Published in final edited form as:

ChemMedChem. 2011 January 3; 6(1): 49–53. doi:10.1002/cmdc.201000377.

Chemotherapeutic Evaluation of a Novel Synthetic Tubulysin Analogue-Dendrimer Conjugate in C26 Tumor Bearing Mice

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Keywords

Tubulysin; dendrimer; drug delivery; antitumor; chemotherapy; colon carcinoma

The tubulysins, first isolated by the Höfle/Reichenbach group from myxobacterial cultures,¹ are exceptionally potent cell-growth inhibitors that act by inhibiting tubulin polymerization and thereby induce apoptosis. For this reason, the biosynthesis,² mechanism,³ and anticancer activity⁴ of the tubulysins have been intensively investigated. Tubulysin D (Figure 1), the most potent of the tubulysins, has activity that exceeds virtually all tubulin modifiers, and in particular is 20- to 10000-fold more cytotoxic than the important, clinically approved anticancer drugs the epothilones, vinblastine, and paclitaxel.^{5,6} Unfortunately, the extraordinary activity of the most potent tubulysins poses considerable complications for chemotherapeutic applications due to toxicity against healthy cells and tissues. Moreover, the tubulysins are difficult to obtain in large quantities from the myxobacteria from which they are isolated, and total synthesis routes are prohibitively difficult due to the fragile and complex nature of the most potent of the naturally occurring tubulysins, which incorporate the labile *N,O*-acetal functionality.⁷ Consequently, both academia and the pharmaceutical industry have focused on the synthesis and evaluation of simpler and more stable tubulysin analogues.⁸ Similarly, significant efforts have been directed to prodrug strategies for selective tumor delivery of tubulysin and its analogues in order to minimize toxicity.^{8q}

Macromolecular drug carriers have proven to be particularly promising for the selective delivery of drugs to tumors.⁹ Carriers such as liposomes, particles, or polymer conjugates can improve drug delivery, and thus drug efficacy, by imparting the desirable properties of the carrier to the drug.¹⁰ Bound or encapsulated drugs exhibit prolonged blood circulation^{10j} increased tumor uptake due to the enhanced permeation-and-retention (EPR) effect,¹¹

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enhanced solubility,¹⁰ⁱ and reduced systemic toxicity. Dendrimers, a highly branched class of polymer, are of particular interest as drug carriers. The stepwise synthesis of dendrimers allows for control over architecture and physicochemical properties while incorporating diversified functional handles for attaching different groups such as solubilizing polyethylene glycol chains (PEG), drug molecules, or imaging agents.¹² Previous work by the Fréchet group has focused on exploring polymer-drug delivery with different dendritic architectures^{12c,e,s,u} and drug release mechanisms.^{12a-c,s,t} In a seminal work by Fréchet and coworkers, Doxorubicin (DOX), a chemotherapeutic drug, was successfully attached to the dendrimer by an acyl hydrazone bond and evaluated in mice bearing C26 colon carcinomas.^{9b}

Herein we describe the synthesis of a novel synthetic tubulysin analogue that contains a ketone moiety for conjugation to a dendrimer via an acid labile hydrazone linker, which has previously been shown to form drug conjugates that are stable in the blood stream but release their payload in endosomal compartments.¹³ The dendrimer conjugate is characterized both *in vitro* and *in vivo* and represents the first report on polymeric drug delivery vehicles utilizing synthetic tubulysin analogues.^{8q}

The design and synthesis of a tubulysin analogue appropriate for dendrimer delivery was based on our work^{8a} and work of others^{8m} establishing that the synthetically challenging and highly labile *N,O* acetal of the most potent tubulysins could be replaced with a simple and highly stable *N*-methyl group with minimal reduction in cytotoxicity. Moreover, structure activity relationships from the our^{8a} and other^{8j} labs also established that the tubuphenylalanine (Tup) portion of tubulysin D (Figure 1) can be extensively modified or truncated with only modest decreases in activity. With this knowledge we conceived and synthesized tubulysin analogue **3** (Scheme 1) from intermediate **1**, for which we had previously developed a synthetic route that proceeds in >40% overall yield from commercial materials.^{8b} Analogue **1** was coupled with 4-acetylphenethylamine (**2**) using PS-carbodiimide to give analogue **3** in 84% yield (Scheme 1). Analogue **3** can readily be prepared in large quantities, is considerably more stable than the most potent tubulysin natural products that incorporate the *N,O* acetal, and also contains a ketone functionality to serve as a handle for coupling to a dendrimer carrier via the acyl hydrazone bond.

The tubulysin analogue-dendrimer conjugate was prepared using the polymeric carrier^{12s}**4**, which is a 40 kDa PEGylated dendrimer with a polypeptide core. This dendrimer scaffold is obtained in a total of ten high yielding steps, and the periphery is decorated with hydrazides for coupling the ketone of tubulysin analogue **3** to form acyl hydrazone linkage (Scheme 2). Dendrimer **4**, after treatment with trifluoroacetic acid to remove the *N*-Boc groups, is then coupled with tubulysin ketone **3** in methanol with trifluoroacetic acid. The reaction progress was followed by ¹H NMR and was complete within 24 h, at which point the reaction was quenched by addition of triethylamine. Evaporation and subsequent purification by size exclusion chromatography resulted in pure tubulysin analogue-dendrimer conjugate **5** in 36% yield as an off white solid.

The release rates of tubulysin analogue **3** from the tubulysin analogue-dendrimer conjugate **5** in blood and endosomal compartments is a critical parameter for the selective delivery of **3** to tumor tissue. The rate at which **3** was released from the carrier was therefore measured at both pH 7.4 (1X PBS without Mg²⁺/Ca²⁺) and 5.0 (100 mM sodium acetate), which corresponds to the pH in blood and endosomal compartments, respectively. As shown in Figure 2, while **3** is released at pH 5.0, no observable release occurs at pH 7.4. These results support our original design of the carrier for release of the toxic tubulysin analogue after cellular endocytosis with little or no release during circulation in the blood.

The cytotoxicities of tubulysin analogue **3** and conjugate **5** were next measured in C26 murin colon carcinoma cells. As a reference, the toxicity of tubulysin D was also determined and found to be 0.14 ± 0.3 nM. Tubulysin analogue **3** had a reduced toxicity ($IC_{50} = 20 \pm 7.4$ nM) when compared to tubulysin D, which, we anticipated, would translate *in vivo* and allow for more favorable dosing conditions. Similarly, conjugate **5** exhibited a further reduction in toxicity ($IC_{50} = 1.50 \pm 0.01$ μ M), however, a significant increase in toxicity is expected upon endocytosis and hydrazone hydrolysis. It should be noted that while analogue **3** has limited water solubility, making *in vivo* application difficult, once conjugated to the dendrimer, **5** is readily water soluble.

To evaluate the toxicity of tubulysin analogue **3** and conjugate **5** *in vivo*, each compound was administered by i.v. delivery to healthy female Balb/C mice. The free analogue **3** was administered at 10 and 20 mg/kg. Immediate death resulted from higher doses; this is likely related to the low solubility of analogue **3** in aqueous systems, which leads to aggregation of **3** in the bloodstream with dire consequences. In contrast, soluble conjugate **5** could be administered at doses of up to 165 mg analogue-**3**/kg, without any sign of toxicity. Higher doses were not attempted for safety reasons after consideration of the viscous nature of highly concentrated polymer solutions.

The chemotherapeutic efficacy of tubulysin analogue **3** and conjugate **5** were next evaluated in C26 tumor bearing mice. Female Balb/C mice inoculated with C26 tumors were treated with a PBS control, **3** (10 and 20 mg /kg), or **5** (165 mg analogue 3/kg). Mice administered the free drug **3** exhibited signs of distress upon injection, again believed to be a side effect of compound aggregation. The injection proved fatal for two mice in the 20 mg/kg treatment group. In contrast, mice treated with **5** suffered no adverse effects during injection. Similarly, none of the treatment groups showed significant weight loss as a result of treatment related toxicity. The free tubulysin analogue did not extend the lifetimes of tumored mice or delay tumor growth (Figure 3, Table 1). However, mice given the polymeric formulation of the tubulysin analogue showed a 172% tumor growth delay (TGD) ($p=0.001$) and a median survival time of 38 days ($p<0.0001$) with three of the eight mice tumor-free at the conclusion of the study (Figure 4).

In conclusion, we have designed a novel, completely synthetic tubulysin analogue **3** capable of conjugation to a dendrimer through an acyl hydrazone linkage. Although **3** did exhibit greater stability, lower toxicity, and could be synthesized more readily than naturally occurring tubulysins, solubility was a limiting factor for its *in vivo* use. In contrast, its dendrimer conjugate **5** is highly water soluble and contains a payload of over ten percent by weight of the tubulysin analogue. Tumored mice treated with the maximum tolerated dose of analogue **3** suffered moderate to severe distress after injections and exhibited no therapeutic benefit compared to a PBS control. However, mice treated with a single dose of over ten times the amount of polymer-bound conjugate **5** showed no signs of toxicity, exhibited a substantially longer lifespan (90% increase in average), and showed a 37% survival rate. While this high dose of the dendrimer tubulysin conjugate was well tolerated, multiple dosing regimens at lower quantities as well as investigation of more potent tubulysin analogues are currently being pursued. To the best of our knowledge, this is the first example of polymeric drug delivery utilizing a synthetic tubulysin analogue.

Acknowledgments

JAE acknowledges the support of the National Science Foundation (CHE-0742565). FCS and JMJD thank the NIH (RO1EB 002047 and in part R01-EB005824) for the financial support of this work. GKD thanks the Swedish Funding Agencies (SAPS/VR).

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for the evaluation of folic acid coupling to tubulysins isolated from natural sources see: **ref 8h**. (ii) for the evaluation of polymers conjugates to tubulysins isolated from natural sources see **ref 8f**.

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IC₅₀ = 0.025 nM in SW480 cells

Figure 1.
Tubulysin D structure and cytotoxicity^{8a}

Figure 2.
Selective release of tubulysin analogue 3.

Scheme 1.
Synthesis of tubulysin analogue **3** for dendrimer delivery.

Scheme 2.
Synthesis of conjugate **5** (12 wt% loading of tubulysin analogue **3**).

Figure 3.
Impact on tumor growth over time

Figure 4.
Antitumor efficacy in C26 colon carcinoma

Table 1

Chemotherapeutic Efficacy of Tubulysin Ketone in C26 Colon Carcinoma

Treatment Group	No. mice	Dose (mg/kg)	Mean TGD (%)	Median survival time (days)	TRD	LTS
PBS	8	-	-	20 ± 1.1	0	0
3	8	10	17 ± 2.7	21 ± 3.2	0	0
3	8	20	17 ± 1.8	23 ± 7.9	2	0
5	8	165	172 ± 13 ^[a]	38 ± 14 ^[b]	0	3

TGD- tumor growth delay, calculated from growth to 500 mm³; TRD-treatment related deaths; LTS- long term survivors.

^[a] Compared to PBS, P = 0.001.

^[b] Compared to PBS, P < 0.0001