manuskryptu (10%).

(podpis)

Łódź, 12.06.2019

(miejsce, data)

Mgr Maciej Studzian

Katedra Biofizyki Molekularnej

Wydział Biologii i Ochrony Środowiska

Uniwersytet Łódzki

ul. Pomorska 141/143, 90-236 Łódź

Oświadczenie

Oświadczam, że w pracy:

Gorzkiewicz M., Jatczak-Pawlik I., Studzian M., Pułaski Ł., Appelhans D., Voit B., Klajnert-Maculewicz B. (2018), Glycodendrimer nanocarriers for direct delivery of fludarabine triphosphate to leukemic cells: improved pharmacokinetics and pharmacodynamics of fludarabine, Biomacromolecules, 19(2):531-543 mój udział polegał na planowaniu i wykonaniu części doświadczeń, częściowej analizie i interpretacji wyników (8%),

Gorzkiewicz M., Deriu M.A., Studzian M., Janaszewska A., Grasso G., Pułaski Ł., Appelhans D., Danani A., Klajnert-Maculewicz B. (2019), Fludarabine-Specific Molecular Interactions with Maltose-Modified Poly(propyleneimine) Dendrimer Enable Effective Cell Entry of the Active Drug Form: Comparison with Clofarabine, Biomacromolecules, 20(3):1429-1442 mój udział polegał na planowaniu i wykonaniu części doświadczeń, częściowej analizie i interpretacji wyników (10%).

M. Studzian

(podpis)

Łódź, 06.06.2019 r.
(miejsce, data)

Dr Adam Buczkowski
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ul. Pomorska 165, 90-236 Łódź

Oświadczenie

Oświadczam, że w pracy **Gorzkiewicz M., Buczkowski A., Appelhans D., Voit B., Pułaski Ł., Pałecz B., Klajnert-Maculewicz B. (2018), Poly(propyleneimine) glycodendrimers non-covalently bind ATP in a pH-and salt-dependent manner-model studies for adenosine analogue drug delivery, International Journal of Pharmaceutics, 544(1):83-90** mój udział polegał na planowaniu i wykonaniu części doświadczeń, częściowej analizie i interpretacji wyników, częściowym przygotowaniu manuskryptu (20%).

A. Buczkowski
(podpis)

Łódź 6.06.2019

(miejsce, data)

Prof. dr hab. Bartłomiej Pałecz

Katedra Chemii Fizycznej

Wydział Chemii

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Oświadczenie

Oświadczam, że w pracy **Gorzkiewicz M., Buczkowski A., Appelhans D., Voit B., Pułaski Ł., Pałecz B., Klajnert-Maculewicz B. (2018), Poly(propyleneimine) glycodendrimers non-covalently bind ATP in a pH-and salt-dependent manner-model studies for adenosine analogue drug delivery, International Journal of Pharmaceutics, 544(1):83-90** mój udział polegał na planowaniu części doświadczeń oraz zatwierdzeniu ostatecznej wersji manuskryptu (3%).

(podpis)

Łódź 10.09.2019
(miejsce, data)

Dr Izabela Jatczak-Pawlik
Katedra Biofizyki Ogólnej
Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki
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Oświadczenie

Oświadczam, że w pracy **Gorzkiewicz M., Jatczak-Pawlik I., Studzian M., Pułaski Ł., Appelhans D., Voit B., Klajnert-Maculewicz B. (2018), Glycodendrimer nanocarriers for direct delivery of fludarabine triphosphate to leukemic cells: improved pharmacokinetics and pharmacodynamics of fludarabine, Biomacromolecules, 19(2):531-543** mój udział polegał na planowaniu i wykonaniu części doświadczeń, częściowej analizie i interpretacji wyników (10%).

Izabela Jatczak-Pawlik
(podpis)

Łódź, 5 czerwca 2019
(miejsce, data)

Dr hab. Anna Janaszewska
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Wydział Biologii i Ochrony Środowiska
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Oświadczenie

Oświadczam, że w pracy **Gorzkiewicz M., Deriu M.A., Studzian M., Janaszewska A., Grasso G., Pułaski Ł., Appelhans D., Danani A., Klajnert-Maculewicz B. (2019), Fludarabine-Specific Molecular Interactions with Maltose-Modified Poly(propyleneimine) Dendrimer Enable Effective Cell Entry of the Active Drug Form: Comparison with Clofarabine, Biomacromolecules, 20(3):1429-1442** mój udział polegał na planowaniu części doświadczeń (3%).

(podpis)

Dresden, 07.08.2019

(place, date)

Dr. Dietmar Appelhans

Leibniz-Institut für Polymerforschung Dresden e.V.

Hohe Straße 6, 01069 Dresden, Germany

Statement

I hereby declare that in the article:

Gorzkiewicz M., Buczkowski A., Appelhans D., Voit B., Pułaski Ł., Pałecz B., Klajnert-Maculewicz B. (2018), Poly(propyleneimine) glycodendrimers non-covalently bind ATP in a pH-and salt-dependent manner-model studies for adenosine analogue drug delivery, International Journal of Pharmaceutics, 544(1):83-90 my contribution was based on the synthesis of the dendrimers and revising the manuscript (4%),

Gorzkiewicz M., Jatczak-Pawlik I., Studzian M., Pułaski Ł., Appelhans D., Voit B., Klajnert-Maculewicz B. (2018), Glycodendrimer nanocarriers for direct delivery of fludarabine triphosphate to leukemic cells: improved pharmacokinetics and pharmacodynamics of fludarabine, Biomacromolecules, 19(2):531-543 my contribution was based on the synthesis of the dendrimers and revising the manuscript (4%),

Gorzkiewicz M., Deriu M.A., Studzian M., Janaszewska A., Grasso G., Pułaski Ł., Appelhans D., Danani A., Klajnert-Maculewicz B. (2019), Fludarabine-Specific Molecular Interactions with Maltose-Modified Poly(propyleneimine) Dendrimer Enable Effective Cell Entry of the Active Drug Form: Comparison with Clofarabine, Biomacromolecules, 20(3):1429-1442 my contribution was based on the synthesis of the dendrimers and revising the manuscript (2%),

Gorzkiewicz M., Appelhans D., Boye S., Lederer A., Voit B., Klajnert-Maculewicz B. (2019), Effect of the structure of therapeutic adenosine analogues on stability and surface electrostatic potential of their complexes with poly(propyleneimine) dendrimers, Macromolecular Rapid Communications, 40(15):1900181 my contribution was based on the synthesis of the dendrimers, the design of the experiments, partial analysis and interpretation of the data and revising the manuscript (10%).

.....

(signature)

12.8.2019

(place, date)

Prof. Brigitte Voit

Leibniz-Institut für Polymerforschung Dresden e.V.

Hohe Straße 6, 01069 Dresden, Germany

Statement

I hereby declare that in the article:

Gorzkiewicz M., Buczkowski A., Appelhans D., Voit B., Pułaski Ł., Pałecz B., Klajnert-Maculewicz B. (2018), Poly(propyleneimine) glycodendrimers non-covalently bind ATP in a pH-and salt-dependent manner-model studies for adenosine analogue drug delivery, International Journal of Pharmaceutics, 544(1):83-90 my contribution was based on the final approval of the manuscript (3%),

Gorzkiewicz M., Jatczak-Pawlik I., Studzian M., Pułaski Ł., Appelhans D., Voit B., Klajnert-Maculewicz B. (2018), Glycodendrimer nanocarriers for direct delivery of fludarabine triphosphate to leukemic cells: improved pharmacokinetics and pharmacodynamics of fludarabine, Biomacromolecules, 19(2):531-543 my contribution was based on the final approval of the manuscript (3%),

Gorzkiewicz M., Appelhans D., Boye S., Lederer A., Voit B., Klajnert-Maculewicz B. (2019), Effect of the structure of therapeutic adenosine analogues on stability and surface electrostatic potential of their complexes with poly(propyleneimine) dendrimers, Macromolecular Rapid Communications, 40(15):1900181 my contribution was based on the final approval of the manuscript (2%).

(signature)

Dresden 12.8.2019

(place, date)

Dr. Susanne Boye

Leibniz-Institut für Polymerforschung Dresden e.V.

Hohe Straße 6, 01069 Dresden, Germany

Statement

I hereby declare that in the article **Gorzkiewicz M., Appelhans D., Boye S., Lederer A., Voit B., Klajnert-Maculewicz B. (2019), Effect of the structure of therapeutic adenosine analogues on stability and surface electrostatic potential of their complexes with poly(propyleneimine) dendrimers, Macromolecular Rapid Communications, 40(15):1900181** my contribution was based on the design and execution of the part of the experiments, partial analysis of the data and partial preparation of the manuscript (10%).

.....

(signature)

Dresden 7.08.2019
(place, date)

Dr. Albena Lederer

Leibniz-Institut für Polymerforschung Dresden e.V.

Hohe Straße 6, 01069 Dresden, Germany

Statement

I hereby declare that in the article **Gorzkiewicz M., Appelhans D., Boye S., Lederer A., Voit B., Klajnert-Maculewicz B. (2019), Effect of the structure of therapeutic adenosine analogues on stability and surface electrostatic potential of their complexes with poly(propyleneimine) dendrimers, Macromolecular Rapid Communications, 40(15):1900181** my contribution was based on the design of the part of the experiments and revising the manuscript (3%).

(signature)

Turin, 5th June 2019
(place, date)

Prof. Marco Agostino Deriu

Department of Mechanical and Aerospace Engineering (DIMEAS), Politecnico di Torino
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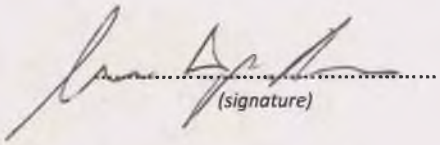
Tel: +39 011 0906944
Fax: +39 011 0906999
e-mail: marco.deriu@polito.it

Statement

I hereby declare that in the article **Gorzkiewicz M., Deriu M.A., Studzian M., Janaszewska A., Grasso G., Pułaski Ł., Appelhans D., Danani A., Klajnert-Maculewicz B. (2019), Fludarabine-Specific Molecular Interactions with Maltose-Modified Poly(propyleneimine) Dendrimer Enable Effective Cell Entry of the Active Drug Form: Comparison with Clofarabine, Biomacromolecules, 20(3):1429-1442** my contribution was based on the design and execution of the part of the experiments, partial analysis and interpretation of the data and partial preparation of the manuscript (10%).



(signature)

LUGANO
04/20/2019
(place, date)

Dr. Gianvito Grasso
SUPSI-DTI
IDSIA-Dalle Molle Institute for Artificial Intelligence
CH-6928 Manno, Switzerland

Statement

I hereby declare that in the article **Gorzkiewicz M., Deriu M.A., Studzian M., Janaszewska A., Grasso G., Pułaski Ł., Appelhans D., Danani A., Klajnert-Maculewicz B. (2019), Fludarabine-Specific Molecular Interactions with Maltose-Modified Poly(propyleneimine) Dendrimer Enable Effective Cell Entry of the Active Drug Form: Comparison with Clofarabine, Biomacromolecules, 20(3):1429-1442** my contribution was based on the execution of the part of the experiments and partial analysis of the data (3%).

(signature)

Manno, 04.06.2019

(place, date)

Prof. Andrea Danani

SUPSI-DTI

IDSIA-Dalle Molle Institute for Artificial Intelligence

CH-6928 Manno, Switzerland

Statement

I hereby declare that in the article **Gorzkiewicz M., Deriu M.A., Studzian M., Janaszewska A., Grasso G., Pułaski Ł., Appelhans D., Danani A., Klajnert-Maculewicz B. (2019), Fludarabine-Specific Molecular Interactions with Maltose-Modified Poly(propyleneimine) Dendrimer Enable Effective Cell Entry of the Active Drug Form: Comparison with Clofarabine, Biomacromolecules, 20(3):1429-1442** my contribution was based on the design of the part of the experiments and final approval of the manuscript (2%).

(signature)