Drug Delivery

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An Aptamer Intrinsically Comprising 5-Fluoro-2′-deoxyuridine for Targeted Chemotherapy**

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Abstract: *An aptamer specifically binding the interleukin-6 receptor and intrinsically comprising multiple units of the nucleoside analogue 5-fluoro-2′-deoxyuridine can exert a cytostatic effect direcly on certain cells presenting the receptor. Thus the modified aptamer fulfils the requirements for active drug targeting in an unprecedented manner. It can easily be synthesized in a single enzymatic step and it binds to a cell surface receptor that is conveyed into the lysosome. Upon degradation of the aptamer by intracellular nucleases the active drug is released within the targeted cells exclusively. In this way the aptamer acts as a prodrug meeting two major prerequisites of a drug delivery system: specific cell targeting and the controlled release of the drug triggered by an endogenous stimulus.*

Aptamers are in vitro selected oligonucleotides that specifically bind to a target like certain cell surface proteins and thus they are applicable for therapeutic purposes as tools for active drug targeting. Aptamer delivery is typically realized by covalent or noncovalent coupling, and multiple steps are required to directly link aptamers to drugs or to assemble aptamer-equipped drug nanocarriers. Importantly, aptamer–drug conjugates remain ineffective if the drug cargo cannot be released from an endocytotic vesicle or if it is converted into an inactive metabolite by lysosomal degradation. Efforts have been made to ensure intracellular release of drugs typically used for targeted therapy, for example, siRNAs and anthracyclines.[1] A key step can be the careful choice of a therapeutic that does not need additional support for endosomal escape as required for gene-silencing siRNAs, for example.[2]

Therapeutic nucleoside and nucleobase analogues represent such a reasonable class of drugs.[3] One of these compounds, 5-fluorouracil (5-FU), has been used as a cytostaticum in cancer therapy and in the treatment of various other diseases for more than fifty years.[4] The capability of 5-FU relies upon three major effects concerning the vitality or proliferation of affected cells (Figure S1).[5] Incorporation of 5-FU metabolites into RNA and DNA (FUTP and FdUTP) leads to hampered RNA maturation[6] and DNA cleavage, respectively.[7] The most significant impact of 5-FU occurs after its conversion to the deoxyribonucleotide (FdUMP), a strong inhibitor of thymidylate synthase (TS), the key enzyme of the de novo biosynthesis of thymidine.[8]

To circumvent side effects, 5-FU can be administered as a prodrug, which is converted into an active form only in specific types of cells, e.g. prostate cancer cells.[9] Active drug targeting represents another strategy to overcome the effects on nontarget cells. Currently nanoparticles, nanogels, and nanopolymers are in the main focus for active 5-FU delivery.[10] Here we report about the application of a cell-specific cytotoxic aptamer that can be prepared in a one-step enzymatic reaction by incorporating multiple units of a nucleoside analogue directly into an aptamer that targets a cytokine receptor. The controlled release of the active drug inside the target cells is initiated by intracellular nucleolytic hydrolysis of the aptamer. In our approach, 5-fluoro-2′-deoxyuridine (5-FUdR), the unphosphorylated precursor of FdUMP, becomes part of the aptamer molecule itself, replacing all the uridines (30 per molecule) in the original aptamer. We demonstrate cell-specific growth inhibition and cell death after exposure to this modified aptamer.

Recently we reported the selection of the RNA aptamer AIR-3 which specifically binds to the human interleukin-6 receptor (hIL-6R) with a dissociation constant in the nanomolar range.[11] Upon binding to the receptor on hIL-6R-positive cells, the aptamer is internalized by means of endocytosis. Such receptor-mediated endocytosis events occur due to receptor recycling. In most cases, the intracellular pathway of ligand-triggered receptors results in the degradation of the tethered ligand or even the whole receptor/ligand complex in a lysosomal process.[12]

Cytotoxic nucleoside and nucleobase analogues such as 5-FUdR are advantageous drugs for receptor-mediated active targeting as they benefit from this intracellular turnover. We suppose that the controlled release of the drug inside the target cells is mediated through naturally occuring degradation by lysosomal nucleases.[13] The hydrolysis of the aptamer would yield 5-FUdR, which could escape the lysosome by active nucleoside transporters, e.g. ENT3,[14] which normally serve as recycling gateways for lysosomally degraded nucleic acids (Figure 1).

We prepared a 5-FUdR-modified variant of AIR-3 by transcription in the presence of 5-FdUTP instead of UTP using the T7 RNA polymerase variant Y639F[15] (Figure 1 a). The resulting modified aptamer AIR-3-FdU still bound effectively and specifically to hIL-6R, albeit with decreased affinity ($K_d = 151 \pm 3$ nM) in comparison to the unmodified aptamer ($K_d = 21 \pm 3$ nM; Figure 2). Therefrom it can be assumed that replacement of all 30 uridines in AIR-3 by 5-FUdR does not affect the overall tertiary structure remarkably. As AIR-3 and AIR-3-FdU bind to their target in

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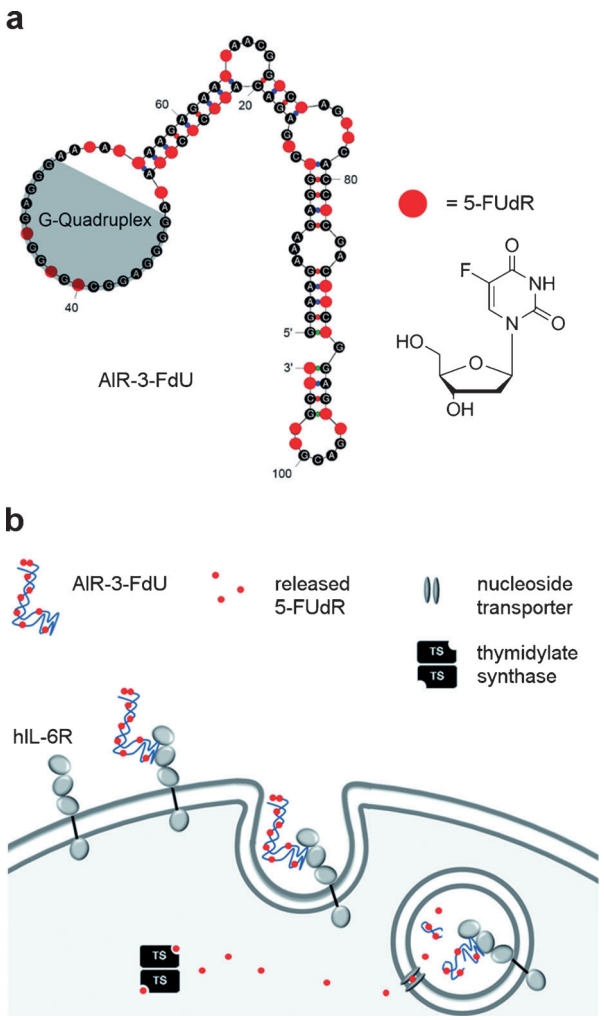


Figure 1. The principle of drug delivery by AIR-3-FdU. a) 2D structure of AIR-3-FdU using the structure prediction of AIR-3 as a template. Each aptamer molecule contains 30 units of 5-FUdR. Gray shaded area covers the minimal binding motif AIR-3A that could be shown to build up a G-quadruplex.[11] b) The 5-FUdR-modified aptamer (AIR-3-FdU) binds to the human interleukin-6 receptor on the cell surface and is internalized. Lysosomal degradation of the aptamer leads to the release of 5-FUdR, which is further phosphorylated to FdUMP, a potent inhibitor of thymidylate synthase (TS) which itself is responsible for dTMP and thus DNA biosynthesis.

a comparable manner, their minimal binding motifs can be assumed to fold in the same fashion of a parallel G-quadruplex, which comprises only three uridines within a 19 nt region. In the aptamer (106 nt) several double-strand regions, which fold into a bulged stem,[16] support the proper formation of the G-quadruplex, allowing the incorporation of modified nucleosides.[17]

The stem structure should not be affected by the nucleoside analogue 5-FUdR since it is capable of the same Watson–Crick base-pairing as the replaced canonical nucleoside.[18] We also constructed a variant of AIR-3 bearing 5-fluorouridine (5-FUR) instead of 5-FUdR. The binding of this modified aptamer (AIR-3-FU; $K_d = 74 \pm 8$ nM) was similar to that of the initial aptamer (Figure S2). In contrast to AIR-3-

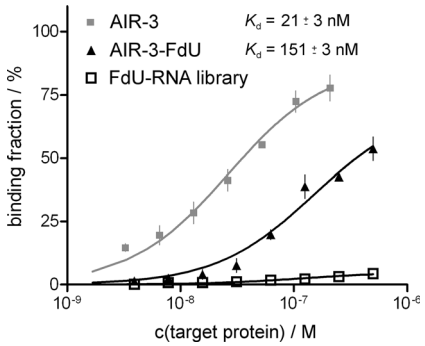


Figure 2. Binding analysis of AIR-3-FdU. Concentration-dependent binding of AIR-3-FdU was determined by a filter retention assay. The affinity of the modified aptamer (triangles) was slightly decreased compared to the unmodified aptamer AIR-3 (gray squares). The 5-FUdR-modified initial SELEX-library served as negative control (white squares).

FdU, however, this variant had no detectable cytotoxic effects.

Using confocal laser scanning microscopy (cLSM), we affirmed that an essential amount of internalized aptamer is conveyed towards the lysosome (Figure 3c). This is in accordance with previous experiments showing that AIR-3, as well as its truncated form AIR-3A, bind to BaF3_hIL-6R

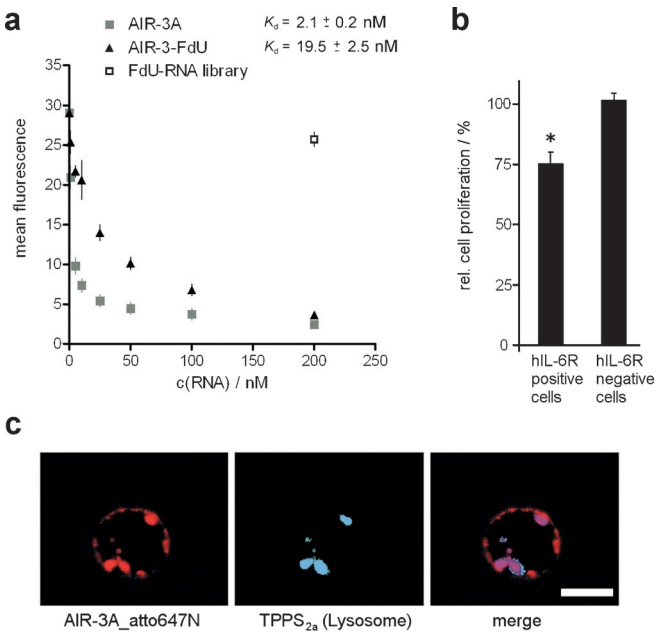


Figure 3. Cellular binding, uptake, and cytotoxicity on BaF3 hIL-6R cells. a) Binding analysis of AIR-3-FdU, FdU-RNA library, and AIR-3A (positive control) on hIL-6R-presenting BaF3 cells. The fluorescence was determined by flow cytometry from the second stain of labeled AIR-3A. Binding was indicated by concentration-dependent decrease in fluorescence. b) Antiproliferative effect of AIR-3-FdU on hIL-6R-presenting cells. Either hIL-6R-positive or -negative BaF3 cells were exposed to AIR-3-FdU. Subsequently the cells were washed and cultured in drug free medium. Proliferation was determined from two independent experiments, both conducted in triplicate ($n = 6$). Asterisk indicates statistical significance of $p < 0.05$. c) Live-cell visualization of AIR-3A_atto647N within BaF3_hIL-6R by cLSM. Scale bar: 10 μm.

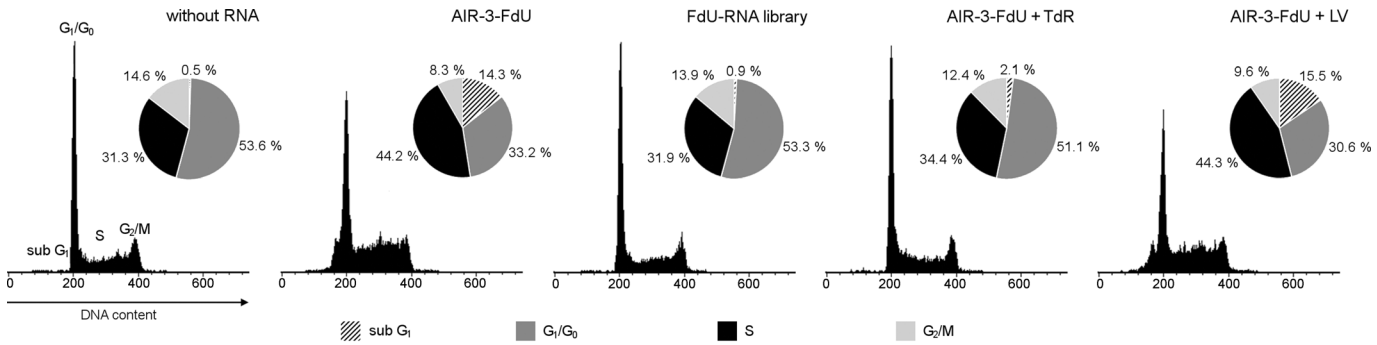


Figure 4. Cell cycle analyses of BaF3_hIL-6R cells after AIR-3-FdU exposure. The cells were exposed to 100 nM AIR-3-FdU for 3 h, washed, and subsequently cultured in drug-free media for 4 h. The cell cycle distribution was analyzed by flow cytometry. Given are the mean values from a duplicate experiment. Cells that were exposed to AIR-3-FdU exhibited enriched S-phase fractions and additionally apoptotic cells appearing as a sub G1 fraction. The FdU-RNA library served as a negative control. Addition of 2′-deoxythymidine (TdR) compensated the effect on the cell cycle. Addition of leucovorin (LV) slightly increased the number of apoptotic cells.

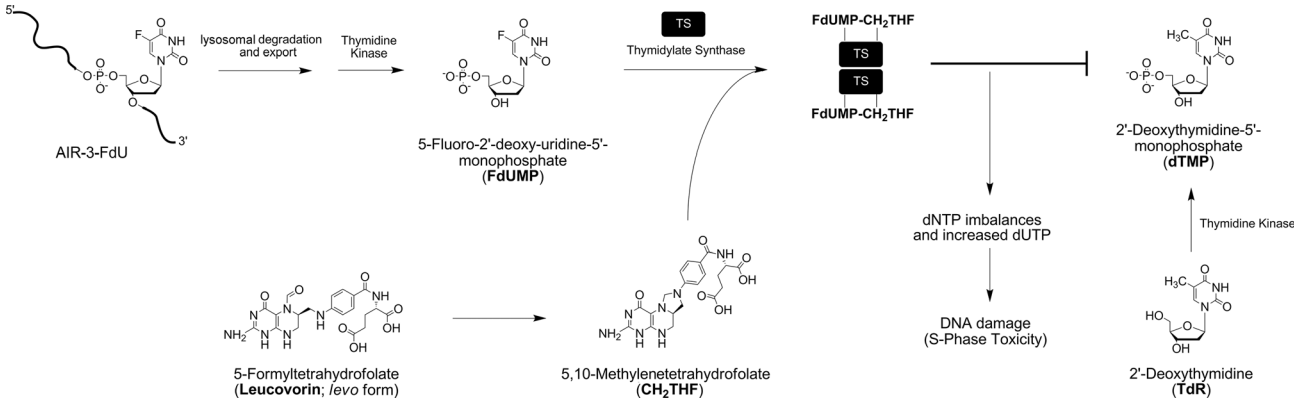
cells. Our data suggest that the aptamer can be used for the delivery of different cargos like fluorescent dyes, proteins, and photosensitizers coupled to the aptamer.[19]

AIR-3-FdU also binds to BaF3_hIL-6R cells with a K_d value of about 20 nM (Figure 3a). When we exposed these cells to 250 nM AIR-3-FdU we could observe a significant decline of 25% in the proliferation of hIL-6R-presenting cells upon incubation with aptamer (Figure 3b). To further prove that the effect was not based on extracellular degradation products of the RNA we performed the control experiment with BaF3 cells lacking hIL-6R, where no effect was shown.

To analyze whether this antiproliferative effect could be attributed to one of the aforementioned antimetabolites of 5-FU, we measured the distribution of the cell cycle phases after the AIR-3-FdU treatment. The 5-FUdR-modified aptamer caused enhanced S-phase population with a fraction increase from $31.3 \pm 1.8\%$ (untreated cells) to $44.2 \pm 1.1\%$ (Figure 4). Additionally we observed a fraction of apoptotic cells (hypodiploid cells marked as sub G1 with $14.3 \pm 0.5\%$). S-phase arrest is a typical FdUMP-generated effect due to its inhibition of TS. BaF3 cells were affected by free 5-FUdR in the same way, when they were exposed to concentrations of 5 μM for at least 5 h.[20] The S-phase arrest could be prevented when the experiment was conducted in the presence of 10 μM 2′-deoxythimidine (TdR), an antidote to FdUMP. Under these conditions AIR-3-FdU did not cause any change in the cell cycle fraction pattern compared to the untreated cells. We thus reason that 5-FdUMP is the active antimetabolite within the cell, hampering the thymidine de novo synthesis by TS inhibition. The depletion of 2′-deoxythymidine-5′-monophosphate (dTMP) led to slowed progression in the S-phase (DNA reduplication) and DNA damage upon nucleotide imbalances. These effects could be compensated by an external salvage source for dTMP such as TdR.[21] We also tried to enhance the antiproliferative effect by addition of leucovorin (LV), a precursor of the reduced-folate cofactor 5,10-methylenetetrahydrofolate (CH_2THF) which is the co-substrate of TS. However, LV did not have a significant effect on the proliferation but slightly enhanced the amount of apoptotic cells ($15.5 \pm 0.8\%$). Alone, neither TdR nor LV had any effect on the cells.

These results imply that FdUMP is the main active antimetabolite causing TS inhibition within the affected cells (Scheme 1). The observed apoptosis can either result from 5-FUdR incorporation into DNA or from incorporation of 2′-deoxyuridine into the DNA as a downstream result of dTMP depletion. Regardless, TS inhibition seems to be the central point of action because of two other facts: 1) FdUMP is by far the most effective metabolite with a K_i value of 5 nM for TS inhibition.[22] 2) The aptamer did not show any cellular effect when it was equipped with 5-FU-ribonucleosides instead of 5-FU-2′-deoxyribonucleosides. For 5-FU-ribonucleoside drugs (as well as for the free base 5-FU) the conversion to FdUMP is less efficient as several metabolic steps are involved (Figure S1), resulting in higher IC_{50} values for 5-FU drugs. Although AIR-3-FdU is stable enough in the conducted cell culture experiments for future in vivo applications it would be advantageous to have an aptamer at hand with enhanced resistance to serum nucleases. Promising results were obtained by the use of 2′F-modified cytidines in AIR-3-FdU. These derivatives showed an increased half-lifetime of up to several hours in serum containing media. However, this modification seems to decrease the antiproliferative efficacy probably due to less pronounced lysosomal degradation.

In conclusion, a new strategy for aptamer-mediated drug delivery was accomplished by incorporating a cytotoxic nucleoside analogue into a receptor-binding aptamer. The internalized aptamer is transported to the lysosome. We hypothesize that the aptamer is subsequently degraded to nucleosides, thereby releasing 5-FUdR, which can be phosphorylated by thymidine kinase within the cell. The resulting 5-FdUMP, a strong TS inhibitor, leads to a decline in cell proliferation and thus cell death. Noteworthy this cytotoxic aptamer can be synthesized emzymatically in one step. We anticipate that our concept can be adopted for other aptamers that are internalized by means of a cell surface receptor that is guided to the lysosome as well as to other nucleoside analogues, e.g. gemcitabine. DNA aptamers might even be beneficial over RNA aptamers in terms of stability against nucleases and since 5-FUdR is structurally more closely related to TdR than to uridine. With the example reported here, diseases related to elevated malignant cell levels in



Scheme 1. Proposed mechanism for AIR-3-FdU cytotoxicity. The 5-FdUR-modified aptamer AIR-3-FdU is internalized by hIL-6R-presenting cells. Hydrolysis by lysosomal nucleases generates free 5-FUdR, which is phosphorylated yielding the thymidylate synthase (TS) inhibitor FdUMP. Irreversible inhibition of TS occurs upon the formation of a covalent ternary complex of TS with FdUMP and the co-substrate CH_2THF leading to S-phase toxicity upon depletion of dTMP an indispensable precursor in DNA biosynthesis. The effect can be abrogated by exogenous TdR, a dTMP precursor. Leucovorin, a precursor of the CH_2THF enhances the effect.

plasma such as lymphoproliferative disorders (e.g. plasma cell leukemia, multiple myeloma) and Castleman's disease might be the most suitable for the clinical application of this method.[23]

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