Bioorganic & Medicinal Chemistry Letters 23 (2013) 2872–2875

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Design considerations for PAMAM dendrimer therapeutics

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ARTICLE INFO

Article history:
Received 9 January 2013
Revised 21 March 2013
Accepted 22 March 2013
Available online 1 April 2013

Keywords:
Cancer
Drug delivery
Dendrimers
Endocytosis
Cleavable linkers

ABSTRACT

We have previously shown that methotrexate (MTX) conjugated to a cancer-specific poly amido amine (PAMAM) dendrimer has a higher therapeutic index than MTX alone. Unfortunately, these therapeutics have been difficult to advance because of the complicated syntheses and an incomplete understanding of the dendrimer properties. We wished to address these obstacles by using copper-free click chemistry to functionalize the dendrimer scaffolds and to exploring the effects of two dendrimer properties (the targeting ligand and drug linkage) on cytotoxicity. We conjugated either ester or amide-linker modified MTX to dendrimer scaffolds with or without folic acid (FA). Because of multivalency, the FA and MTX functionalized dendrimers had similar capacities to target the folate receptor on cancer cells. Additionally, we found that the ester- and amide-linker modified MTX compounds had similar cytotoxicity but the dendrimer–ester MTX conjugates were much more cytotoxic than the dendrimer–amide MTX conjugates. These results clarify the impact of these properties on therapeutic efficacy and will allow us to design more effective polymer therapeutics.

Because of their controllable properties and carrying capacity, synthetic polymers have been used for a variety of biomedical applications.[1–3] Despite these advantages, many polymer therapeutics have suffered setbacks because of their complex syntheses and our limited understanding of the properties responsible for the therapeutic efficacy.[4,5] A more complete understanding of the interactions between synthetic polymers and biological systems is necessary to more rationally design the next generation of synthetic polymer therapeutics.

Dendrimers are branched, synthetic polymers with controllable chemical topology that have been used for a broad range of biomedical applications including drug delivery, gene transfection, and tissue engineering.[6] Because of their low polydispersity and tunable chemical properties, dendrimers are well suited for mechanistic studies on polymer properties and polymer interactions with biological systems.[7] Our early studies demonstrated that folic acid (FA) conjugated to a generation 5 (G5) polyamidoamine (PAMAM) dendrimer could target the folate receptor (FR) on cancer cells to deliver therapeutics in a cell-specific manner.[8–10] Importantly, these studies demonstrated that cell-specific targeting greatly enhanced the therapeutic index of the dendrimer–methotrexate (dendrimer–MTX) conjugates compared to MTX alone. Our early studies used a sequential syntheses strategy to engineer the dendrimer–MTX conjugates. Due to inefficiencies in the synthesis, there were inconsistencies in the amount of FA and MTX conjugated to the dendrimer scaffold.[11] While these early studies showed the potential of this approach, the complex synthesis and a limited understanding of dendrimer properties have slowed the advancement of these polymer therapeutics. To overcome some of the synthetic obstacles, we have employed copper-free strain-promoted alkyne azide cycloaddition (Cu-free click chemistry) to functionalize the dendrimer scaffold.[12–15] The high strain of the cyclooctyne ring enhances the alkyne reactivity enabling rapid reaction with azides under mild conditions without the need for copper catalysts. The efficiency and selectivity of this class of reactions will likely improve the functionalization of dendrimer scaffolds by increasing yields and conjugate consistency.[5]

Herein, we describe the synthesis of dendrimer–MTX conjugates using Cu-free click chemistry with variations in the type of MTX linkage (ester or amide) and targeting ligand (FA or MTX). Using a human epithelial cancer model, we evaluated the effects of these dendrimer properties on therapeutic efficacy. Extending our prior studies to biological systems, we show that both FA and MTX on the dendrimer scaffold can mediate cell-specific delivery of therapeutics. Additionally, we demonstrate that the linker-modified MTX compounds had similar cytotoxicity, but when the modified-MTX compounds were conjugated to the dendrimer scaffold, there were dramatic differences in therapeutic efficacy.

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http://dx.doi.org/10.1016/j.bmcl.2013.03.088

Synthesis and characterization of linker modified-MTX compounds and dendrimer-MTX conjugates

We recently reported the development of a G5 PAMAM dendrimer scaffold that can be functionalized using Cu-free click chemistry.[12] The linker-modified MTX compounds and the dendrimer–MTX conjugates were synthesized as shown in Scheme 1. First, we modified MTX with either an amide-linker (γ-N3-aMTX, **8**) or an ester-linker (γ-N3-eMTX, **10**). To retain its functionality, MTX was modified on the terminal glutamyl functional group preserving the structural integrity of the pteridine ring and p-aminobenzoic acid (Fig. S1).[16] Because of the structural similarity between compounds **8**, **10**, and γ-azido modified FA, we synthesized the dendrimer–MTX conjugates by simultaneous reaction with FA (Scheme 1). The dendrimer–MTX conjugates were characterized by ^{1}H NMR and had an average of 6 MTX and the FA-dendrimer conjugates had an average of 3 FA (Fig. S1).

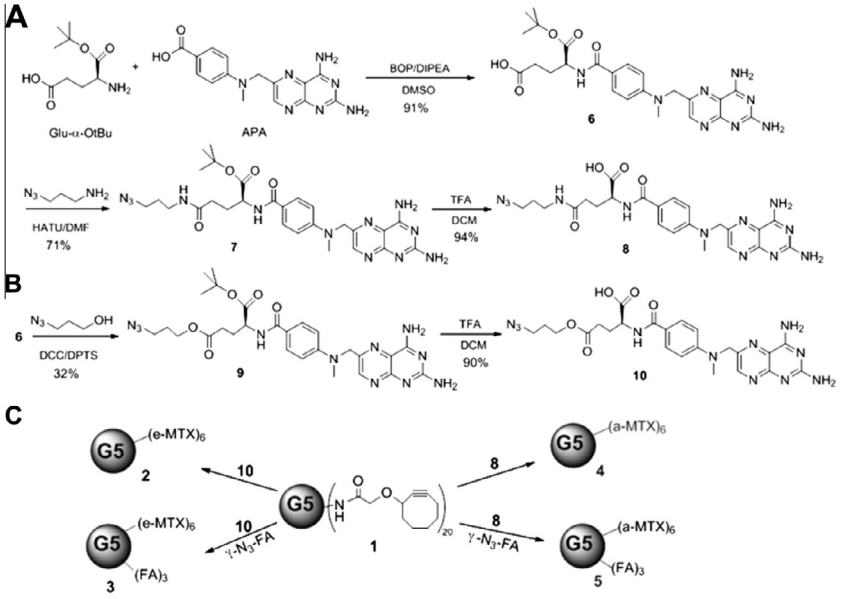
Drug conjugation to polymer scaffolds can be achieved using stable or cleavable linkages depending on the desired biological effect. We have used amide and ester bonds to conjugate therapeutics to the dendrimer scaffold. Amide bonds are known to be extremely stable while ester linkages can be hydrolyzed enzymatically or in acidic or basic environments. On small molecules, ester linkages hydrolyze quickly and are not as stable compared with similar amide linkages. Because our approach relies on active targeting and endocytosis, the drug should not be released under normal physiological conditions that exist outside of the cell. We monitored the stability of the dendrimer–MTX conjugates in physiologic buffers (PBS; pH 7.4) using UPLC as previously described.[17] Briefly, the four conjugates (**2–5**) were dissolved in PBS and incubated at 37 °C. At the time points indicated, samples were analyzed to quantify the amount of free MTX released from the dendrimer–MTX conjugates. There was no evidence of MTX release from the dendrimer–amide MTX (dendrimer–aMTX) conjugates (**4** and **5**) over 72 h (Fig. S2). The dendrimer-ester MTX (dendrimer–eMTX) conjugates (**2** and **3**) also demonstrated excellent stability, releasing approximately 2.3% of the total MTX over 72 h (Fig. S2). This amount of MTX would not induce cytotoxicity in the biological experiments. These results demonstrate that the MTX ester and amide linkages are stable on the dendrimer scaffold in physiologic buffers and can be used to deliver MTX to cancer cells.

Biological evaluation of linker-modified MTX compounds

To evaluate the therapeutic efficacy of the linker-modified MTX compounds and the dendrimer–MTX conjugates, we used the Annexin V/7-Aminoactinomycin D (An/7AAD) apoptosis assay and the FR overexpressing human KB cancer cell line.[18] KB cells were incubated with 100 nM of the linker-modified MTX compounds and free MTX as previously described.[10] At 24 h, KB cells treated with the linker-modified MTX compounds (**8** and **10**) and free MTX all had evidence of apoptosis with both the ester- and amide-linker MTX compounds treated cells showing approximately a 100% increase in apoptosis and the MTX treated cells showing a 200% increase in apoptosis (Fig. 1). At 48 h, the difference in apoptosis between MTX and the linker-modified MTX compounds was more evident (Fig. 1). Importantly, the modified MTX compounds retained their cytotoxicity and the ester- and amide-linker-modified MTX compounds had similar efficacy.

Biological evaluation of dendrimer-MTX conjugates

After confirming that the linker-modified MTX compounds had similar activity, we then evaluated the efficacy of the dendrimer–MTX conjugates. The cytotoxicity of MTX largely depends upon its ability to inhibit dihydrofolate reductase (DHFR) in the cytoplasm of cells.[19] One challenge for synthetic polymers is to efficiently deliver therapeutic payloads to the cytoplasm of cells. Cationic polymers can deliver therapeutics to the cytoplasm but are inherently non-specific and can be cytotoxic themselves.[20,21] To address the issue of cell-specificity, we used FA or MTX as targeting ligands for the dendrimer scaffold. Although this approach greatly enhances cell specificity because it relies on receptor-mediated endocytosis, it results in intracellular compartmentalization of the therapeutic payload that can lead to changes in therapeutic efficacy. Based on previous studies, we hypothesized that the dendrimer–eMTX conjugates would hydrolyze in the endosome allowing MTX to escape into the cytoplasm where it could inhibit the DHFR.[22] In contrast, due to the stability of the amide linkage, we hypothesized that the dendrimer–aMTX conjugates would not hydrolyze in the endosome, trapping MTX and diminishing its ability to inhibit cytoplasmic DHFR. We evaluated the cytotoxicity of



Scheme 1. Synthesis of linker-modified MTX and dendrimer–MTX conjugates.

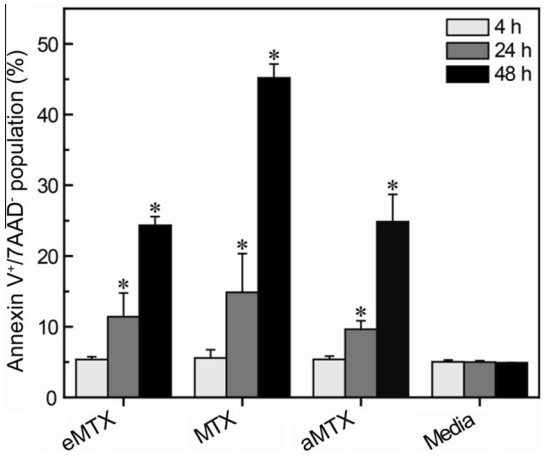


Figure 1. Therapeutic activity of linker-modified MTX compounds. KB cells were treated with 100 nM of MTX, γ-N3-aMTX (**8**) and γ-N3-eMTX (**10**) and analyzed for apoptosis at 4, 24, and 48 h. Apoptotic cells were defined as AnV+/7AAD− and assessed in relation to a 5% threshold for media control cells. Significance of a 1-sided paired student *t*-test recorded (p <0.05), for treatments compared to media control. Results are representative of three independent experiments.

100 nM of the dendrimer–MTX conjugates (**2**–**5**) at 4, 24, and 48 h. In contrast to the linker-modified MTX compounds, the dendrimer–MTX conjugates had striking differences in their cytotoxicity. At all of the time points evaluated, the dendrimer–eMTX conjugates were more cytotoxic compared to the dendrimer–aMTX conjugates (Fig. 2). The dendrimer–eMTX 2 demonstrated the greatest efficacy inducing a ~800% increase in apoptosis relative to the media control. Similarly, the FA targeted, dendrimer–eMTX conjugate (FA dendrimer–eMTX **3**) induced a ~400% increase in apoptosis relative to the media control. Both of the dendrimer–aMTX conjugates (**4** and **5**) had much less activity with only the FA dendrimer–aMTX 5 conjugate inducing a small amount of apoptosis at 48 h (Fig. 2).

Comparison of FA and MTX as targeting ligands

After evaluating the therapeutic activity of the dendrimer–MTX conjugates, we compared the targeting ability of MTX and FA on the dendrimer scaffold. To evaluate their targeting ability in relation to the therapeutic activity of the dendrimer conjugates, we used free FA to block receptor-mediated endocytosis and assessed the cytotoxicity of the FA and MTX-targeted dendrimer conjugates. High concentrations of free FA for short periods of time (30 min–1 h) inhibits FR-mediated uptake of conjugates without affecting the cytotoxicity of MTX (data not shown). KB cells were incubated with 100 nM FA for 30 min to saturate the FR and then treated with 100 nM of the dendrimer–MTX conjugates for an additional 6 h. After treatment for 6 h, the cells were washed and fresh media was added. After 48 h, the cells were assayed for apoptosis using the An/7AAD assay as described above. Saturation of the FR with free FA inhibited the cytotoxicity of all of the dendrimer–MTX conjugates confirming that the conjugates internalize through the FR (Fig. 3). In agreement with our other findings, only cells treated with the dendrimer–eMTX (**2** and **3**) showed signs of apoptosis (Fig. 3). At the time points examined, the FA and MTX-targeted dendrimer conjugates had similar therapeutic activity that could be blocked to a similar extent with free FA. These results are in agreement with recent SPR studies demonstrating that the multivalent capacity of the dendrimer scaffold facilitates high avidity receptor–ligand interactions even when using low-affinity receptor–ligand pairs like MTX-FR.[23]

Therapeutics can be selectively delivered to diseased cells and tissues by incorporating targeting ligands on the polymer scaffold. This selectivity can be further enhanced because polymer scaffolds provide the opportunity for multivalent interactions to enhance binding avidity.[24] Previous studies have shown that MTX binds the FR with 100-fold lower affinity than FA.[25] Using surface plasmon resonance spectroscopy (SPR), we recently showed that decorating the dendrimer surface with MTX greatly enhances the conjugate affinity for the FR.[23] Building on these studies, we demonstrate in biological systems that both FA and MTX dendrimer-conjugates can deliver therapeutic payloads through the FR receptor with similar efficiency. Further studies are under way to validate these findings in vivo. By using MTX as the targeting ligand and therapeutic, the synthetic scheme for these dendrimer therapeutics is greatly simplified which we anticipate will facilitate further advancement of these therapeutics.

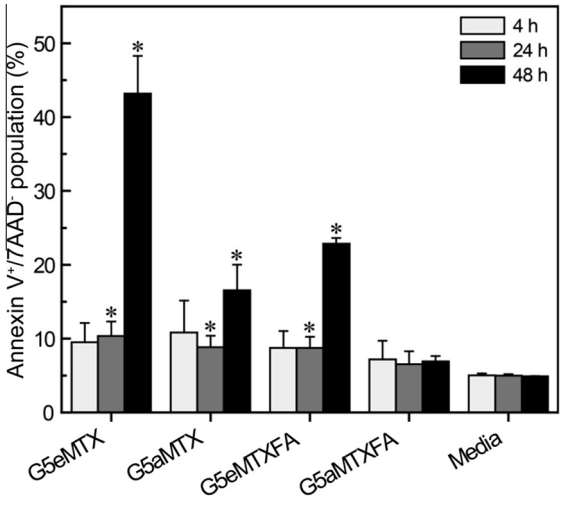


Figure 2. Therapeutic activity of dendrimer–MTX conjugates. KB cells were treated with 100 nM of the dendrimer–MTX conjugates (**2**–**5**) and analyzed for apoptosis at 4, 24, and 48 h. Apoptotic cells were defined as AnV+/7AAD− and assessed in relation to a 5% threshold for media control cells. Significance of a 1-sided paired student *t*-test recorded (p <0.05), for treatment groups compared to media control. Results are representative of three independent experiments.

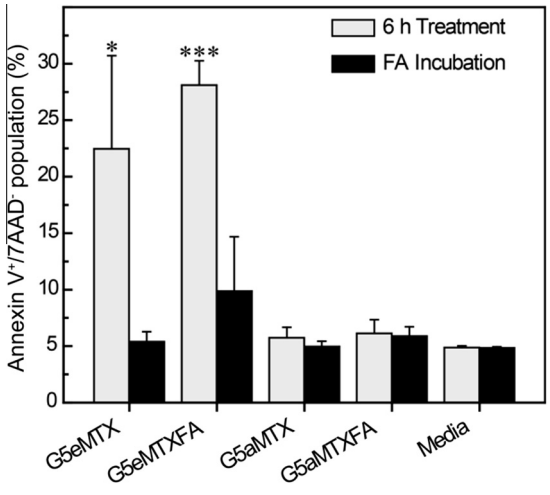


Figure 3. Competition experiments to compare folate receptor targeting of dendrimer FA and MTX ligands. KB cells were incubated with or without 100 nM free FA for 30 min and then treated with 100 nM of the dendrimer–MTX conjugates (2–5) for 6 h. After 6 h, cells were washed and media was replaced. KB cells were assayed at 48 h for AnV+/7AAD− as a marker of apoptosis as above. Apoptotic cells were defined as AnV+/7AAD− and assessed in relation to a 5% threshold for media control cells. Significance of a 1-sided paired student *t*-test recorded ($^{*}p$ <0.05 and $^{**}p$ <0.01), for treatment groups versus media control. Results are representative of three independent experiments.

We and others have shown that therapeutics can be incorporated on the dendrimer scaffold through either complexation or conjugation.[26–28] Our group has been exploring both approaches to deliver therapeutics balancing circulating stability with therapeutic efficacy. The ideal polymer therapeutic would have excellent stability in circulation as it is delivered to its biological target where it would have enhanced therapeutic activity. Unfortunately, for targeted therapeutics, there is a tradeoff between stability and efficacy with enhanced stability achieved at the expense of efficacy because of reductions in the intracellular release of the therapeutic payload. Using SPR and in vitro enzyme assays, our group recently reported that an amide dendrimer–MTX was able to inhibit the DHFR and had some cytotoxicity at high concentrations and extended incubation times. Although these results were provocative, the study did not systematically compare different linkages for MTX and how the linkages impacted efficacy. In this study, we found that the ester dendrimer–MTX conjugates were much more cytotoxic than the amide-dendrimer–MTX conjugates even though the ester- and amide-modified MTX compounds had similar activity. These results provide evidence that the polymer scaffold and the route of internalization can greatly impact the efficacy of polymer therapeutics.

In summary, this study provides several important insights into the design of polymer therapeutics. Specifically, we have shown that (1) Cu-free click chemistry can be used to efficiently functionalize dendrimer scaffolds, (2) dendrimers decorated with several of the low-affinity ligand MTX can be used for cell-specific targeting of the FR as well as the therapeutic, and (3) although the ester and amide-modified MTX compounds have similar cytotoxicity, the ester-linked dendrimer–MTX conjugates have much greater cytotoxicity than the amide-linked dendrimer–MTX conjugates. These results highlight the effects the polymer scaffold and the route of internalization can have on therapeutic activity and emphasize the importance of systematically evaluating polymer properties in biological systems. These findings have important implications for engineering polymer therapeutics and we anticipate these findings will facilitate their translation to the clinics.

Acknowledgments

This project has been funded with funds from the National Cancer Institute, National Institutes of Health, under award CA119409. S.N.G. was supported by an Inaugural Grant from the University of Michigan Cardiovascular Center.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.03.088.

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