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Host-Guest Tethered DNA Transducer: ATP Fueled Release of a Protein Inhibitor from Cucurbit[7]uril

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Abstract

Host-guest complexes are emerging as powerful components in functional systems with applications ranging from materials to biomedicine. In particular, CB7 based host-guest complexes have received much attention for the controlled release of drugs due to the remarkable ability of CB7 toward binding input molecules in water with high affinity leading to displacement of CB7 from included pharmacophores (or from drug loaded porous particles). However, the release of bound guests from CB7 in response to endogenous biological molecules remains limited since the input biomolecule needs to have the appropriate chemical structure to bind tightly into the CB7 cavity. Herein we describe a synthetic transducer based on self-assembling DNA-small molecule chimeras (DCs) that is capable of converting a chosen biological input, adenosine triphosphate (ATP; that does not directly bind to the CB7 host) into functional displacement of a protein inhibitor that is bound within the CB7 host. Our system—which features the first example of a covalent CB-DNA conjugate—is highly modular and can be adapted to enable responsiveness to other biologically/clinically relevant stimuli via its split DNA aptamer architecture.

Graphical Abstract

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Supporting Information.

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Detailed synthesis and characterization of DCs, Structures of control and precursor ODNs and ATP aptamer, Fluorescence binding of AO, CB7 and **1**, Surface plasmon resonance experiment for the binding of **1** and CA-II, Michaelis-Menten curve for *p*-NPA hydrolysis catalyzed by CA-II

INTRODUCTION

Cucurbituril (CB[n]) based host-guest systems is a flourishing area of supramolecular chemistry that is making its mark with exciting applications in materials, catalysis, sensors, and biomedicine.^{1–6} In particular, the heptameric family member, CB7, has received considerable attention because it is nicely soluble in water (30 mM), forms ultratight and selective complexes with guests,^{7–10} and is not toxic¹¹. These remarkable attributes have led to the incorporation of CB7 as key components in stimuli-responsive nanomaterials^{12–14} and molecular switches^{15–18} that undergo guest release in the presence of photochemical, electrochemical, and small molecule inputs.

One impetus for the development of CB7 containing systems is for the transport and input-triggered release of pharmacophores.^{19–26} However, achieving guest release from CB7 (or other CB) complexes using relevant biomolecule triggers remains a formidable challenge. The current state-of-the-art is to: a) use covalent enzymatic transformations that can catalyze the formation of a product which is a strong CB7 binder^{27,28} or b) use biomolecules that have been identified to have the appropriate chemical structure to bind tightly into the CB cavity.^{29,30} We were keen to develop a non-covalent strategy that uses synthetic signal transducers to enable the release of tightly bound protein inhibitors from a CB7 host in the presence of a pertinent biomolecule input. Importantly, in this approach the trigger itself does not directly displace the guest from the CB7 host, but rather induces a cascade of constitutional changes that ultimately release guest. This strategy is expected to be broadly applicable to input-responsive drug release technologies since a variety of biomolecules could be used as the triggering agent.

With this article we describe such an approach where a key bio-molecule, adenosine 5'-triphosphate (ATP), is used as a stimuli that leads to the release of a carbonic anhydrase II (CA-II)³¹ inhibitor **1** (that is tethered to a hydrophobic tail) from a CB7 host. ATP was chosen as the input molecule since it is a coenzyme used by Nature to turn ON the activity of an array of proteins (e.g., topoisomerases and kinases). Further, intracellular ATP concentrations (1–10 mM) are much higher than in the extracellular milieu (< 0.4 mM).^{32,33} Thus ATP-input derived release mechanisms provide an attractive approach for releasing pharmacophores within cells,^{33,34} especially aggressive cancer cells that have greater metabolic activity.³⁵

In brief, a DNA machine composed of a pair of self-assembling DNA small molecule chimeras (DCs)^{36–44}—based on a split ATP binding DNA aptamer—acts as a synthetic transducer that converts the ATP input signal into functional displacement of inhibitor **1** from the CB7 host via a duplex induced intramolecular host-guest interaction (see Scheme 1). Since a key feature of this blueprint is an aptamer domain selected for ATP, it is envisioned that alternative aptamers against other biomolecule targets can be exploited to develop host-guest derived DC transducers that are triggered via those biomarkers.

The main component of our design is DC **2** that is composed of one half of an ATP binding DNA aptamer⁴⁵ (residues 1–13) tethered to a CB7 head-group at the 5' position. It was expected that addition of the second component, Janus molecule **1**, that contains an adamantane head-group (for strong CB7 binding) and a benzenesulfon-amide moiety (that is a prototypical CA-II inhibitor) will result in the host-guest complex DC **2**•**1**. In this complex, the adamantane moiety of **1** is included into the CB7 head-group of DC **2** rendering the benzenesulfonamide unit of **1** too sterically encumbered for optimal CA-II inhibition as CA-II has a deep (15 Å) and conical active site. The third component is DC **3** that is composed of the other half of the ATP binding aptamer sequence (residues 14–27) decorated with an adamantane head-group on the 3' position. Since the two DCs are composed of a split aptamer sequence, they are not complementary to each other and hence no significant duplex hybridization should occur in the absence of ATP. Further the intermolecular CB7/adamantane host-guest interaction between DC **2** and DC **3** should be significantly attenuated due to electrostatic repulsion between the negatively charged DNA backbones. Therefore even in the presence of DC **3** inhibitor **1** is expected to remain inactive as it is included in the CB7 host of DC **2**. However, upon addition of ATP the two DCs are expected to form an ATP-templated non-canonical duplex (vide infra for details), with the 5' terminus of DC **2** positioned in proximity to the 3' terminus of DC **3**. This duplex architecture should, in turn, lead to the formation of a strong intramolecular host-guest interaction between the CB7 head-group of DC **2** and the adamantane moiety of DC **3** resulting in the eviction of **1**. Non-sequestered **1** is thereby available for optimal inhibition of CA-II.

RESULTS AND DISCUSSION

The full synthesis and characterization of DC **2** and DC **3** are provided in the SI. However, it is noteworthy to mention that attaching functional elements to CBs, especially in a well-defined mono-functionalized manner, remains a formidable challenge. Indeed, to the best of our knowledge, DC **2** is the first report of a covalent DNA conjugate with any member of the CB family. Briefly, hexynyl modified DNA precursor was reacted with monoazido functionalized CB7⁴⁷ via the copper catalyzed azide-alkyne click reaction (Cu-AAC), in the presence of chelating ligand TBTA, to afford DC **2** (Figure 1A). After desalting and purification by RP-HPLC (Figure 1B), DC **2** was characterized using MALDI-TOF (found MW = 5614.24 Da, calculated MW = 5616.13 Da, for [DC **2** + Et₃NH]⁺).

The ATP-induced self-assembled duplex architecture shown in Scheme 1 was designed based on the NMR structure of the parent 27-mer ATP binding aptamer.⁴⁶ These studies revealed—when AMP was used as the binding ligand—the aptamer folds into a hairpin-type

conformation containing a series of stems and loops with two AMP molecules binding to the aptamer via hydrogen bonding and intercalation (see Figure S-7). Thus, we hypothesized that dissecting the ATP binding aptamer into two halves and attaching appropriate adamantyl and CB7 head-groups would result in an ATP responsive self-assembled duplex as illustrated in Scheme 1. In order to ascertain that the core sequences of the split ATP binding aptamer do indeed self-assemble into a duplex in the presence of ATP, fluorescence quenching studies were conducted with oligodeoxynucleotides (ODNs) **4** and **5** (Figure 2A). These ODNs contain the same sequence as DC **2** and DC **3**, however, instead of CB7 and adamantane headgroups ODN **4** and ODN **5** are tethered to a fluorophore (6-FAM) and a dark quencher (dabcyl), respectively. When a mixture of ODNs **4** and **5** was titrated with ATP a significant quenching of the 6-FAM fluorescence of ODN **4** was observed; 1 mM ATP lead to 80% quenching (Figure 2B and C).^{48,49} These results provide evidence for the ATP-induced formation of a heterodimer complex where the 6-FAM of ODN **4** is in proximity to the dabcyl moiety of ODN **5**. Further, a fluorescence quenching titration was conducted to estimate the hybridization affinity of the two core ODN sequences in the presence of 1 mM ATP. The quencher strand ODN **5** was titrated into a solution of the fluorescent strand ODN **4** (Figure 2D). Nonlinear regression analysis, using a 1:1 binding model resulted in a dissociation constant (K_d) of $5.2 \pm 3.7 \times 10^{-9}$ M. These fluorescence-quenching studies clearly indicate that ODN domains of DC **2** and DC **3** are capable of forming a tight heterodimer in the presence of 1 mM ATP.

A second requisite in our design is that two-faced inhibitor **1** should have a significantly higher affinity (i.e., lower K_d) for CB7 over CA-II. Based on the typical affinities of CB7 toward adamantanes and benzenesulfonamides toward CA-II, we hypothesized that adamantane-linked benzenesulfonamides should be superior at binding CB7 over CA-II.^{50–53} We investigated the binding interaction between **1** and CB7 using a fluorescence competition assay (Figure S-8), where the fluorescent probe acridine orange (AO) was displaced from the cavity of the CB7 host as the concentration of **1** increased. A K_d of $5.3 \pm 0.5 \times 10^{-10}$ M was determined for the CB7•**1** interaction. Next, the binding interaction of **1** with CA-II was probed using surface plasmon resonance. Here CA-II was immobilized on a CM5 chip via amine coupling chemistry following surface activation with EDC and NHS. From the kinetic binding assay (Figure S-9) a K_d of 4.8×10^{-7} M was obtained. Importantly, the affinity for **1** to CB7 is ca. three orders of magnitude stronger than that for **1** to CA-II.

With the above mentioned preliminary experiments indicating that (a) the core ODNs can self-assemble in the presence of ATP and (b) CB7 is a superior host for **1** than CA-II, we performed CA-II Inhibition studies with various solutions of **1**. First the ability of free **1** to inhibit the activity of CA-II was investigated. Enzyme inhibition was probed by following the CA-II induced initial reaction velocity for the hydrolysis of *p*-nitrophenyl acetate (to afford *p*-nitrophenolate, *p*-NPA, that absorbs at 405 nm) versus increasing inhibitor **1** concentrations (Figure 3A). From this experiment an inhibition constant (K_i) for **1** was determined to be $5.2 \pm 0.3 \times 10^{-7}$ M, which is consistent with the SPR binding data. Next, the ability of the DC **2**•**1** complex to inhibit CA-II was tested. These experiments indicated that the K_i of DC **2**•**1** ($6.5 \pm 0.7 \times 10^{-6}$ M) is one order of magnitude larger than that of the

naked inhibitor **1**.⁵⁴ Since the DC **2•1** complex effectively hinders the CA-II inhibition ability of **1**, this complex can serve as the OFF state for the system.

Subsequently, a solution of **1**, DC **2** and DC **3** was tested for CA-II inhibition (Figure 3B) and a K_i of $3.6 \pm 0.4 \times 10^{-6}$ M was determined. This K_i value is close to the value of the DC **2•1** complex (*vide supra*) suggesting that—in the absence of ATP—the adamantane head-group of DC **3** does not significantly bind to the CB7 unit of DC **2** in order for displacement of **1**. In marked contrast, a solution of **1**, DC **2** and DC **3** in the presence of 1 mM ATP exhibits a smaller K_i value of $7.9 \pm 0.7 \times 10^{-7}$ M which is close to the K_i of free **1** with CA-II. Taken together, these studies establish that in the presence of ATP, inhibitor **1** is released from the host cavity of DC **2** that revives its CA-II inhibitory activity.

In order to clearly illustrate the ATP induced OFF-ON switch, an enzyme inhibition assay with a fixed equimolar concentration (2 μ M) of **1**, DC **2** and DC **3** was conducted (Figure 4). At this concentration, CA-II should be effectively inhibited by **1** but should remain largely active in the presence of the DC **2•1** complex (since the concentration is higher than the K_i of **1** and lower than the K_i of DC **2•1** complex). As is evident from inspection of this bar graph, in the absence of inhibitor **1**, CA-II shows a high initial velocity for hydrolyzing *p*-NPA (normalized to 100%). As a positive control addition of free **1** to CA-II, leads to a decrease in CA-II activity (dropping to 28% of the original activity). When the DC **2•1** complex was added to CA-II, the CA-II activity was not significantly inhibited (81%), implying that the DC **2•1** complex hinders binding of **1** optimally to CA-II. Addition of DC **3** to the DC **2•1** complex shows only a modest decrease in CA-II activity (70%) indicating that these two DCs do not interact significantly in the absence of ATP. Importantly, addition of 1 mM ATP to the DC **2•1** + DC **3** solution leads to a dramatic decrease in CA-II activity (38%).

Importantly, control studies were conducted to ascertain that the above results were indeed due to the mechanism illustrated in Scheme 1. First, we investigated the effect of the DNA core and CB7 components of DC **2**. When ODN **6**, containing the same sequence as DC **2** but lacking the CB7 head-group, was added to inhibitor **1** and then incubated with CA-II, no revival of CA-II activity was observed (Figure S-10). However, when unmodified CB7 was used, the activity of CA-II was revived. These findings indicate that the CB7 host moiety is essential for sequestering **1** from inhibiting CA-II. Additionally, control experiments were undertaken to insure that ATP was not interacting separately with the various components of the system. In particular, CA-II activity was probed when 1 mM ATP was added only to (a) CA-II, (b) CA-II + **1**, and (c) CA-II + **1** + DC **2**. In Figure 5A, as bar graph A shows, ATP by itself is not a CA-II inhibitor. Further, ATP alone does not have an appreciable effect on the inhibitory capacity of **1** (bar graph B), and ATP does not directly release **1** from the CB7 cavity of DC **2** (bar graph C).

The last set of controls gauged whether the DC transducer is selective for ATP. Hence, the activity of other nucleoside 5'-triphosphates, to trigger release of **1** from the DC **2•1** + DC **3** system, was tested. As illustrated in Figure 5B, CTP, TTP, and GTP (all at 1 mM concentration) show no significant decrease in CA-II activity when added to solutions

containing DC **2•1** + DC **3**. These experiments illustrate that the host-guest derived DC transducer is selectively triggered by addition of ATP.

CONCLUSIONS

We have reported a pair of CB7 and adamantane linked DCs derived from a split ATP binding DNA aptamer that serves as a synthetic transducer capable of selectively converting an ATP input signal into functional displacement of a CB7 guest (protein inhibitor) from the CB7 host. The displacement is thought to take place via a combination of ATP induced duplex formation and intramolecular host-guest interactions. To the best of our knowledge, this is the first report of a covalent DNA-CB conjugate. Moreover, this work shows proof-of-concept that supramolecular systems can be developed wherein a salient biomolecule trigger—that is not capable of directly displacing a guest from CB7—can nevertheless be used to trigger guest release. We are in the process of investigating DC transducers for other relevant biomolecule inputs.

EXPERIMENTAL PROCEDURES

Unless otherwise stated, all chemicals and solvents were purchased from Sigma Aldrich or Fisher Scientific and used without further purification. Compound **1** was prepared using the procedure described in the literature.⁵⁶ Carbonic Anhydrase II (human recombinant, expressed in *E. coli*, buffered aqueous solution) was purchased from Sigma-Aldrich. The precursor oligodeoxynucleotide (ODN) sequences (Figure S-11) used in this study were synthesized by the W. M. Keck Foundation Biotechnology Resource Laboratory located at Yale University, using standard automated solid phase synthesis. All modification and labelling reagents used to prepare these ODNs, including 5'-Fluorescein Phosphoramidite, 3'-Dabcyl CPG, 5'-Hexynyl Phosphoramidite and 3'-PT-Amino-Modifier C6 CPG, were purchased from Glen Research. All ODNs were purified with sephadex resin microspin G-25 columns (GE healthcare) followed by chromatographic separation using RP-HPLC. RP-HPLC purification of ODNs was carried out using a Varian Prostar HPLC system, equipped with an Agilent PLRP-S 100 Å 5 µm 4.6 × 250 mm reverse phase column. The column was maintained at 65 °C for all runs. The flow rate was set at 1 mL/min. A gradient composed of two solvents (Solvent A is 0.1 M TEAA (aq) in 5% acetonitrile and solvent B is 100% acetonitrile) was used (Table S-1). UV absorption was monitored at 260 nm.

The concentrations of purified ODNs were quantified based on their UV absorption at 260 nm and their molar extinction coefficients obtained by nearest neighbor calculations. Mass spectral data was acquired using a Bruker Autoflex III matrix-assisted laser desorption time-off-flight mass spectrometer (MALDI-TOF MS) with positive ion and linear detection modes. For all ODN samples, a solution of 2',4',6'-Trihydroxyacetophenone (25 mg/mL) in 1:1 water/acetonitrile with 0.1% trifluoroacetic acid was used as the matrix. MALDI samples were prepared by combining 1 µL of ODN solution (1 mM in H₂O) and 1 µL of matrix solution.

Fluorescence binding studies

All fluorescence titrations were conducted on a Varian Cary Eclipse Fluorescence Spectrophotometer. To estimate the dissociation constant (K_d) of ODN **4** and ODN **5** in the presence of 1 mM ATP, the quencher strand ODN **5** was titrated into a solution of the fluorescent strand ODN **4** in 20 mM sodium phosphate buffer with 5 mM $MgCl_2$, pH 7.3. The excitation wavelength was set to 485 nm. Emission was monitored at 520 nm. The K_d was determined via non-linear regression analysis (using OriginPro 2017 software) by fitting to the following equation ⁵⁷:

$$I = I_{max} + (I_{min} - I_{max}) \frac{C_F + C_Q + K_d - \sqrt{(C_F + C_Q + K_d)^2 - 4C_F C_Q}}{2C_F} \quad (1)$$

The values for I_{max} and I_{min} are determined as the maximum and minimum values from the plot. The total fluorescence of the system I is then plotted vs. concentration of the quencher strand, C_Q , given a total fluorescent strand concentration C_F .

A Fluorescence competition binding assay was conducted to assess the binding between CB7 and **1**. First, binding affinity of CB7 and fluorescent probe acridine orange (AO) was established by adding CB7 into 2 μM AO solution in 20 mM sodium phosphate buffer with 5 mM $MgCl_2$, pH 7.3 (Figure S-12). Subsequently, the displacement of CB7 from the fluorescent probe with **1** was investigated by adding **1** into 1.3 μM AO and 2 μM CB7. The data was fitted to a competitive binding mode⁵⁸ in OriginPro 2017 software.

Surface Plasmon Resonance binding assay

The CA-II binding experiment was performed on a Biacore T200 SPR instrument. Series S CM5 chips containing carboxymethylated dextran (GE Healthcare) were used for all measurements. CA-II protein was immobilized using amine coupling chemistry at room temperature. PBS-P+ (pH 7.4, containing 0.5% surfactant P20) buffer was used as immobilization buffer. Using a flow rate of 10 $\mu L/min$, the chip surface was activated with a 7 min injection of 0.4 M EDC and 0.1 M NHS, followed by 7 min injection of 100 $\mu g/mL$ CA-II protein in 10 mM sodium acetate (pH 5.0). Unreacted NHS groups were blocked with 7 min injection of ethanolamine. As a result, CA-II was immobilized on the chip to 6000 response unit. One flow cell of the chip was left as a reference surface without protein immobilization.

For kinetic analysis, 3% DMSO in PBS-P+ was used as running buffer. Inhibitor **1** was 2-fold diluted from 2.0 μM to 0.125 μM and injected for 50 seconds at a flow rate of 30 $\mu L/min$ and dissociation was monitored for 3 minutes. Each cycle of analyte injection was followed by a 1 minute injection of running buffer as a washing step. Data sets were blank correlated and analyzed with the Biacore T200 evaluation software using a 1:1 binding model.

CA-II enzyme activity assay

The CA-II enzyme activity assay was conducted by dissolving CA-II (1×10^{-7} M) in assay buffer (20 mM sodium phosphate buffer with 5 mM MgCl_2 , pH 7.3), and incubating in a 96-well plate with a dilution series of inhibitors for 30 min at R.T. The colorimetric assay was initiated by adding substrate *p*-nitrophenylacetate (*p*-NPA), and the UV-absorption change at 405 nm was recorded (which corresponds to the production of *p*-nitrophenolate) by using a PerkinElmer EnSpire Multimode Plate Reader. Normalization of the initial reaction velocity was performed by assigning a maximum value of 1 to the initial reaction velocity obtained from a CA-II esterase activity assay in the absence of any inhibitor for each series of experiments. Specific initial reaction velocities in the presence of inhibitors in these studies were converted to proportions of this value (ranging from 0 to 1). To estimate the K_i of inhibitor complexes, the normalized initial reaction velocity (V) was plotted against the corresponding inhibitor concentration (I), and the K_i (the inhibition constant of the enzyme–inhibitor complex) value was obtained by fitting, via nonlinear regression, to equation

$$V = \frac{K_m + [S]}{[S] + K_m \left(1 + \frac{[I]}{K_i}\right)} \quad (2)$$

Here, $[S]$ is the total concentration of the substrate *p*-NPA, which is 1.0 mM. The Michaelis–Menten constant K_m (the substrate concentration that gives a reaction rate equal to one-half the maximum rate) was first obtained for *p*-NPA hydrolysis catalyzed by CA-II in the absence of inhibitors by fitting the data (Figure S-13) to the Michaelis-Menten equation 3:

$$V_0 = \frac{V_{max} [S]}{K_m + [S]} \quad (3)$$

The K_m value was calculated to be 2.52 ± 0.26 mM, and V_{max} was 0.125 ± 0.003 O.D./min.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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54. While the DC **2•1** complex is a much weaker inhibitor than inhibitor **1** alone, the reason that the DC **2•1** complex still has a small inhibition capacity is likely due to the low (but non zero) binding affinity between the CB7•**1** portion of the DC **2•1** complex with the enzyme active site. Indeed, when the CA-II activity was probed using non-DNA attached (i.e., unmodified) CB7 in the presence of inhibitor **1** (see Figure S-10), the unmodified CB7•**1** complex shows a similar inhibition strength as the DC **2•1** complex.
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Figure 1.

(A) Synthesis of DC 2 via the CuAAC reaction. (B) RP-HPLC trace of hexynyl modified DNA starting material (green line) and product DC 2 (violet line); absorbance measured at 260 nm. The inset shows the MALDI-TOF spectrum for DC 2.

Figure 2.

(A) ATP-induced self-assembly of ODNs **4** and **5** resulting in a fluorescence-quenched heterodimer **4•5**. (B) Fluorescence titration of ODN **4** and ODN **5** (both at 1 μ M) with increasing concentration of ATP (0–2 mM) in 20 mM sodium phosphate buffer with 5 mM MgCl_2 , pH 7.3. The excitation $\lambda = 485$ nm. (C) The decrease in the emission profile of **4** (at 520 nm) with increasing ATP concentration. (D) Fluorescence titration of quencher strand ODN **5** into a solution of ODN **4** (1×10^{-7} M) in the presence of 1 mM ATP in 20 mM sodium phosphate buffer with 5 mM MgCl_2 , pH 7.3.

Figure 3.

(A) CA-II esterase activity upon addition of naked inhibitor **1** (red line) and an equimolar concentration of DC **2** and **1** (blue line). (B) CA-II activity with an equimolar mixture of **1**, DC **2** and DC **3** without ATP (green line) or with 1 mM ATP (blue line). All measurements were carried out in 20 mM sodium phosphate buffer with 5 mM $MgCl_2$, pH 7.3. Results are the mean $\pm$ SD of three independent experiments. The data were fit to a competitive inhibition model.⁵⁵

Figure 4.

CA-II inhibition with inhibitor **1** and varying DC combinations (all at 2 μ M). The concentration of ATP was 1 mM. All measurements were carried out in 20 mM sodium phosphate buffer with 5 mM MgCl_2 , pH 7.3. Results are the mean $\pm$ SD of three independent experiments.

Figure 5.

(A) CA-II enzyme activity with **1** (2 μ M) alone or with DC **2** in the absence/presence of 1 mM ATP. (B) CA-II inhibition with equimolar (2 μ M) inhibitor **1**, DC **2** and DC **3** in the absence and presence of 1 mM NTPs. All measurements were carried out in 20 mM sodium phosphate buffer with 5 mM MgCl_2 , pH 7.3. Results are the mean $\pm$ SD of three independent experiments.

Scheme 1.

A DNA-small molecule chimera (DC) transducer based on a split DNA aptamer that converts an ATP input into functional release of a CA-II inhibitor **1** from the CB7 host.