Antitumor Agents

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An Aptamer–Doxorubicin Physical Conjugate as a Novel Targeted Drug-Delivery Platform**

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The active targeting of drugs in a cell-, tissue-, or disease-specific manner represents a potentially powerful technology with widespread applications in medicine, including the treatment of cancers. In a typical approach, a drug and a ligand are complementarily functionalized to allow for covalent or noncovalent (for example, biotin–streptavidin) conjugation for targeted delivery. The resulting chemical modifications of the drugs and/or the ligands may adversely affect the safety and efficacy profile of the drugs and the binding characteristics of the ligands, thereby resulting in less efficacious drug–ligand conjugates. It would be desirable to develop simple but effective targeted drug-delivery strategies that do not require chemical modification of the drug or the ligands.

Recently our group and other investigators have used nucleic acid ligands or aptamers for therapeutic and diagnostic targeted-delivery applications.[1–6] Aptamers are structured single-stranded DNA or RNA molecules that can specifically bind to small molecules,[7] peptides and proteins,[8] and oligosaccharides[9] with high affinity and specificity.

Here we report a novel strategy for the targeted delivery of doxorubicin (Dox) to cancer cells through the formation of an aptamer–Dox physical conjugate. Dox is a well-known anticancer drug which has shown efficacy against a range of neoplasms, including acute lymphoblastic and myeloblastic leukemias, malignant lymphomas, soft tissue and bone sarcomas, and breast, ovarian, prostate, bladder, gastric, and bronchogenic carcinomas. This widely used oncology drug is also associated with dose-dependent cardiotoxicities, including dilated cardiomyopathy and congestive heart failure, which make the development of targeted Dox-delivery systems of particular importance.[10] Dox is known to intercalate within the DNA strand due to the presence of flat aromatic rings in this molecule. Other closely associated drugs, such as donarubicin, have also been shown to be intercalated into a double helix of DNA.[11] Since aptamers are known to form tertiary conformations with short double-stranded regions through intramolecular base pairing,[12] we hypothesized that doxorubicin may intercalate into these double-stranded regions and form a physical complex with the aptamers through noncovalent intercalation, a process requiring no modification of the drug or the aptamer (Figure 1). We postulated that the resulting aptamer–Dox

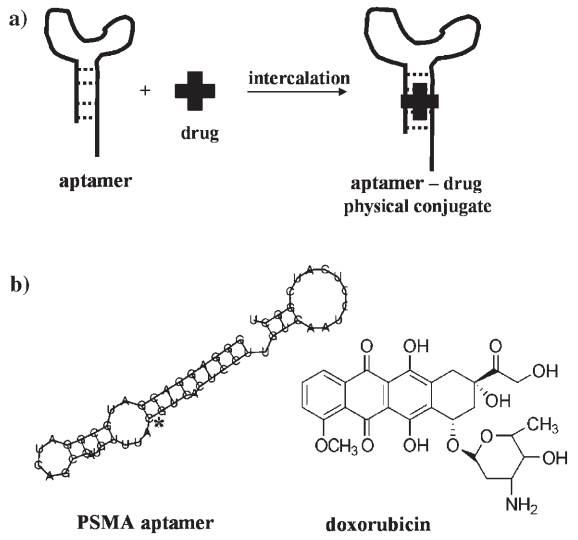

Figure 1. a) Physical-conjugate formation between an aptamer and a model drug. b) 2D structure of the A10 PSMA aptamer as predicted by M fold program and the chemical structure of doxorubicin.

physical conjugate may be used for the targeted delivery of Dox by taking advantage of the aptamer's binding specificity to its target antigen. In this study, we have examined the feasibility of this concept by using the A10 2′-fluoropyrimidine RNA aptamer[13] that binds to the prostate-specific membrane antigen (PSMA) with high affinity and specificity.

The two-dimensional structure of the A10 PSMA[13] aptamer used herein was predicted by the M fold program.[14] It is well known that the anthracycline class of drugs, including Dox, have fluorescence properties that become quenched after intercalation into DNA.[15,16] To examine whether such intercalation also occurs within the RNA aptamer, we carried out binding studies between the A10 PSMA aptamer and Dox. Fluorescence spectroscopy was used to examine the binding of doxorubicin to the A10 PSMA aptamer. Sequential decreases in the native fluorescence spectrum of Dox were observed when a fixed concentration of

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

Dox was incubated with an increasing molar ratio of the A10 PSMA aptamer, results consistent with the intercalation of Dox within the A10 PSMA aptamer (Figure 2). Dox is known to preferentially bind to double-stranded 5′-GC-3′ or 5′-CG-3′

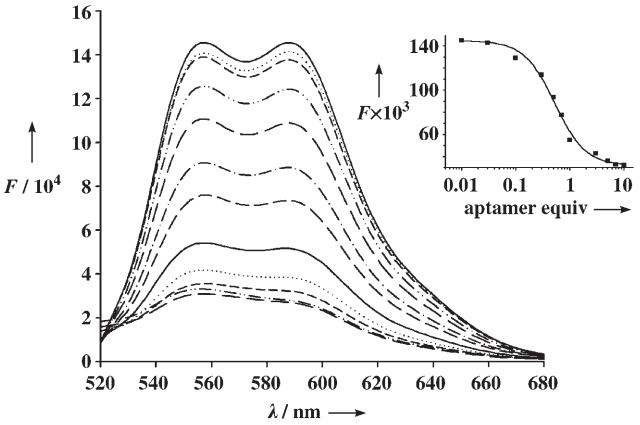

Figure 2. Fluorescence spectra of doxorubicin solution (1.5 μM) with increasing molar ratios of the aptamer (from top to bottom: 0, 0.01, 0.03, 0.1, 0.3, 0.5, 1, 3, 5, 7, and 10 equiv). Inset: A Hill plot for the aptamer titration (K_d = 0.6 μM; 0.52 equiv of the aptamer).

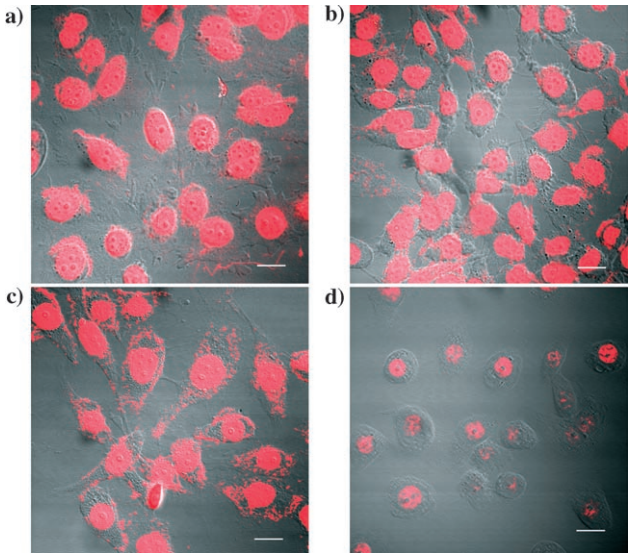

Figure 3. Confocal laser scanning microscopy images (superimposed images of fluorescence and transmittance) of LNCaP (a and c) and PC3 (b and d) cells after treatments of 1.5 μM free doxorubicin (a and b) and of 1.5 μM aptamer–Dox physical conjugate (c and d) for 2 h. Scale bars: 20 μm.

sequences,[17,18] and evaluation of the predicted A10 aptamer secondary structure reveals one possible site for Dox intercalation, as marked by an asterisk in Figure 1b. The incubation of Dox with the A10 aptamer results in maximal quenching of the Dox fluorescence at approximately 1:1.2 molar equivalence of Dox to aptamer, a result suggesting that Dox makes a physical conjugate with the A10 aptamer by intercalating into its predicted CG sequence (Figure 2). The inset of Figure 2 shows a Hill plot of fluorescence quenching as a function of increasing aptamer concentration. The dissociation constant (K_d = 600 nM) of the aptamer–Dox physical conjugate was derived from this figure and suggests a spontaneously formed stable physical conjugate. The stability of the aptamer–Dox conjugate was further confirmed by high-pressure liquid chromatography (HPLC) where the conjugate peak appeared at a different elution time from those of the native aptamer and Dox (data not shown). A study of the release of Dox from the aptamer–Dox physical conjugate over time was conducted by using a dialysis tube (see Figure S1 in the Supporting Information). Upon dialysis, more than 80% Dox release was observed in 6 h with zero-order kinetics, which suggests that the Dox molecule is released from the conjugate beyond the concentration of its dissociation constant by simple diffusion.

To evaluate the feasibility of the aptamer–Dox physical conjugate as a targeted drug-delivery platform, we performed in vitro binding and uptake studies with the LNCaP prostate epithelial cells which express the target PSMA protein on their plasma membrane. We used the PC3 prostate epithelial cells which do not express any detectable level of the PSMA protein as a negative control (Figure 3). Our confocal laser scanning microscopy data demonstrate that while free Dox readily diffuses through the plasma membrane of LNCaP and PC3 cells with equal efficiency (Figure 3a and b), there is a remarkable specificity in the uptake of the aptamer–Dox conjugate by LNCaP but not PC3 cells (Figure 3c and d), as marked by strong nuclear fluorescence that is consistent with the intercalation of Dox within the genomic DNA.

The mechanisms of uptake of the aptamer–Dox physical conjugate and the free Dox by LNCaP cells appear distinct.[19] Unlike free Dox, which exclusively stains the nuclei, the aptamer–Dox conjugate demonstrates both nuclear and cytosolic staining, with the latter predominately in the form of punctuate granules that are consistent with compartmentalization of the Dox within endosomes (Figure 3c). This pattern of cytosolic staining is consistent with receptor-mediated endocytic uptake of the aptamer–Dox physical conjugate, which results after binding of the conjugate to the PSMA protein on the LNCaP plasma membrane. The aptamer–Dox physical conjugate failed to produce any cytosolic staining of the PC3 cells, a result consistent with the lack of PSMA protein expression in these cells (Figure 3d). The weak fluorescence staining of the PC3 nuclei after incubating with the aptamer–Dox conjugate is likely attributable to a small amount of free Dox that may be present in the media bathing the cells. Indeed, the LNCaP- and PC3-binding data suggest that the majority of Dox remains in the form of a complex with the aptamer, thereby demonstrating the stability of the aptamer–Dox physical conjugate over time in the culture media. Furthermore, the data demonstrate the ability of an aptamer to retain its binding characteristics while the Dox is intercalated within it, so allowing the targeted delivery of Dox to the cells that express the aptamer target. We next compared the binding characteristics of an equimolar concentration of the aptamer–Dox physical conjugate to the free aptamer in LNCaP-binding assays.[20] By using quantitative PCR amplification of the LNCaP-bound aptamers, about 84% of the binding ability

of the A10 aptamer was shown to be retained by the aptamer–Dox physical conjugate (see Figure S2 in the Supporting Information). This result also demonstrates the ability of an aptamer to retain its binding characteristics while the Dox is intercalated within it, thereby allowing the targeted delivery of Dox to the cells that express the aptamer target.

The targeting specificity of the aptamer–Dox physical conjugate was next quantified by flow cytometry experiments (Figure 4). The data demonstrate near-identical staining of LNCaP and PC3 cells after treatment of these cells with free Dox (Figure 4a and b). However, when LNCaP and PC3 cells were incubated with the aptamer–Dox physical conjugate, there was a significant enhancement in the fluorescence signal from LNCaP cells as compared to that from PC3 cells (FL2 log intensity for LNCaP was 123 ± 4.66 versus 35 ± 1.79 for PC3; mean $\pm$ standard error (SE), number of samples (N) = 4), which validates the targeting specificity of the aptamer–Dox physical conjugate (Figure 4c). Taken together, our microscopy and flow cytometry data demonstrate the proof of concept for the feasibility of the aptamer–Dox physical conjugate to serve as a novel drug-delivery platform for a variety of applications in oncology.

We next examined whether the targeted delivery of the aptamer–Dox physical conjugate to LNCaP cells results in enhanced cellular cytotoxicity[21] compared to that in PC3 control cells. We first demonstrated in escalating-dose studies that the cytotoxicity of free Dox to LNCaP and PC3 cells is equipotent (data not shown). At a dose where the cytotoxicity of free Dox had reached a plateau near its maximum, we compared the cytotoxic efficacy of free Dox (5 μM) to that of the aptamer–Dox physical conjugate (5 μM), as well as that of free aptamer without Dox, on LNCaP and PC3 cells by MTT assay. The data demonstrate that while the cytotoxicity of free Dox is equipotent against LNCaP and PC3 cells, the cytotoxicity of the aptamer–Dox conjugate is significantly enhanced against the targeted LNCaP cells as compared to the nontargeted PC3 cells (cellular viability: $52.8\% \pm 1.73$ LNCaP versus $75.2\% \pm 1.19$ PC3; mean $\pm$ SE, $N = 5$; probability value (p) < 0.005; Figure 5). Interestingly, the data demonstrate a near-equipotent cytotoxicity of the aptamer–Dox physical conjugate to the LNCaP cells as compared to that of free Dox. The free aptamer without Dox had no inherent cytotoxicity to LNCaP or PC3 cells (Figure 5). Our data suggest that, after endocytic uptake, the aptamer–Dox physical conjugate releases the Dox molecules inside the LNCaP cells, possibly due to the aptamer–Dox dissociation constant favoring the release of Dox because of the relatively negligible concentrations of Dox inside the cells. Alternatively, the release of Dox from the aptamer–Dox physical conjugate may occur through gradual degradation of the aptamer by endonucleases in the lysosomes after cellular uptake. We postulate that a combination of these factors may contribute to the observed findings. In contrast to the dramatic cytotoxic effects of the aptamer–Dox complex to the LNCaP cells, the cytotoxicity of this physical-conjugate system to PC3 cells was significantly less pronounced, a result consistent with the lack of PSMA expression in PC3 cells.

In conclusion, by exploiting the ability of anthracycline drugs to intercalate between bases of polynucleotides, a novel

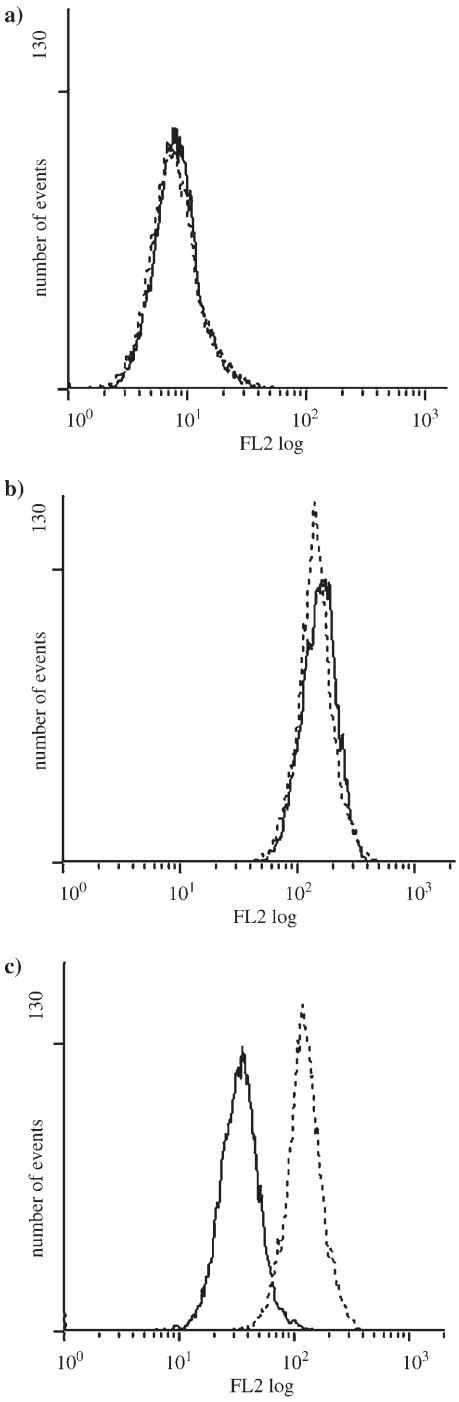

Figure 4. Flow cytometry histogram profiles of LNCaP (dotted line) and PC3 (solid line) cells obtained after treatments with a) nothing, b) 1.5 μM free doxorubicin, and c) 1.5 μM aptamer–Dox physical conjugate. FL2 log = fluorescence intensity of FL2 sensor (band pass filter, 575 nm).

physical conjugate was made between the anticancer drug Dox and the A10 RNA aptamer that binds to the PSMA protein on the surface of prostate cancer cells. The stability and efficacy of this conjugate to serve as a novel drug-delivery platform was further demonstrated in vitro. Our data suggest that the aptamer–Dox physical conjugate is stable in the cell-culture medium and could differentially and with high

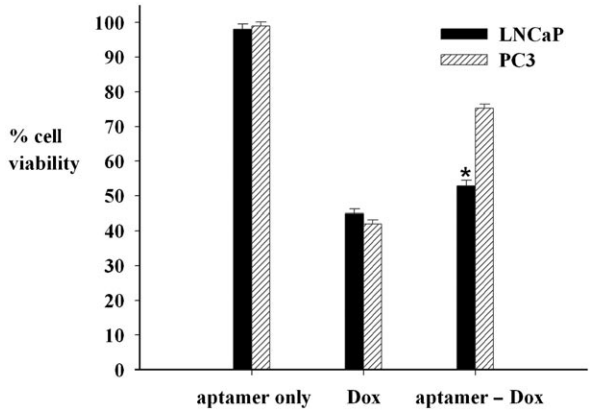

Figure 5. Growth-inhibition assay (MTT) results for prostrate cancer cell lines LNCaP and PC3 after 2 h of incubation with free doxorubicin (5 μM) and the physical conjugate (5 μM) and 24 h of subsequent incubation. * indicates the LNCaP result that is significantly different from that with PC3 cells ($p < 0.005$, $N = 5$).

efficiency target the PSMA-expressing LNCaP cells. The specificity of the system was further demonstrated by the lack of targeting of the PSMA-negative PC3 cells. We expect that the small size of the aptamer–Dox physical-conjugate system ($\approx$18 kD) as compared to that of similar antibody-based immunoconjugates ($\approx$150 kD) may facilitate the rapid vascular extravasation and intratumoral penetration of the former, thereby making it a therapeutically effective drug-delivery system for in vivo applications.[22] The much larger nanoparticle drug-delivery systems compensate for their size disadvantage through their capacity to release a large payload of drug in a controlled manner, a property that makes each ligand–target biorecognition a potentially relevant therapeutic event. Each of these systems may contribute in a unique way to our arsenal of cancer-therapy options in the future.[23] Furthermore, these systems may be combined such that a targeted nanoparticle–aptamer bioconjugate system may deliver distinct drugs through encapsulation within the nanoparticles and through intercalation within the aptamers, with the result of a temporally distinct release of two or more drugs for combination chemotherapy.[1–3] We anticipate that the aforementioned aptamer–drug platform technology based on the intercalation of anthracyclines within the bases of aptamers may be utilized in distinct ways to develop novel targeted therapeutic modalities for more effective cancer chemotherapy. In the next phase of our studies, we will be evaluating the in vivo efficacy of the aptamer–Dox physical conjugate in animal models of cancer.

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