Drug Delivery Hot Paper

International Edition: DOI: 10.1002/anie.201804156
German Edition: DOI: 10.1002/ange.201804156

Floxuridine Homomeric Oligonucleotides "Hitchhike" with Albumin In Situ for Cancer Chemotherapy

Cheng Jin[+], Hui Zhang[+], Jianmei Zou, Yan Liu, Lin Zhang, Fengjie Li, Ruowen Wang, Wenjing Xuan, Mao Ye, and Weihong Tan**

Abstract: *Automated attachment of chemotherapeutic drugs to oligonucleotides through phosphoramidite chemistry and DNA synthesis has emerged as a powerful technology in constructing structure-defined and payload-tunable oligonucleotide–drug conjugates. In practice, however, in vivo delivery of these oligonucleotides remains a challenge. Inspired by the systemic transport of hydrophobic payloads by serum albumin in nature, we report the development of a lipid-conjugated floxuridine homomeric oligonucleotide (LFU20) that "hitchhikes" with endogenous serum albumin for cancer chemotherapy. Upon intravenous injection, LFU20 immediately inserts into the hydrophobic cave of albumin to form an LFU20/albumin complex, which accumulates in the tumor by the enhanced permeability and retention (EPR) effect and internalizes into the lysosomes of cancer cells. After degradation, cytotoxic floxuridine monophosphate is released to inhibit cell proliferation.*

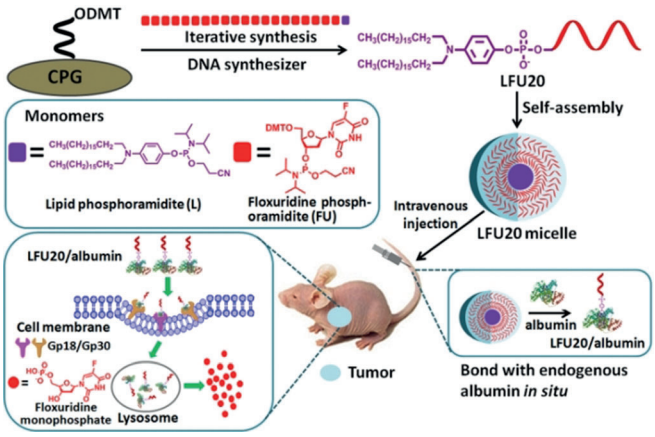
Solid-phase synthesis of nucleic acids is an example of automated and modular molecular synthesis.[1] This technology can generate oligonucleotides from nucleoside phosphoramidites with high yields. As such, almost any small molecule can be incorporated into oligonucleotides to generate advanced nucleic acids with novel properties, but only under the following conditions: 1) The corresponding phosphoramidites are available, and 2) the small molecules are stable during DNA synthesis and subsequent treatment. Based on these principles, various molecular drugs have been engineered as building blocks to synthesize therapeutic oligonucleotides with well-defined molecular structure and tunable payload.[2] Among these drug modules, therapeutic nucleoside and nucleobase analogues show advantages because of their excellent stability during DNA synthesis.[3]

Fluorouracil, one of the most notable therapeutic nucleobase analogues, has been approved as an antimetabolite drug in the treatment of cancers and other diseases.[4] Recently, floxuridine was employed to construct fluoropyrimidine polymer (F10) and to pair with guanosine in a DNA polyhedral nanosystem for cancer therapy.[3c,5] However, in spite of these advances, drug-incorporated oligonucleotides face challenges in systemic delivery.[6] Conventional methods focus on chemical modifications with active targeting ligands and conjugation with nanoparticles, which accumulate in the tumor by the EPR effect. However, these technologies have their limitations, such as complicated preparation and poor safety profiles.[7] Therefore, facile and effective carriers for in vivo delivery of drug-incorporated oligonucleotides are still needed.[8]

Serum albumin is the most abundant serum protein (about 40 $mg\,mL^{-1}$)[9] and has an attractive circulation half-time ($t_{1/2}$) of about twenty days.[10] It is also well-known as a natural transporter of poorly water-soluble molecules (for example, lipids and cholesterol) in plasma.[3d,11] Remarkably, as a delivery carrier for chemotherapy, the biological safety of serum albumin outperforms that of most other carriers.[11] Therefore, various hydrophobic payloads, for instance, paclitaxel (Abraxane) and perflutren (Optison), have been designed to bond with albumin for cancer therapy and for contrast enhancement during ultrasound imaging, respectively.[12] Apart from hydrophobic payloads, Levemir (insulin detemir), a man-made human insulin analogue with a hydrophobic alkyl chain at the terminus, was designed to bond with endogenous serum albumin for longer circulation time.[13]

Following the concept of these examples, we developed a LFU20 able to "hitchhike" with endogenous albumin for cancer therapy (Scheme 1). A lipid tail with two octadecyl chains inserts into the hydrophobic core of albumin by hydrophobic interactions.[6,8a,14] As such, upon intravenous injection, LFU20 noncovalently bonds with albumin in situ to form an LFU20/albumin complex. LFU20/albumin accumulates in the tumor by the EPR effect and internalizes into the lysosomes of cancer cells by the Gp18/Gp30-mediated pathway. After enzymatic degradation, floxuridine monophosphate is released to inhibit cell proliferation.[5,15]

[*] C. Jin,[+] H. Zhang,[+] J. Zou, Y. Liu, L. Zhang, F. Li, W. Xuan, Prof. M. Ye, Prof. W. Tan
Molecular Science and Biomedicine Laboratory, State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, College of Life Sciences, Aptamer Engineering Center of Hunan Province, Hunan University
Changsha, Hunan, 410082 (China)
E-mail: yemaocsu@hotmail.com
tan@chem.ufl.edu
Prof. R. Wang, Prof. W. Tan
Institute of Molecular Medicine, Renji Hospital, Shanghai Jiao Tong University School of Medicine, and College of Chemistry and Chemical Engineering, Shanghai Jiao Tong University, Shanghai, (China)
Prof. R. Wang, Prof. W. Tan
Department of Chemistry and Department of Physiology and Functional Genomics, Center for Research at the Bio/Nano Interface, Health Cancer Center, UF Genetics Institute and McKnight Brain Institute, University of Florida
Gainesville, FL 32611-7200 (USA)
[+] These authors contributed equally to this work.
Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/anie.201804156.



Scheme 1. Illustration of the solid-phase synthesis of LFU20, self-assembly of LFU20 to a micellar nanostructure, noncovalent interaction of LFU20 with endogenous albumin after intravenous injection, and the subsequent cancer therapy of LFU20.

LFU20 was generated by iterative synthesis of one lipid and twenty floxuridine phosphoramidites on a DNA synthesizer (Supporting Information, Figure S7). After deprotection and purification, LFU20 was obtained with acceptable yields (>70%) and high purity (97%) (Supporting Information, Figures S8 and S10). In DPBS buffer, LFU20 was self-assembled into amphiphilic polymeric micelles (Figure S12). However, LFU20 DNA micelles can be disrupted by serum albumin, which has strong binding affinity for the hydrophobic lipid tail (Figure 1a). As shown in Figure 1b, 0.5 mM bovine serum albumin (BSA) induces the complete conversion of 10 μM LFU20 micelles to LFU20/albumin complex (lanes 4 and 5). In contrast, FU20, without a lipid tail at the 5′-terminus, has no obvious interaction with BSA (lanes 1 and 2), demonstrating that the lipid tail is essential for the noncovalent interaction between LFU20 and BSA.

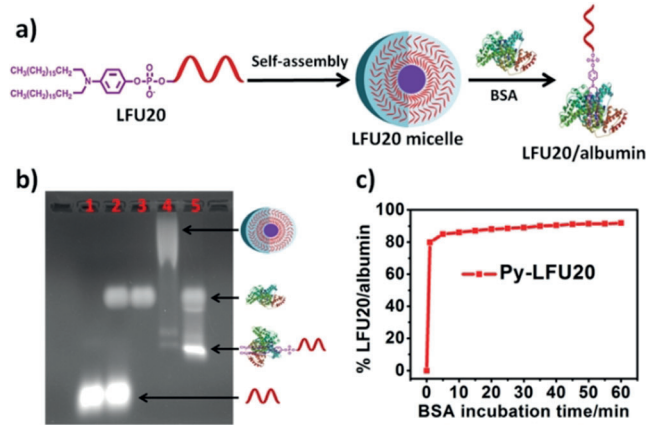


Figure 1. a) Illustration of LFU20 self-assembly into DNA micellar structure and noncovalent interaction with serum albumin. b) 2% agarose gel electrophoresis analysis of noncovalent interaction between 10 μM FU20 or LFU20 with 0.5 mM BSA. Lane 1, FU20; lane 2, FU20 + BSA; lane 3, BSA; lane 4, LFU20 micelles; and lane 5, LFU20 micelles + BSA. Because of their large particle size (about 43 nm), LFU20 micelles exhibit a tailed band in lane 4. LFU20/albumin migrates faster than albumin in lane 5, which can be explained by the presence of negatively charged LFU20. c) Time-dependent analysis of the percentage of Py-LFU20 in the albumin-bound state after incubation with 0.5 mM BSA.

To further investigate the kinetics of LFU20 interaction with albumin, pyrene molecules, which have excimer fluorescence in the aggregated state and monomer fluorescence in the dissociated state, were used to monitor the formation of the LFU20/albumin complex. As shown in Figure 1c and the Supporting Information, Figure S13, upon the addition of BSA, almost all Py-LFU20 micelles had dissociated within one minute to form Py-LFU20/albumin. Py-G8-LFU12 micelles, which have intermolecular G-quadruplexes in the micellar corona to stabilize the micellar structure,[14] showed only 48% formation of Py-G8-LFU12/albumin, even after one hour of incubation (Figure S13f). Apart from BSA, 0.5 mM human serum albumin (HSA) and 10% mice blood also induce effective formation of LFU20/albumin complex (Supporting Information, Figures S14 and S15). Since the concentration of albumin in blood is about 0.52–0.75 mM,[16] which is greater than 0.5 mM, we can conclude that the intravenous injection of LFU20 DNA micelles into the body will result in their complete and immediate conversion to LFU20/albumin complex.

Having confirmed the noncovalent interaction of LFU20 DNA with serum albumin in vitro, we further investigated the cellular endocytosis of the LFU20/albumin complex. As a negatively charged biomacromolecule, FU20 (without lipid tail) cannot internalize into cytoplasm individually (Figure 2a). LFU20 DNA anchors onto the cell membrane in DPBS solution (without serum albumin), even after two hours of incubation (Figures 2a and S16). However, in

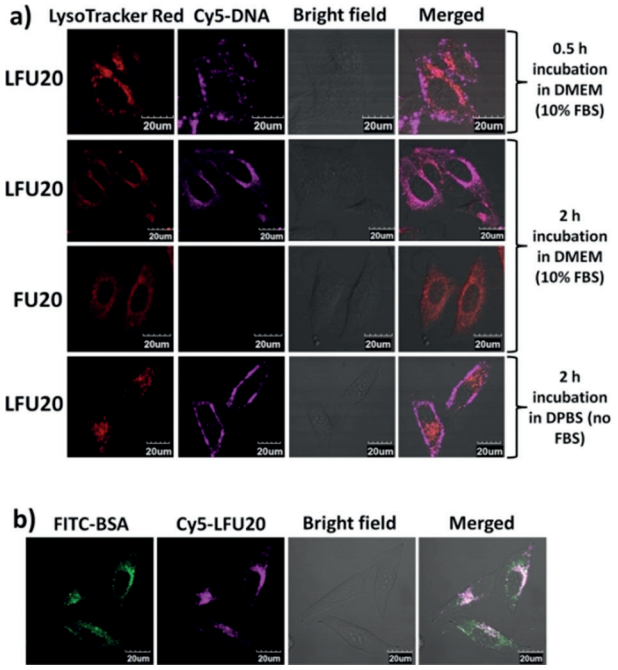


Figure 2. a) Confocal microscopy fluorescence images of HeLa cells treated with 1 μM Cy5-labeled FU20 or LFU20 in DMEM culture medium (10% FBS) or DPBS buffer at 37°C. Lysosome was stained by DND-99. b) Colocalization investigation of HeLa cells treated with 1 μM FITC-labeled BSA and Cy5-labeled LFU20 in DMEM culture medium (10% FBS) for 2 h at 37°C.

DMEM culture medium (10% fetal bovine serum (FBS)), an obvious and time-dependent internalization of LFU20 into HeLa cells was observed (Figure 2a). The lipid tail plays an important role in the interaction between LFU20 and living cells. In DPBS solution, LFU20 inserts into the hydrophobic area of the cell membrane by hydrophobic interactions. However, in DMEM culture medium (10% FBS), LFU20 bonds with serum albumin to form an LFU20/albumin complex, resulting in cellular endocytosis (Supporting Information, Figure S19). This phenomenon also indicates that LFU20 binds more strongly to serum albumin than to the cell membrane. Fluorescence colocalization with Lysotracker red (DND-99) demonstrated the transport of internalized LFU20/albumin to the lysosomes (Pearson correlation factor: 0.81; Figure 2a).

To further assess whether LFU20/albumin shares an endocytosis pathway in common with serum albumin, a fluorescence colocalization assay of Cy5-labeled LFU20/albumin and FITC-labeled BSA was performed. As shown in Figure 2b, fluorescence of the two channels shows a high overlap ratio with a Pearson correlation factor of 0.83. Previous literature has reported that dye-modified BSA internalizes into cells by the Gp18/Gp30-associated pathway.[17] Consistent with these findings, our results suggest that the formation of the LFU20/albumin complex avoids membrane anchoring based on the blocking of the lipid tail into albumin, instead favoring cellular internalization by the Gp18/Gp30-mediated pathway (Figure S19, Supporting Information).

Since LFU20 contains twenty tandem floxuridine modules, a universal antimetabolite drug used in the treatment of several cancers, the performance of LFU20 in inhibiting cell proliferation was examined. Four control groups were used, FU20, free floxuridine, DNA with twenty repeated thymidine (T20), and lipid-conjugated T20 (LT20). LFU20 clearly decreased cell proliferation with an IC_{50} value of 1.58 μM. However, FU20 showed only about 38% inhibition ratios, even at the concentration of 10 μM (Figure 3). Compared with FU20, LFU20/albumin showed stronger cellular internalization efficacy, indicating more release of drugs in HeLa cells. Neither T20 nor LT20 had appreciable cytotoxicity, demonstrating that the therapeutic efficacy of LFU20 could be attributed to the incorporation of floxuridine, not the lipid group. Free floxuridine showed an IC_{50} value of 23.07 μM. Because LFU20 contains twenty floxuridine modules, the standardized IC_{50} value of LFU20 after calculation is 31.6 μM, which is somewhat greater than that of free floxuridine. Compared to LFU20/albumin, which is a negatively charged macromolecular complex (molecular weight > 70 kDa), small molecular floxuridine has a stronger ability to internalize into cells; therefore, it is reasonable that LFU20 would show lower in vitro inhibition ratios than those of free floxuridine. Additionally, compared to FU20, LFU20 also exhibits more efficient inhibition of the cell proliferation of HepG2 and U-2 OS cells (Supporting Information, Figure S22).

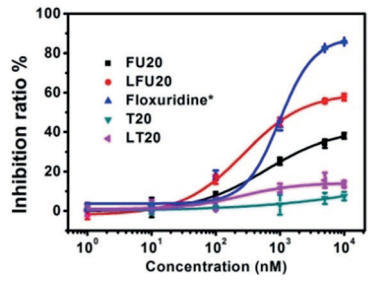


Figure 3. Inhibition ratios of FU20, LFU20, free floxuridine, T20, and LT20 to HeLa cells. Samples were diluted with DMEM culture medium (10% FBS) to the corresponding concentration, followed by addition to 96-well plates. Cells were cultured for an additional 48 h prior to cell viability assay. The concentration of free floxuridine is twenty-fold higher than that of the label on the *X* axis.

Next, in vivo fluorescence imaging of tumor-implanted nude mice intravenously injected with Cy5-FU20 or Cy5-LFU20 was studied. In vivo distribution plays a critical role in the anticancer efficacy of drug-incorporated oligonucleotides. As shown in Figure 4, Cy5-FU20 was quickly eliminated from the body owing to its small molecular size. Although most Cy5-LFU20/albumin was also eliminated from the body, an obvious fluorescence signal in the tumor was still observed, even after 48 h. Upon injection into mice through the tail vein, LFU20 micelles dissociated to form LFU20/albumin complex with longer circulation time and stronger penetration through tumor tissue compared to FU20.[8a] This resulted in the better performance of LFU20 in accumulating in tumor tissue compared to FU20.

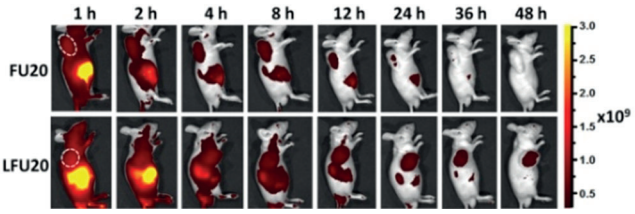


Figure 4. In vivo fluorescence imaging of tumor-implanted BALB/c nude mice intravenously injected with Cy5-FU20 or Cy5-LFU20.

Encouraged by in vitro therapeutic efficacy and passive accumulation in tumor, an in vivo evaluation of therapeutic efficacy was performed. Tumor-implanted BALB/c nude mice with tumor volumes of about 70 mm^3 were intravenously injected with LFU20, FU20, floxuridine, or PBS. As shown in Figure 5a,b, LFU20 was the most efficient drug for the inhibition of tumor growth compared with the others. Free floxuridine exhibited poorer therapeutic efficacy compared to that of LFU20, which is an inverse result compared with the in vitro results. This could be explained by the faster elimination of the small-molecule floxuridine from the body when compared to albumin-bound LFU20.[9] Finally, hematoxylin and eosin (H&E) staining of tumor sections was also used to evaluate in vivo therapeutic efficacy (Figure 5c). Cancer cells from the free floxuridine and FU20-treated groups showed well-defined nuclear structure with features similar to those of the PBS-treated group. On the other hand, in the LFU20-treated group, obvious nuclear shrinkage of cancer cells was apparent, suggesting that LFU20 had induced efficient cell apoptosis in tumor.

In summary, a new strategy for in vivo delivery of floxuridine homomeric oligonucleotides was accomplished by incorporating a hydrophobic lipid tail, allowing LFU20 to

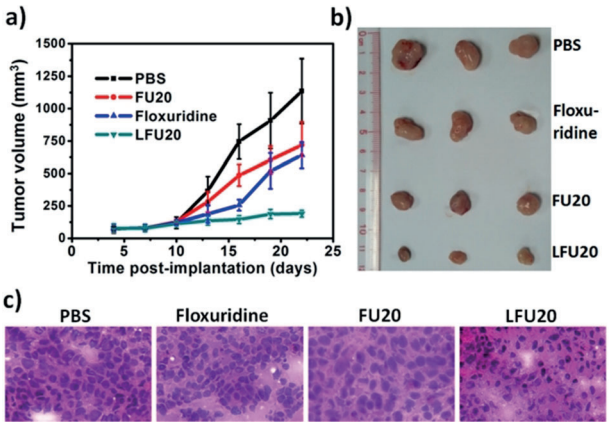


Figure 5. In vivo evaluation of LFU20 as an anticancer agent. a) Tumor volumes of each group. b) Photograph of tumors dissected from nude mice on day 22. c) Microscopic images of tumor sections stained with H&E. Images were cropped from 40× images to make the shape of cell nuclei clearer.

hitchhike with endogenous serum albumin. The internalized LFU20/albumin is transported to the lysosomes. After degradation, cytotoxic floxuridine monophosphate is released, leading to a decrease in cell proliferation. For in vivo cancer therapy, LFU20/albumin accumulates in the tumor by the EPR effect and exhibits more effective therapeutic efficacy than that shown by the control groups. Notably, LFU20 can be synthesized automatically on a DNA synthesizer with high yields, well-defined molecular structure, and tunable payloads. Additionally, other nucleoside analogues, for example, gemcitabine (Gemzar),[18] can be incorporated into lipid-conjugated oligonucleotides individually or combined with floxuridine for synergistic chemotherapy.

Acknowledgements

This work is supported by NSFC grants (NSFC 21521063 and NSFC 21327009) and by NIH GMR35127130 and NSF 1645215.

Conflict of interest

The authors declare no conflict of interest.

Keywords: albumin · cancer chemotherapy · drug delivery · floxuridine · lipid-conjugated oligonucleotides

[1] a) R. I. Letsinger, J. L. Finnan, G. A. Heavner, N. B. Lunsford, *J. Am. Chem. Soc.* **1975**, *97*, 3278–3279; b) R. L. Letsinger, W. B. Lunsford, *J. Am. Chem. Soc.* **1976**, *98*, 3655–3661.

[2] a) H. Asanuma, X. Liang, H. Nishioka, D. Matsunaga, M. Liu, M. Komiyama, *Nat. Protoc.* **2007**, *2*, 203–212; b) R. Wang, C. Wang, Y. Cao, Z. Zhu, C. Yang, J. Chen, F.-L. Qing, W. Tan, *Chem. Sci.* **2014**, *5*, 4076–4081; c) R. Wang, C. Jin, X. Zhu, L. Zhou, W. Xuan, Y. Liu, Q. Liu, W. Tan, *J. Am. Chem. Soc.* **2017**, *139*, 9104–9107; d) H. B. Liu, J. M. Gao, S. R. Lynch, Y. D. Saito, L. Maynard, E. T. Kool, *Science* **2003**, *302*, 868–871; e) P. S. Nelson, M. Kent, S. Muthini, *Nucleic Acids Res.* **1992**, *20*, 6253–6259.

[3] a) R. Wang, G. Zhu, L. Mei, Y. Xie, H. Ma, M. Ye, F.-L. Qing, W. Tan, *J. Am. Chem. Soc.* **2014**, *136*, 2731–2734; b) S. Kruspe, U. Hahn, *Angew. Chem. Int. Ed.* **2014**, *53*, 10541–10544; *Angew. Chem.* **2014**, *126*, 10711–10715; c) Q. Mou, Y. Ma, G. Pan, B. Xue, D. Yan, C. Zhang, X. Zhu, *Angew. Chem. Int. Ed.* **2017**, *56*, 12528–12532; *Angew. Chem.* **2017**, *129*, 12702–12706; d) P. Dua, S. Sajeesh, S. Kim, D.-K. Lee, *Nucleic Acid Ther.* **2015**, *25*, 180–187.

[4] D. B. Longley, D. P. Harkin, P. G. Johnston, *Nat. Rev. Cancer* **2003**, *3*, 330–338.

[5] W. H. Gmeiner, W. Debinski, C. Milligan, D. Caudell, T. S. Pardee, *Future Oncol.* **2016**, *12*, 2009–2020.

[6] H. Liu, K. D. Moynihan, Y. Zheng, G. L. Szeto, A. V. Li, B. Huang, D. S. Egeren, C. Park, D. J. Irvine, *Nature* **2014**, *507*, 519–522.

[7] B. R. Meade, K. Gogoi, A. S. Hamil, C. Apergi, A. Berg, J. C. Hagopian, A. D. Springer, A. Eguchi, A. D. Kacsinta, C. F. Dowdy, A. Presente, P. Loenn, M. Kaulich, N. Yoshioka, E. Gros, X.-S. Cui, S. F. Dowdy, *Nat. Biotechnol.* **2014**, *32*, 1256–1261.

[8] a) S. M. Sarett, T. A. Werfel, L. Lee, M. A. Jackson, K. V. Kilchrist, D. B. Sieders, C. L. Duvall, *Proc. Natl. Acad. Sci. USA* **2017**, *114*, E6490–E6497; b) J. Winkler, *Future Med. Chem.* **2015**, *7*, 1721–1731.

[9] F. Kratz, *J. Controlled Release* **2008**, *132*, 171–183.

[10] a) C. L. Anderson, C. Chaudhury, J. Kim, C. L. Bronson, M. A. Wani, S. Mohanty, *Trends Immunol.* **2006**, *27*, 343–348; b) J. Kim, W. L. Hayton, J. M. Robinson, C. L. Anderson, *Clin. Immunol.* **2007**, *122*, 146–155.

[11] M. T. Larsen, M. Kuhlmann, M. L. Hvam, K. A. Howard, *Mol. Cell. Ther.* **2016**, *4*, 3–3.

[12] a) N. Desai, V. Trieu, Z. W. Yao, L. Louie, S. Ci, A. Yang, C. L. Tao, T. De, B. Beals, D. Dykes, P. Noker, R. Yao, E. Labao, M. Hawkins, P. S. Shiong, *Clin. Cancer Res.* **2006**, *12*, 1317–1324; b) J. L. Cohen, J. Cheirif, D. S. Segar, L. D. Gillam, J. S. Gottdiener, E. Hausnerova, D. E. Bruns, *J. Am. Coll. Cardiol.* **1998**, *32*, 746–752.

[13] A. Dornhorst, H. J. Lueddeke, S. Sreenan, C. Koenen, J. B. Hansen, A. Tsur, L. L. Hallin, P. S. Grp, *Int. J. Clin. Pract.* **2007**, *61*, 523–528.

[14] C. Jin, X. Liu, H. Bai, R. Wang, J. Tan, X. Peng, W. Tan, *ACS Nano* **2017**, *11*, 12087–12093.

[15] W. H. Gmeiner, P. J. Gearhart, Y. Pommier, J. Nakamura, *Future Oncol.* **2016**, *12*, 2183–2188.

[16] J. Lu, S. C. Owen, M. S. Shoichet, *Macromolecules* **2011**, *44*, 6002–6008.

[17] a) A. M. Merlot, D. S. Kalinowski, D. R. Richardson, *Front. Physiol.* **2014**, *5*, 299; b) J. E. Schnitzer, J. Bravo, *J. Biol. Chem.* **1993**, *268*, 7562–7570.

[18] a) H. A. Burris, M. J. Moore, J. Andersen, M. R. Green, M. L. Rothenberg, M. R. Modiano, M. C. Cripps, R. K. Portenoy, A. M. Storniolo, P. Tarassoff, R. Nelson, F. A. Dorr, C. D. Stephens, D. D. Hoff, *J. Clin. Oncol.* **1997**, *15*, 2403–2413; b) T. Conroy, F. Desseigne, M. Ychou, O. Bouche, R. Guimbaud, Y. Becouarn, A. Adenis, J.-L. Raoul, S. G. Bourgade, C. Fouchardiere, J. Bennouna, J.-B. Bachet, F. K. Akouz, D. P. Verge, C. Delbaldo, E. Assenat, B. Chauffert, P. Michel, C. M. Grillot, M. Ducreux, U. G. Digestives, P. Intergrp, *N. Engl. J. Med.* **2011**, *364*, 1817–1825.

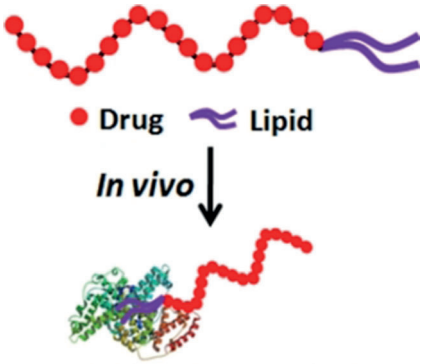
Manuscript received: April 8, 2018
Revised manuscript received: May 8, 2018
Version of record online: ■■ ■■, ■■■■

Communications

Drug Delivery

C. Jin, H. Zhang, J. Zou, Y. Liu, L. Zhang, F. Li, R. Wang, W. Xuan, M. Ye,* W. Tan* ——— ■■■■–■■■■

Floxuridine Homomeric Oligonucleotides "Hitchhike" with Albumin In Situ for Cancer Chemotherapy



Hitchhiker's guide to the bloodstream: A new strategy for in vivo delivery of floxuridine homomeric oligonucleotides was accomplished by incorporating a hydrophobic lipid tail at the 5′-terminus, allowing LFU20 to hitchhike with endogenous serum albumin. The LFU20/albumin complex accumulates in the tumor by the EPR effect and inhibits tumor growth.