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The self-assembly of a camptothecin-lysine nanotube

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ABSTRACT

A simple, low molecular weight camptothecin-lysine conjugate is reported to self-assemble into nanotubes with diameters of 70–100 nm and a drug loading level of 60.5%. The nanotubes exhibited promising in vitro cytotoxicity against cancer cell lines A549, NCI-H460 and NCI-H23. The release of active camptothecin was highly dependent on conjugate concentration, temperature and pH of the solution.

The selective delivery of high doses of anticancer drugs to tumor sites without side effects remains as an important goal in cancer therapy.[1] Nanotechnology is emerging as a potential strategy to reduce the dose-limiting toxicity of many chemotherapeutic agents.[2] Linking anticancer drugs to excipient nanoscale carriers[3] exploits the enhanced permeability and retention (EPR) effect to achieve selective transport to tumor tissue.[4] This effect allows nanoscale materials to accumulate in tumor tissues via the abnormally leaky blood vessels at these sites. The majority of nanoscale materials used as inert carriers are polymers,[5] solid nanoparticles,[2c,3b,6] and liposomal aggregates,[7] which bind the drug via noncovalent encapsulation[6a,8] or covalent conjugation.[7a,9] The drug loading level of these constructs is often limited by the size of the inert carrier, which often dominates the mass of the conjugated drug, requiring larger quantities to be administered.[5c,5e] Thus, decreasing the mass of the carrier is necessary to increase the effective drug loading of the nanomedicine. Higher drug loadings have been achieved by the multivalent attachment of the drug to carriers such as dendrimers.[5b,10] An alternative approach creates the nanoscale carrier via the self-assembly of a suitable derivative of the drug.[7b,11] This strategy offers a potential to achieve higher loading levels by using smaller, drug-containing building blocks as precursors.[12]

Camptothecin (CPT) is a natural quinoline alkaloid with potent anti-tumor activity that functions through the inhibition of DNA topoisomerase I.[13] However, the clinical application of CPT is limited due to its poor aqueous solubility, the instability of the E-lactone ring and nonselective biodistribution.[14] Previously, we reported that the assembly of CPT-dipeptide conjugates produced well-defined nanotubes in PBS and human serum.[11c] These nanostructures exhibited enhanced resistance to hydrolytic deactivation and showed high in vitro potency against several human cancer cell types. In this work, we report that a smaller CPT-Lysine conjugate (**A**) assembles into water soluble, uniform nanotubes (Fig. 1) having a high drug loading (≈60.5%) and also exhibits favorable in vitro cytotoxicity and cellular uptake against several tumor cell lines. Furthermore, the potential hydrolytic breakdown products of CPT-lysine **A** would be succinic acid and lysine, which are both classified as 'generally recognized as safe' (GRAS) by the FDA.

CPT-Lysine **A** was prepared via on-resin modification of the ε-amino group of N_{α}-Fmoc-lysine with CPT, linked through a 20-*O*-succinic acid linkage[15] (Scheme S2). In contrast to free CPT, which was insoluble in water (0.003 mg/mL),[16] CPT-Lysine **A** exhibited excellent aqueous solubility (5.14 mg/mL in PBS, pH = 7.4; 12.1 mg/mL in water). The self-assembly of **A** was explored by transmission electron microscopy (TEM) in PBS and pure water. A sample of **A**, incubated at 10 mM in PBS for 72 h, then diluted to 1 mM, exhibited an array of nanotubes displaying diameters ranging from 70 to 100 nm and lengths of several micrometers by TEM imaging (Fig. 2a, S2). When shorter incubation times were employed prior to imaging, a range of intermediates that preceded the formation of well-defined nanotubes could be observed.[17] For example, after 24 h at 10 mM, short, incompletely formed

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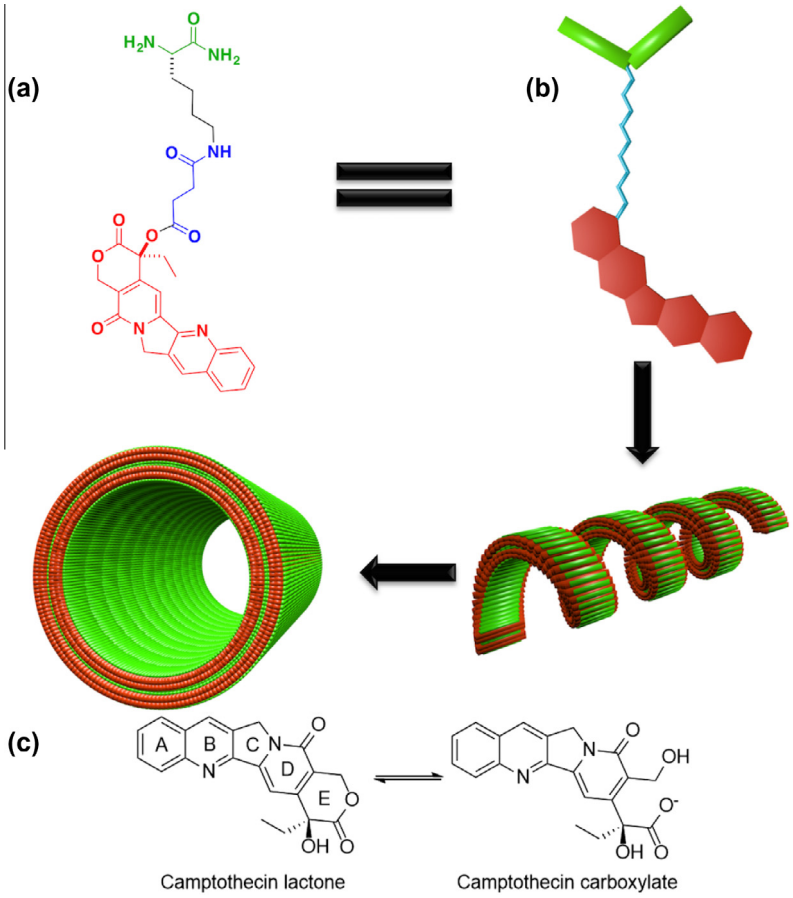

Figure 1. Structure and self-assembly of CPT-Lysine **A**. (a) CPT-lysine **A**. (b) Progressive helical winding and nanotube formation. (c) Hydrolysis of E lactone ring in camptothecin.

nanotubes (<500 nm) were present (Fig. 2b). Under these conditions, partially formed nanotubes, coiled ribbons and helical tapes could be discerned in addition to the fully formed nanotubes. The critical aggregation concentration (CAC) of freshly dissolved solutions of **A** in PBS and H_2O, prepared without preincubation, was 160 and 200 μM respectively, measured using the solvochromatic dye Nile Red (Fig. S4).[18] Solutions of **A** at concentrations in this range (250 μM in PBS), imaged without prior incubation at 10 mM, displayed primarily non-specific aggregation. In pure water (10 mM for 72 h, then diluted to 1 mM, pH 7), **A** formed parallel arrays of nanotubes with decreased diameters (~40 nm) (Fig. 2c). The aligned packing and decreased diameters of the nanotubes can be attributed to the reduced charge screening that takes place in pure water.[19]

The thickness of the nanotube walls (~7.5 nm), as measured by TEM imaging, suggested a double bilayer structure comprised of ~4 molecules of **A** in an extended conformation (1.7 nm) (Fig. 2a (inset), S2). The zeta potential of nanotube **A** in PBS was 19.7 mV, due to the positive ammonium head group of **A**. The UV–Vis spectra of **A** revealed bands at 350 and 368 nm in PBS that were decreased in amplitude and slightly red-shifted compared with solutions measured in TFE, indicative of *J*-type aggregation of the CPT chromophores in PBS (Fig. 2d).[20] Fourier transform infrared spectra of a sample of **A**, prepared in PBS (20 mM), then lyophilized and redissolved in D_2O, exhibited a peak at 1650 cm^{-1}, indicative of the lack of any β-sheet interactions within the assembly (Fig. S1).

The self-assembly of **A** sequesters the hydrophobic CPT structure within the hydrophobic regions of the nanotubes, thereby protecting the 20-*O*-succinyl linkage from the hydrolytic aqueous environment. Free CPT undergoes a reversible, pH-dependent ring-opening of the lactone ring in water, producing the carboxylate form, which has been shown to be toxic.[21] Although hydrolytic cleavage of the 20-*O*-succinyl linkage is required to produce active CPT, competing hydrolysis of the lactone ring of CPT has a detrimental impact on its biological activity.[21c] Accordingly, we explored the capability of the nanotube structure to enhance the stability of the CPT lactone and the 20-*O*-succinyl linkage to hydrolytic cleavage in PBS. The release of CPT from the nanotubes formed from **A** was measured by HPLC over one week as a function of concentration in PBS at 37 °C. Under these conditions, hydrolytic cleavage of 20-*O*-succinyl linkage produced free CPT, observed in both the lactone and carboxylate states.[22] However, the carboxylate form of **A**, which would be formed by CPT-lactone hydrolysis, could not be detected at any concentration or time point. In contrast, exposure of **A** to borate buffer at pH 9 for 6 h, produced free CPT-carboxylate and the carboxylate form of **A**, clearly observable by hplc analysis (Fig. S8). For example, after 3 days at 1 mM in PBS, 61% of CPT was released from **A**, observed in both the CPT-lactone (79%) and CPT-carboxylate (21%) forms. The remainder of **A** (39%) was intact as the lactone without any apparent hydrolysis to the carboxylate form (Fig. S5). Although no apparent ring-opening of **A** could be observed, it is not possible to exclude the possibility that ring-opening takes place prior to a more rapid cleavage of the 20-*O*-succinyl linkage, which would also preclude observation of **A**-carboxylate. The rate of hydrolytic release of CPT from **A** depended strongly on concentration (Fig. 3a). Accordingly, as the concentration was increased from

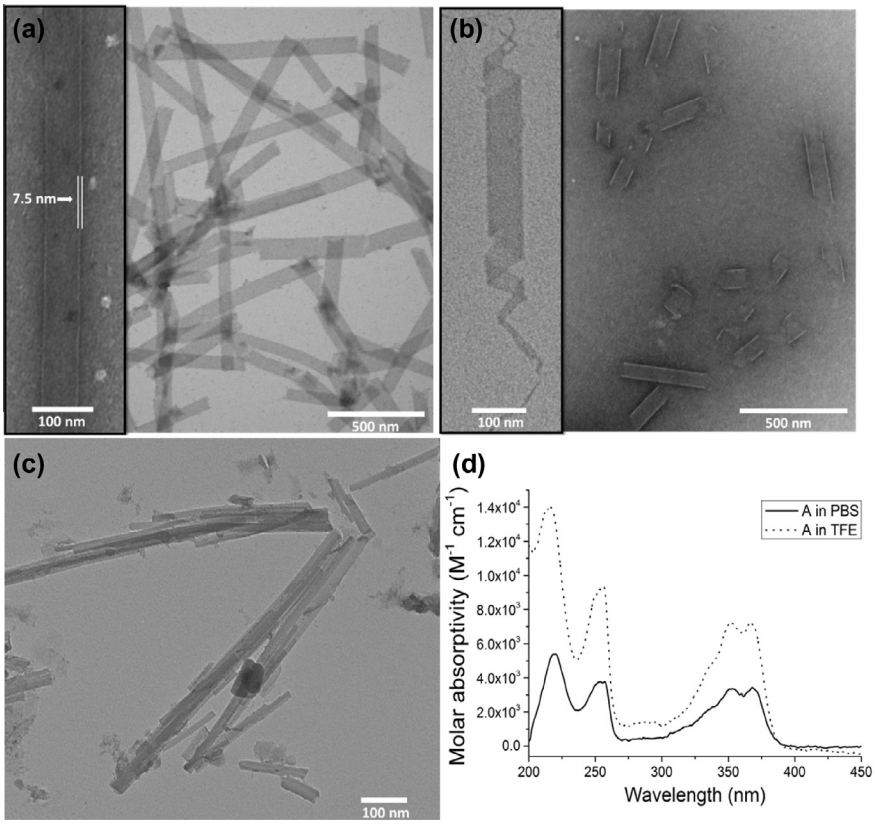

Figure 2. TEM images of **A** in (a) PBS (3 days, pH 7.4), (b) PBS (1 day, pH 7.4) and (c) water (3 days, pH 7.0). Samples were prepared by dissolving **A** in PBS or H_2O (10 mM), then diluting to 1 mM after 1 or 3 days. (d) UV–Vis spectra of **A** in PBS and TFE. Solutions in either PBS or TFE were prepared by diluting a 10 mM concentration to 250 μM after 3 days.

0.1 mM to 10 mM, the amount of free CPT progressively decreased from 70% to 4% after 30 h (Fig. S7). These observations contrast with the hydrolytic instability of free CPT in PBS, which experiences rapid hydrolysis to the inactive carboxylate form within hours.[21a]

The CPT release rates were also studied at lower pH values to replicate the lysosomal and tumor environments, which are more acidic than normal tissues.[3b,23] The pH of the buffer had a large impact on the rate of CPT release. For example, at 0.1 mM in PBS, whereas 55% of the CPT was released from **A** at pH 7.4 (37.5 °C), only 15% was released at pH 6.0. At 1 mM, the rate of release was ~7-fold slower at pH 6.0; however, the CPT-lysine conjugate was stable at 10 mM at either pH value (Fig. 3b, S6). The pH dependence of the hydrolytic cleavage of the 20-*O*-succinyl linkage in the nanotubes was consistent with reports of other CPT conjugates attached via a 20-*O* ester group.[5a,24]

The CPT-lysine **A** was assessed for efficacy against human non-small cell lung cancer (NSCLC) cell lines A549, NCI-460, and NCI-H23, which were selected according to the indications of approved CPT derivative Topotecan.[25] The cytotoxic activity was assayed using MTT-assay over the course of a 96 h incubation period and the IC_{50} values were 1.12 μM, 0.43 μM, 0.12 μM for **A**, and 0.35 μM, 0.19 μM, and 0.07 μM for CPT, respectively (Fig. 4a). CPT exhibits a roughly 2–3 fold higher potency than **A** in all cell

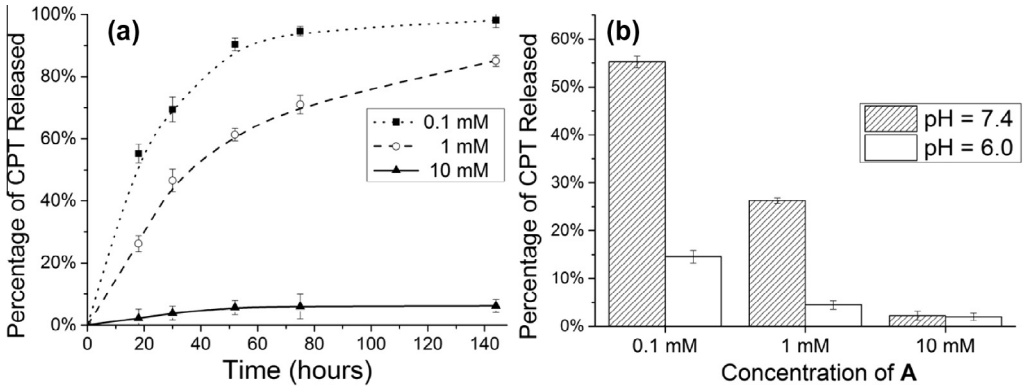

Figure 3. (a) Release of CPT in PBS at 37 °C, pH 7.4 and (b) Release of CPT from compound **A** in PBS (pH = 7.4) and pH = 6 at 37 °C after 15 h. Solutions of **A** were aged in PBS at different concentrations. Percentage of CPT released was monitored by analytical reverse-phase HPLC eluting with a linear gradient of CH_3CN/water containing 0.05 M ammonium acetate in H_2O, pH = 5.5–6.0.

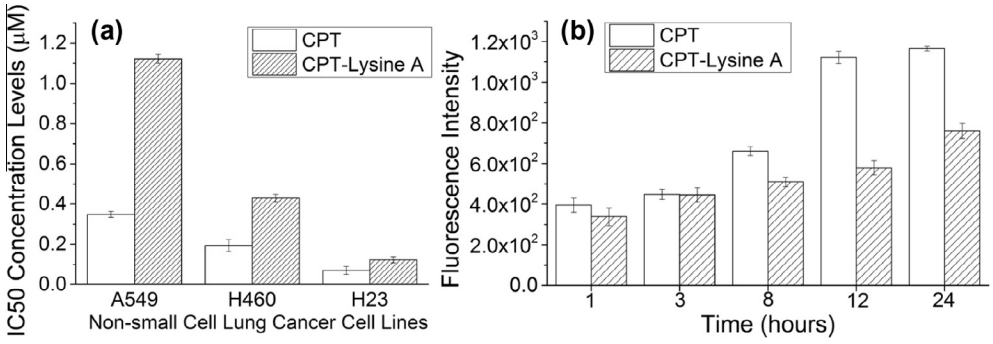

Figure 4. (a) IC_{50} values of **A** and CPT on Non-Small Cell Lung Cancer Cell Lines A549, H460 and H23 (b) Normalized flow cytometry results showing intracellular drug uptake of **A** and CPT on human lung cancer cell lines A549 over 24 h period.

lines; however, it is not used clinically due to its limited aqueous solubility and toxicity. Flow cytometry was performed with the A549 cells to monitor the cellular uptake of **A**, and compared with free CPT. The cellular uptake was monitored at 425 nm, after excitation at 355 nm, over a 24 h period. As shown in Figure 4b, CPT-treated cells exhibited a larger number of fluorescent cells at longer incubations, but the uptake was similar for both **A** and CPT during the first 8 h. The lower cytotoxicity of **A** may be due to the slower cellular uptake and the gradual release of the active CPT component.

In summary, the self-assembly and anticancer activity of a simple CPT-lysine conjugate was reported. The CPT-lysine conjugate assembles into well-defined nanotubes in PBS with diameters ranging from 70 to 100 nm. The nanotube structures provide a hydrophobic environment for the CPT segment, thereby protecting the drug from hydrolytic lactone inactivation and achieving higher aqueous solubility. Hydrolytic cleavage of the 20-*O*-succinyl linkage readily releases active CPT at rates that depend strongly on concentration, temperature and the pH of the solution. The tremendous stability imparted to the CPT drug linkage suggests that drug release occurs from smaller aggregates or monomeric forms of **A**. This possibility creates a potential to induce drug release via a triggered disassembly of the nanotube structure. This strategy also enables the creation of a nanoscale drug delivery vehicle that is comprised primarily of a low molecular weight derivative of CPT, resulting in exceptionally high drug loadings (60.5%), aqueous solubility and increased CPT stability without the need for excipient, macromolecular carriers.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.04.056.

References and notes

1. He, Q.; Shi, J. *Adv. Mater.* **2014**, *26*, 391.
2. (a) Devadasu, V. R.; Bhardwaj, V.; Kumar, M. N. *Chem. Rev.* **2013**, *113*, 1686; (b) Gao, Z.; Zhang, L.; Sun, Y. *J. Controlled Release* **2012**, *162*, 45; (c) Faraji, A. H.; Wipf, P. *Bioorg. Med. Chem.* **2009**, *17*, 2950.
3. (a) Li, R. X.; Shu, C.; Wang, W.; Wang, X. L.; Li, H.; Xu, D. K.; Zhong, W. Y. *J. Pharm. Sci.* **2015**, *104*, 2266; (b) Madhusudhan, A.; Reddy, G. B.; Venkatesham, M.; Veerabhadram, G.; Kumar, D. A.; Natarajan, S.; Yang, M. Y.; Hu, A. R.; Singh, S. S. *Int. J. Mol. Sci.* **2014**, *15*, 8216.
4. (a) Fang, J.; Nakamura, H.; Maeda, H. *Adv. Drug Delivery Rev.* **2011**, *63*, 136; (b) Torchilin, V. *Adv. Drug Delivery Rev.* **2011**, *63*, 131.
5. (a) Cheng, J. J.; Khin, K. T.; Jensen, G. S.; Liu, A. J.; Davis, M. E. *Bioconjugate Chem.* **2003**, *14*, 1007; (b) Feng, X. S.; Pinaud, J.; Chaikof, E. L.; Taton, D.; Gnanou, Y. *J. Polym. Sci., Part B: Polym. Phys* **2011**, *49*, 2839; (c) Fox, M. E.; Guillaudeu, S.; Frechet, J. M.; Jerger, K.; Macaraeg, N.; Szoka, F. C. *Mol. Pharm.* **2009**, *6*, 1562; (b) Kamaly, N.; Yameen, B.; Wu, J.; Farokhzad, O. C. *Chem. Rev.* **2016**; (e) Yurkovetskiy, A. V.; Fram, R. J. *Adv. Drug Delivery Rev.* **2009**, *61*, 1193.
6. (a) Chen, Y.; Chen, H. R.; Zeng, D. P.; Tian, Y. B.; Chen, F.; Feng, J. W.; Shi, J. L. *ACS Nano.* **2010**, *4*, 6001; (b) Tanaka, K.; Kitamura, N.; Morita, M.; Inubushi, T.; Chujo, Y. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5463.
7. (a) Ke, X.; Ng, V. W.; Ono, R. J.; Chan, J. M.; Krishnamurthy, S.; Wang, Y.; Hedrick, J. L.; Yang, Y. Y. *J. Controlled Release* **2014**, *193*, 9; (b) Zhu, W.; Fang, S.; Zhang, Y.; Li, X. *Bioorg. Med. Chem. Lett.* **2015**, *25*, 188.
8. Morgan, M. T.; Nakanishi, Y.; Kroll, D. J.; Griset, A. P.; Carnahan, M. A.; Wathier, M.; Oberlies, N. H.; Manikumar, G.; Wani, M. C.; Grinstaff, M. W. *Cancer Res.* **2006**, *66*, 11913.
9. Shi, M.; Ho, K.; Keating, A.; Shoichet, M. S. *Adv. Funct. Mater.* **2009**, *19*, 1689.
10. (a) Kaminskas, L. M.; McLeod, V. M.; Porter, C. J. H.; Boyd, B. J. *Mol. Pharm.* **2012**, *9*, 355; (b) Isaacman, S.; Buckley, M.; Wang, X.; Wang, E. Y.; Liebes, L.; Canary, J. W. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 1290; (c) Lock, L. L.; LaComb, M.; Schwarz, K.; Cheetham, A. G.; Lin, Y.-A.; Zhang, P.; Cui, H. *Faraday Discuss.* **2013**, *166*, 285.
11. (a) Baek, K.; Hwang, I.; Roy, I.; Shetty, D.; Kim, K. *Acc. Chem. Res.* **2015**, *48*, 2221; (b) Cheetham, A. G.; Zhang, P. C.; Lin, Y. A.; Lock, L. L.; Cui, H. G. *J. Am. Chem. Soc.* **2013**, *135*, 2907; (c) Kim, S. H.; Kaplan, J. A.; Sun, Y.; Shieh, A.; Sun, H. L.; Croce, C. M.; Grinstaff, M. W.; Parquette, J. R. *Chem. Eur. J.* **2015**, *21*, 101; (d) Rosler, A.; Vandermeulen, G. W. M.; Klok, H. A. *Adv. Drug Delivery Rev.* **2012**, *64*, 270; (e) Nagahama, K.; Sano, Y.; Kumano, T. *Bioorg. Med. Chem. Lett.* **2015**, *25*, 2519; (f) Bhardwaj, I.; Jha, D.; Admane, P.; Panda, A. K.; Haridas, V. *Bioorg. Med. Chem. Lett.* **2016**, *26*, 672; (g) Kim, S. H.; Sun, Y.; Kaplan, J. A.; Grinstaff, M. W.; Parquette, J. R. *New J. Chem.* **2015**, *39*, 3225; (h) Hu, X.; Hu, J.; Tian, J.; Ge, Z.; Zhang, G.; Luo, K.; Liu, S. *J. Am. Chem. Soc.* **2013**, *135*, 17617; (i) Lin, Y. A.; Cheetham, A. G.; Zhang, P. C.; Ou, Y. C.; Li, Y. G.; Liu, G. S.; Hermida-Merino, D.; Hamley, I. W.; Cui, H. G. *ACS Nano.* **2014**, *8*, 12690; (j) Sun, Y.; Kaplan, J. A.; Shieh, A.; Sun, H. L.; Croce, C. M.; Grinstaff, M. W.; Parquette, J. R. *Chem. Commun.* **2016**.
12. (a) Shen, Y. Q.; Jin, E. L.; Zhang, B.; Murphy, C. J.; Sui, M. H.; Zhao, J.; Wang, J. Q.; Tang, J. B.; Fan, M. H.; Van Kirk, E.; Murdoch, W. J. *J. Am. Chem. Soc.* **2010**, *132*, 4259; (b) Pei, Q.; Hu, X.; Li, Z.; Xie, Z.; Jing, X. *RSC Adv.* **2015**, *5*, 81499.
13. (a) Pommier, Y. *Nat. Rev. Cancer* **2006**, *6*, 789; (b) Wall, M. E. *Med. Res. Rev.* **1998**, *18*, 299; (c) Wall, M. E.; Wani, M. C.; Cook, C. E.; Palmer, K. H.; Mcphail, A. T.; Sim, G. A. *J. Am. Chem. Soc.* **1966**, *88*, 3888; (d) Thomas, C. J.; Rahier, N. J.; Hecht, S. M. *Bioorg. Med. Chem.* **2004**, *12*, 1585.
14. (a) Burke, T. G.; Mi, Z. H. *Anal. Biochem.* **1993**, *212*, 285; (b) Davis, M. E. *Adv. Drug Delivery Rev.* **2009**, *61*, 1189; (c) Mi, Z. H.; Burke, T. G. *Biochemistry* **1994**, *33*, 10325; (d) Zhou, Y.; Xu, X. L.; Sun, Y.; Wang, H. P.; Sun, H. P.; You, Q. D. *Bioorg. Med. Chem. Lett.* **2013**, *23*, 2974.
15. (a) Greenwald, R. B.; Pendri, A.; Conover, C. D.; Lee, C.; Choe, Y. H.; Gilbert, C.; Martinez, A.; Xia, J.; Wu, D. C.; Hsue, M. *Bioorg. Med. Chem.* **1998**, *6*, 551; (b) Safavy, A.; Georg, G. I.; Vander Velde, D.; Raisch, K. P.; Safavy, K.; Carpenter, M.; Wang, W.; Bonner, J. A.; Khazaeli, M. B.; Buchsbaum, D. *J. Bioconjugate Chem.* **2004**, *15*, 1264.
16. (a) Saha, S. C.; Patel, D.; Rahman, S.; Savva, M. *J. Pharm. Sci.* **2013**, *102*, 3653; (b) Zhang, L. M.; Lu, Z. X.; Li, X. L.; Deng, Y.; Zhang, F. Q.; Ma, C.; He, N. Y. *Polym. Chem.* **2012**, *3*, 1958.
17. Shao, H.; Gao, M.; Kim, S. H.; Jaroniec, C. P.; Parquette, J. R. *Chem. Eur. J.* **2011**, *17*, 12882.
18. Stuart, M. C. A.; van de Pas, J. C.; Engberts, J. B. F. N. *J. Phys. Org. Chem.* **2005**, *18*, 929.

19. Dong, H.; Paramonov, S. E.; Aulisa, L.; Bakota, E. L.; Hartgerink, J. D. *J. Am. Chem. Soc.* **2007**, *129*, 12468.
20. Kasha, M.; Rawls, H. R.; El-Bayoumi, M. A. *Pure Appl. Chem.* **1965**, *11*, 371.
21. (a) Burke, T. G.; Mi, Z. H. *J. Med. Chem.* **1994**, *37*, 40; (b) Fassberg, J.; Stella, V. J. *J. Pharm. Sci.* **1992**, *81*, 676; (c) Hertzberg, R. P.; Caranfa, M. J.; Holden, K. G.; Jakas, D. R.; Gallagher, G.; Mattern, M. R.; Mong, S. M.; Bartus, J. O.; Johnson, R. K.; Kingsbury, W. D. *J. Med. Chem.* **1989**, *32*, 715.
22. Zhao, H.; Lee, C.; Sai, P. K.; Choe, Y. H.; Boro, M.; Pendri, A.; Guan, S. Y.; Greenwald, R. B. *J. Org. Chem.* **2000**, *65*, 4601.
23. (a) Bareford, L. A.; Swaan, P. W. *Adv. Drug Delivery Rev.* **2007**, *59*, 748; (b) Gerweck, L. E.; Seetharaman, K. *Cancer Res.* **1996**, *56*, 1194.
24. (a) Cheng, J.; Khin, K. T.; Davis, M. E. *Mol. Pharm.* **2004**, *1*, 183; (b) Greenwald, R. B.; Pendri, A.; Conover, C.; Gilbert, C.; Yang, R.; Xia, J. *J. Med. Chem.* **1996**, *39*, 1938; (c) Zhao, H.; Rubio, B.; Sapra, P.; Wu, D. C.; Reddy, P.; Sai, P.; Martinez, A.; Gao, Y.; Lozanguiez, Y.; Longley, C.; Greenberger, L. M.; Horak, I. D. *Bioconjugate Chem.* **2008**, *19*, 849.
25. O'Brien, M.; Eckardt, J.; Ramlau, R. *Oncologist* **2007**, *12*, 1194.