# Rational design of a small molecule-responsive intramer controlling transgene expression in mammalian cells

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## ABSTRACT

Aptamers binding proteins or small molecules have been shown to be versatile and powerful building blocks for the construction of artificial genetic switches. In this study, we present a novel aptamer-based construct regulating the Tet Off system in a tetracycline-independent manner thus achieving control of transgene expression. For this purpose, a TetR protein-inhibiting aptamer was engineered for use in mammalian cells, enabling the RNA-responsive control of the tetracycline-dependent transactivator (tTA). By rationally attaching the theophylline aptamer as a sensor, the inhibitory TetR aptamer and thus tTA activity became dependent on the ligand of the sensor aptamer. Addition of the small molecule theophylline resulted in enhanced binding to the corresponding protein *in vitro* and in inhibition of reporter gene expression in mammalian cell lines. By using aptamers as adaptors in order to control protein activity by a predetermined small molecule, we present a simple and straightforward approach for future applications in the field of Chemical Biology. Moreover, aptamer-based control of the widely used Tet system introduces a new layer of regulation thereby facilitating the construction of more complex gene networks.

## INTRODUCTION

Aptamers are short nucleic acids binding ligands with high affinity (1). They can be artificially designed by a method called Sytematic Evolution of Ligands by Exponential Enrichment to recognize a wide variety of ligands ranging from small molecules to intracellular proteins and cell-surface epitopes (1). Upon ligand binding, a conformational change of the aptamers' secondary structure can be induced (2). This has been broadly used to construct molecular sensors detecting the corresponding ligand *in vitro* (3).

Moreover, aptamers have been shown to be capable of regulating gene expression in bacteria and eukaryotes. In 2002, more than 10 years after the first artificial aptamers have been reported (4), natural aptamer domains were discovered as part of so-called riboswitches. Located in the untranslated regions (UTRs) of messenger RNAs (mRNAs), riboswitches affect transcription or translation by a ligand-induced change of mRNA secondary structures (5,6). Moreover, artificial gene regulation systems have been designed by sophisticated incorporation of aptamers in natural systems. In bacteria, the conformational change of an aptamer is used to mask the ribosome-binding site on the mRNA thus affecting translation initiation (7,8). In eukaryotes, ligand binding to the aptamer can result in a stabilized secondary structure, which can be used to block ribosomal mRNA scanning (9,10), dicer processing (11) or splicing (12,13). Also, aptamers fused to ribozymes have been validated as versatile control devices in bacteria (14–17) as well as in eukaryotes (18–21). It has to be noted that even though a vast variety of aptamers is existing, many of these synthetic riboswitches contain the well-characterized theophylline aptamer, which has first been described in 1994 and binds its ligand with a $K_d = 320\,\text{nM}$ (22). Interestingly, this aptamer has a 10 000-fold lower affinity to caffeine, the methylated derivative of theophylline (22).

Additionally, gene expression control was obtained by applying aptamers specific for transcription factors. Often based on a yeast-three-hybrid system, there the aptamer is part of a larger RNA construct and has the mission to recruit an essential transcription factor to the cognate

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promoter region (23–25). Buskirk *et al.* (26) successfully demonstrated, that a tetramethylrhosamine-specific aptamer could regulate such transcription factor recruitment and trigger gene expression in yeast accordingly. However, *in vivo* selection was required to identify a suitable connection sequence linking the RNA domains (26), the specific aptamer–protein interaction site remained elusive (23,26).

Besides their role in artificial gene regulation, protein-binding aptamers have been shown to modify protein functionality *in vitro* and *in vivo* (27–30). Prominent examples include the inhibition of the fruit fly splicing factor B52 (28), yeast RNA polymerase II (29) and reverse transcriptase of HIV-1 (27,30). Such aptamers could also be used to identify small-molecule inhibitors of the target protein by a method known as aptamer displacement (31). Also, aptamers specific for intracellular target proteins (intramers) have been used for protein-function analysis (32).

In this study, we have successfully designed a small molecule-responsive mammalian intramer, which could modulate protein function in a trigger-inducible manner. In such a configuration, the intramer serves as a universal adaptor attenuating protein activity in response to a specific dose of a desired small molecule.

## MATERIAL AND METHODS

### Plasmid design

The functional and the inactive control variants of the TetR aptamer (33) were inserted into 'pRzTheo-miREGFP M5' (kindly provided by Prof. Dr Yokobayashi, UC Davis) (34) by polymerase chain reaction (PCR)-mediated cloning using sequence-specific primers containing the TetR aptamer sequences at their 5′-end (underlined). In brief, immediately after the PCR (Phusion, Finnzyme), the template plasmid was digested using the restriction enzyme DpnI. The aptamer-containing PCR fragments were ligated (T4 DNA Ligase, Fermentas) and transformed into *Escherichia coli* XL10 gold (Stratagene). Single colonies were picked and grown in LB-medium supplemented with $100\,\mu g\,ml^{-1}$ ampicillin (Roth). To confirm successful assembly, the cloned plasmids were isolated (Miniprep Kit, Zymo Research) and sequenced. For the primers used for cloning, see Table 1. The constructed aptamer-harboring plasmids pDAX were denominated as follows: pDA1 (active TetR aptamer), pDA3 (inactive TetR aptamer), pDA4 (theophylline-responsive TetR aptamer) and pDA15 and pDA16 (different connector lengths of theophylline-responsive TetR aptamer).

### Cell culture

Human embryonic kidney cells HEK293-T (American Type Culture Collection, ATCC No. CRL-11268) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) Fetal Calf Serum (FCS) (PAN Biotech; lot no. P231902) and 1% (v/v) penicillin/ streptomycin solution (PAN Biotech). Chinese hamster ovary cells (CHO-K1, ATCC No. CCL-61) were cultivated

in ChoMaster HTS (Cell Culture Technologies) supplemented with 5% (v/v) FCS and 1% penicillin/streptomycin solution. All cell lines were incubated at 37°C in a humidified atmosphere containing 5% $CO_2$.

### Transfection

One day prior to transfection 50 000 cells per well were seeded into a 24-well plate containing 0.5 ml medium. HEK293-T were cotransfected with 800 ng total DNA [pSAM200 (35):pMF111 (36):pDAX = 1:1:14] using a standard $CaPO_4$ transfection protocol. (37) In brief, plasmids were mixed in 12.5 μl sterile water, an equal volume of sterile 0.5 M $CaCl_2$ was added and incubated for 15 min. Thereafter, 25 μl of sterile 2×HBS (100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid pH 7.05; 280 mM NaCl, 1.5 mM $Na_2HPO_4$) was added while vortexing and subsequently added dropwise to the cells. After an incubation time of 4 h, fresh medium supplemented with appropriate concentrations of theophylline was added. CHO-K1 cells were transfected with Fugene 6 (Roche Diagnostics AG) according to the manufacturer's protocol. Reporter gene expression was evaluated 48 h after transfection.

### Colorimetric SEAP assay

Cell culture supernatant was first heat inactivated for 30 min at 65°C and centrifuged at 2000 rpm for 5 min to remove cell debris. A total of 100 μl of 2×SEAP assay buffer (20 mM homoarginine, 1 mM $MgCl_2$, 21% diethanolamine pH 9.8) was mixed with 80 μl of heat-inactivated cell culture supernatant. 20 μl substrate solution [120 mM *p*-nitrophenylphosphate (pNPP, Sigma Chemie)] was added and absorbance was measured at 405 nm (*p*-nitrophenol; pNP) at 37°C for 30 min from which SEAP activity can be calculated as described elsewhere (38).

### Reverse transcription–polymerase chain reaction

HEK293-T cells were cotransfected as described above with plasmids as indicated in Figure 2C. Total RNA was isolated from cells after 48 h using TRIzol Reagent (Invitrogen) following the manufacturer's protocol. Subsequently, RNA was treated with 2 U DNase I (Fermentas) to remove residual DNA contaminations. One microgram DNaseI-treated RNA and SuperScript II RT (Invitrogen) were used to generate cDNAs using specific primers ODA64_GAPDH_rv (5′-GGCATGGAC TGTGGTCATGAG) and ODA66_invitro_rv3 (5′-GGG AGATACGCGTATCAAG) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and TetR aptamers, respectively. Twenty five cycles of PCR were carried out using following primers: ODA63_GAPDH_ fw (5′-ACAGCTACTCCTCGCCTGTG) and ODA64_ GAPDH_rv giving an amplicon length of 87 bp for GAPDH, ODA38-in_vitro_Fw and ODA66_invitro_rv giving amplicon lengths of 72 bp for pDA1/pDA3 and 87 bp for pDA4. PCR products were separated on 3% agarose gels supplemented with ethidium bromide and visualized under UV light.

**Table 1.** Primer sequences used for the construction of the aptamers

| Plasmid name | Primer names and sequences | Template |
|---|---|---|
| pDA1—TetR aptamer | ODA10<br>5'-<u>CACAGACCAGAGAAAAGCTTGATACGCGAAAGGAG</u><br>TTTTTT GGAAAAGCTTGGCACTGGC-3'<br>ODA11<br>5'-Pho-<u>ATGACCCATAACATGCTGCTTGATACGCGTATCTCCCTC</u><br>GATCCCGCGTCCTTTCCACAAG-3' | pRzTheo-miREGFP M5 |
| pDA3—inactive TetR aptamer | ODA11<br>ODA13<br>5'-<u>CACAGACCA**T**AGAAAAGCTTGATACGCGAAAGGAG</u> TTTTTT<br>GGAAAAGCTTGGCACTGGC-3' | pRzTheo-miREGFP M5 |
| pDA4—theophylline-responsive TetR aptamer—2 GC | ODA14<br>5'-<u>GGATACCAGCCGAAAGGCCCTTGGCAGCC</u><br>AGAGAAAAGCTTGATACGC-3'<br>ODA15<br>5'-Pho-CATAACATGCTGCTTGATACG-3' | pDA1 |
| pDA15—theophylline-responsive TetR aptamer—1 GC | ODA30<br>5'-GATACCAGCCGAAAGGCCCTTGGCAGC<br>AGAGAAAAGCTTGATACGC-3'<br>ODA15 | pDA1 |
| pDA16—theophylline-responsive TetR aptamer—3 GC | ODA31<br>5'-GGGATACCAGCCGAAAGGCCCTTGGCAGCCC<br>AGAGAAAAGCTTGATACGC-3'<br>ODA15 | pDA1 |

## TetR protein purification

The open reading frame of TetR was cloned with NdeI/HindIII into pET30a in frame to a 5'-hexahistidine sequence and the plasmid was transformed into *E. coli* BL21/Gold (*DE3*). One millimolar Isopropyl β-D-1-thiogalactopyranoside was added, when the bacterial culture reached $OD_{600} = 0.8$. Cells were harvested by centrifugation after 3–4 h expression at 30°C and resuspended in lysis buffer (25 mM $NaH_2PO_4$, 100 mM NaCl, 10 mM Imidazol pH 8). Cell were lysed by French Press and subsequently loaded onto a 1 ml $Ni^{2+}$-NTA column (GE Healthcare) equilibrated with lysis buffer. TetR was eluted by gradually increasing the imidazole concentration to 500 mM (see Ni-NTA Spin Kit Handbook, www.qiagen.com). The peak fractions were analyzed by SDS–PAGE, pooled and protein was dialyzed in 25 mM $NaH_2PO_4$, 100 mM NaCl, 25% glycerol pH 8 overnight and subsequently stored at −20°C until use.

## RNA preparation

Synthetic DNA template of theo-TetR intramer was PCR-amplified using the following primers: ODA37_in_vitro_rv: TAATACGACTCACTATAGGGAGATAC GCGTATCAAG, ODA38-in_vitro_Fw: CTCCTTTCGC GTATCAAGC and the plasmid pDA4 as a template. The PCR product was *in vitro* transcribed using T7 RNA polymerase (Fermentas) in transcription buffer (40 mM Tris–HCl pH 7.9, 6 mM $MgCl_2$, 10 mM DTT, 10 mM NaCl, 2 mM spermidine, 2 mM ATP, 2 mM CTP, 2 mM GTP and 2 mM UTP). After 4 h of incubation at 37°C the reaction was stopped by addition of one volume of stop buffer [80% (v/v) formamide, 50 mM EDTA pH 8.0, 0.025% (w/v) bromophenolblue and 0.025% (w/v) xylene cyanole]. Subsequently the product was purified
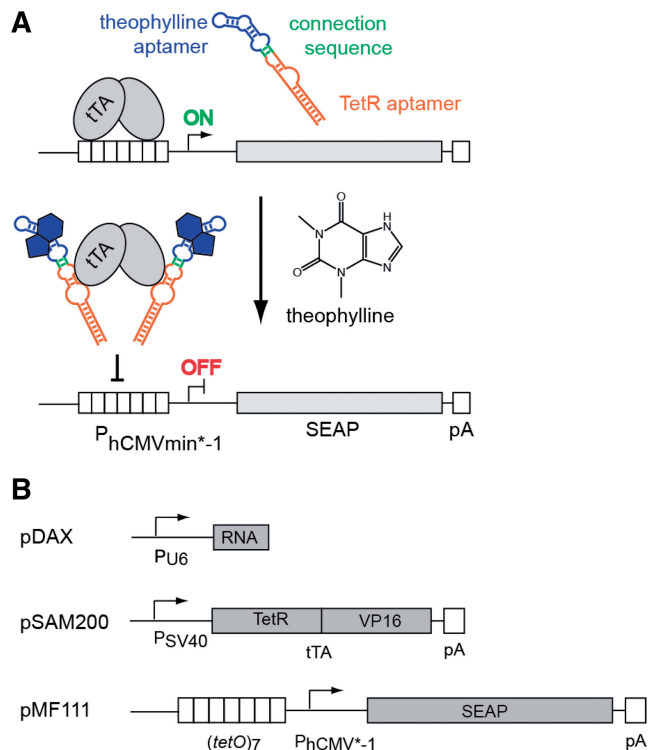


**Figure 1.** (**A**) Proposed mechanism of the theophylline-responsive TetR aptamer. In presence of theophylline the TetR aptamer structure is restored. This allows for specific binding to tTA thus inhibiting transcription initiation. $P_{hCMVmin*-1}$ stands for tetracycline responsive promoter, SEAP for secreted alkaline phosphatase and pA for poly(A)-tail. (**B**) Plasmid constructs used in this work. pDAX encodes either pDA1 (active TetR aptamer), pDA3 (inactive TetR aptamer) or pDA4 (theophylline-responsive TetR aptamer). pSAM200 encodes tTA, pMF111 contains a $P_{hCMV*-1}$-driven SEAP expression unit.
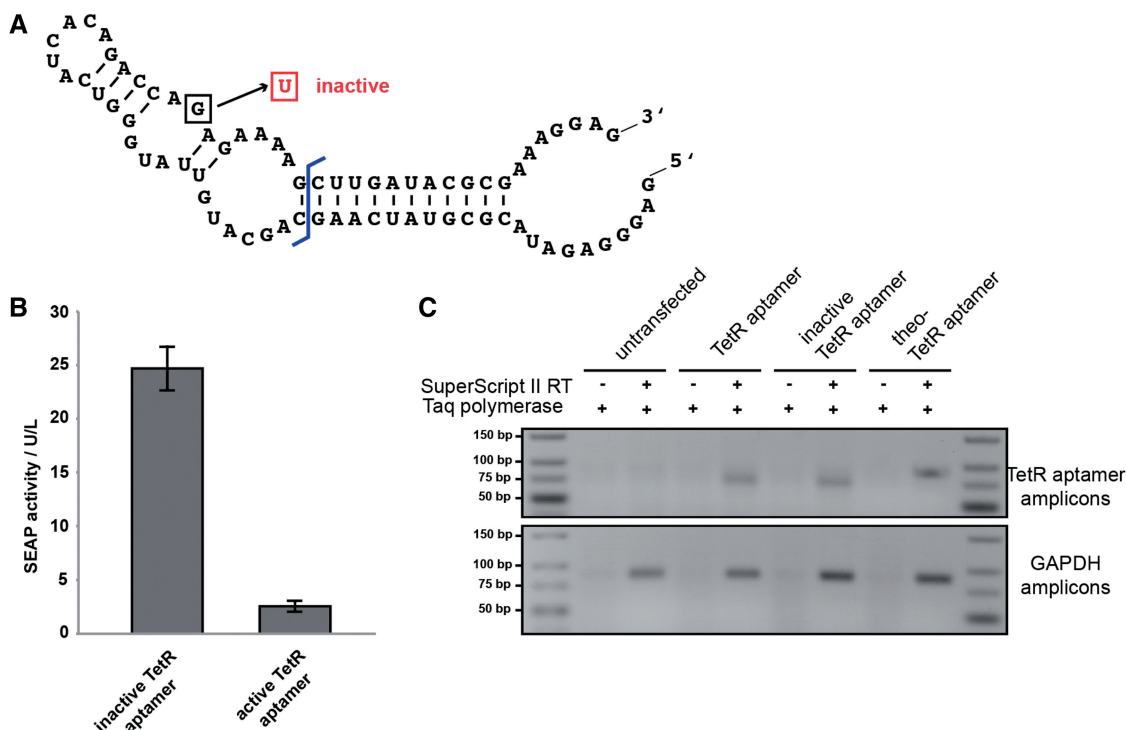
**Figure 2.** The TetR aptamer in mammalian cells. (**A**) Nucleotide sequence and secondary structure of the TetR aptamer variant 12-1R adapted from Hunsicker and colleagues (33). Boxed nucleotide indicates an inactivating point mutation (33). (**B**) Transcription of the TetR aptamer in HEK293-T cells significantly represses reporter gene expression compared to the genetically inactivated control variant. (**C**) Analysis of RNA expression of different TetR aptamer constructs in HEK293-T cells by semi-quantitative RT–PCR showed comparable expression levels of all constructs.

on an 8% denaturing PAGE. Full-length product was excised and isolated from the gel.

### Competition assay

For competition assays, two complementary oligonucleotides ODA68 (Cy5-TTGACACTCTATCATTGATAGAGTTAT) and ODA69 (ATAACTCTATCAATGATAGAGTGTCAA) containing the tet operator sequence were hybridized in 1x annealing buffer [20 mM Tris–HCl (pH 7.8), 5 mM MgCl$_2$ and 50 mM NaCl] by heating to 95°C and slowly cooling down to RT for 30 min. One ninety nanomolar Cy5-labeled DNA was then incubated in electrophoretic mobility shift assays (EMSA) buffer [20 mM Tris–HCl (pH 7.8), 5 mM MgCl2, 50 mM NaCl, 0.5 µg/ml polydI:dC, 20% glycerol] with indicated amounts of protein, RNA aptamers, theophylline and anhydrotetracycline (atc). After a 15 min incubation period at room temperature, samples were electrophoresed at 4°C on a 10% polyacrylamide gel containing 0.5% Tris-borate-EDTA buffer.

### Determination of the equilibrium dissociation constants $K_d$ and $K_c$

Dissociation constant $K_d$ of the His-tag TetR to its DNA operator was determined by quantifying the gel shift assay with ImageJ. Data obtained was fitted to

$$f = m^* C^{n*}(K_d^n + C^n)^{-1} \tag{1}$$

with $f$ = fraction bound, $C$ = concentration of the TetR protein, $n$ = Hill coefficient and $m$ = maximal levels of fraction bound.

Dissociation constants of the competitors $K_c$ of the aptamer variants were determined by quantifying the competition assays with ImageJ. From the data obtained, the concentration of aptamer that disrupts 50% of the protein–DNA complex (IC$_{50}$) was calculated with the following equation (39)

$$f = b + ((m - b)^*(1 + (IC_{50}^* C^{(-n)}))^{-1}) \tag{2}$$

with $f$ = fraction bound, $C$ = concentration of the aptamer variant, $n$ = Hill coefficient and $m$ and $b$ maximal and minimal levels of fraction bound.

The IC$_{50}$ value was then used to calculate $K_c$ using the Lin and Riggs equation (39,40)

$$K_c = (2^* K_d^* IC_{50})^* (2^* P - R - 2^* K_d)^{-1} \tag{3}$$

with $K_d$ = dissociation constant of the His-tag TetR to its DNA operator, $P$ = His-tag TetR concentration and $R$ = labeled DNA operator concentration.

## RESULTS AND DISCUSSION

The modular nature of aptamers enables the rational design of allosteric multi-domain variants such as those containing interconnected sensor and effector domains (41). Upon binding of the trigger to the sensor domain
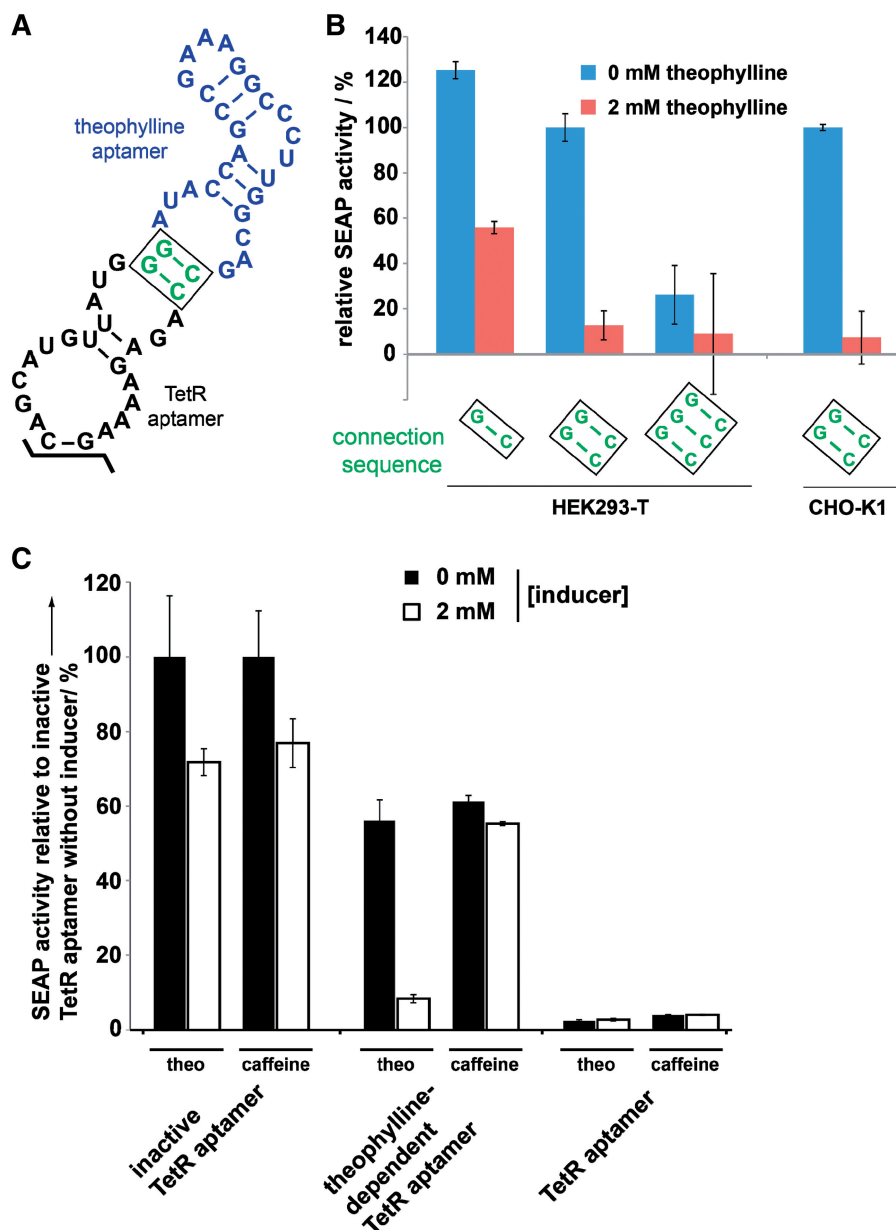
**Figure 3.** *In vivo* characterization of the theo-TetR intramer. (**A**) Nucleotide sequence and secondary structure of the theo-TetR intramer. The 5'- and 3'-stem region is identical to Figure 2A and, thus, denoted as black points. (**B**) Theo-TetR intramer-responsive reporter gene expression levels in the absence (blue column) and in the presence of 2 mM theophylline (red column). The rationally designed connection sequence has a strong influence on the performance of the device. (**C**) Specificity and cytotoxicity of the system. In contrast to theophylline, caffeine does not specifically repress SEAP expression. However, due to their cytotoxicity, both compounds reduce overall gene expression by 25%.

the aptamer structure refolds and unlocks the effector domain for the desired action (41). As a proof-of-principle design for trigger-inducible intramers, we chose the well-characterized theophylline aptamer (22) as the sensor domain and the TetR-*tetO*-inhibiting intramer as effector domain (33,42). In mammalian cells, TetR fused to the VP16 transactivation domain (tTA, tetracycline-dependent transactivator) and the heptameric *tetO* [(*tetO*)₇] fused to a minimal promoter (P$_{hCMV*-1}$, tetracycline-responsive promoter) are known as the TET system (43) and represent frequently used parts for tetracycline-dependent transgene control and synthetic gene networks (44). Therefore, the design of a ligand-responsive intramer would add an additional layer of regulation and expand the use of the TET system by introducing a new inducer molecule, see Figure 1A.

**Transfer of TetR aptamer into mammalian cells**

Evolved in a combined *in vitro*/*in vivo* approach, the TetR aptamer (see Figure 2A for sequence and secondary structure) was shown to induce transcription in *E. coli* by competing with TetR for binding with the *tetO* operator sequence by masking the *tetO*-binding helix-turn-helix
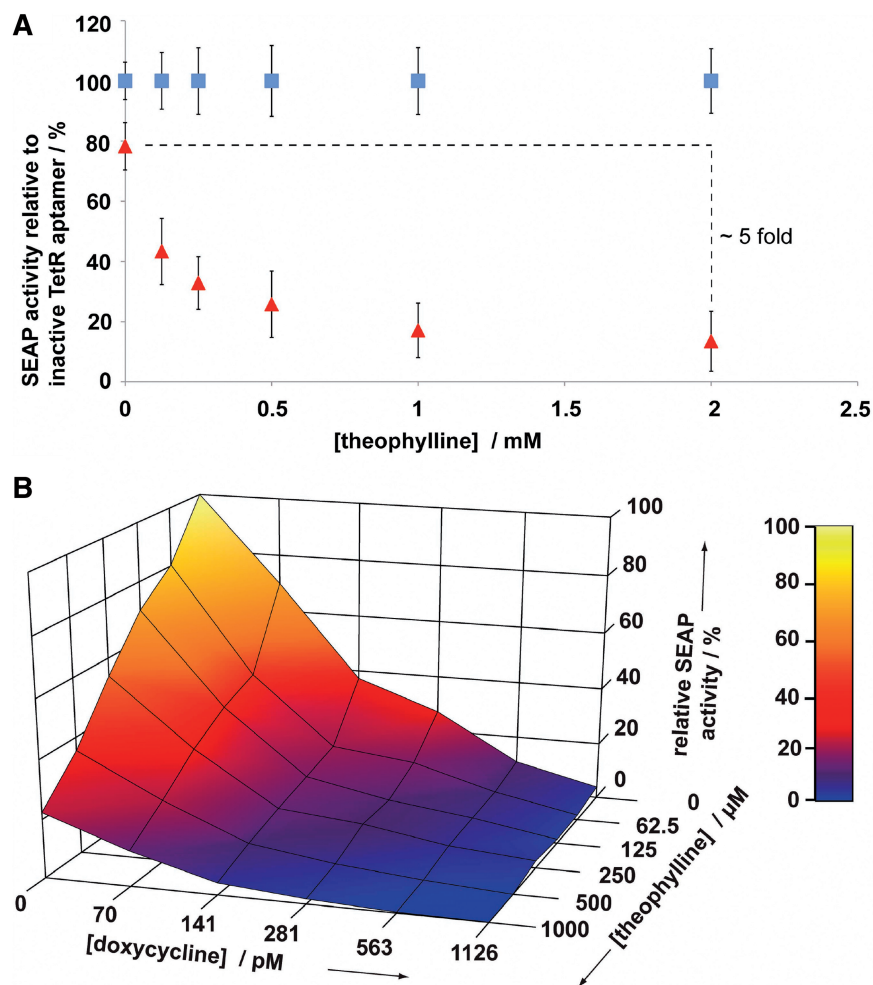
**Figure 4.** (**A**) Theophylline dose-dependent inhibition of SEAP expression of the theo-TetR intramer (red) relative to the inactivated TetR intramer variant (blue) in HEK293-T cells. (**B**) Theophylline and doxycycline cross-titration of the theo-TetR intramer in HEK293-T cells. Intersection points of the grid represent SEAP expression levels measured in duplicates.

motif of TetR (33). Since the flanking stem regions were shown to influence aptamer activity, we chose the best-characterized TetR aptamer variant 12-1R (33) for initial experiments. In a first step, we had to engineer this system for use in mammalian cells. Therefore, the TetR intramer was placed under control of the RNA polymerase III-dependent U6 promoter (pDAX). Since U6 promoter transcripts used in this study are devoid of any export signal corresponding aptamers remain in the nucleus. tTA was expressed constitutively from a second plasmid (pSAM200) while the reporter gene SEAP was driven by $P_{hCMV*-1}$ (pMF111) of a third plasmid (Figure 1B). After binding of tTA to $tetO_7$, gene expression should be induced. Indeed, tTA in combination with an inactivated control TetR aptamer (33) resulted in efficient gene expression. However, with aptamer functionality restored, SEAP expression was shut down (Figure 2B). Semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR)-based control experiments showed that these aptamer variants were expressed at similar levels (Figure 2C), confirming that differences in SEAP

production profiles correlated with changes in aptamer affinity. This basic experiment demonstrated the cross-kingdom interoperability of aptamers. Noteworthy, unlike in bacteria where the TetR aptamer induces gene expression when competing with TetR binding (33), it represses transgene expression in mammalian cells by inhibiting the tTA-$tetO_7$ interaction.

**Theophylline-responsive TetR aptamer in mammalian cells**

In order to obtain a controllable intramer ('theo-TetR intramer'), we replaced its stem–loop region of the TetR aptamer, which is not involved in ligand binding (33), by the theophylline aptamer (22) (Figures 1A and 3A). A central element of this novel RNA construct was the connection sequence linking the two domains. Only the presence of theophylline should induce the defined TetR aptamer structure that is capable of binding the corresponding protein. Using the secondary structure prediction algorithm mfold (45), we designed this connector sequence to be a short unstable stem region that does not interfere with the overall aptamer structure, see Figure 3A.
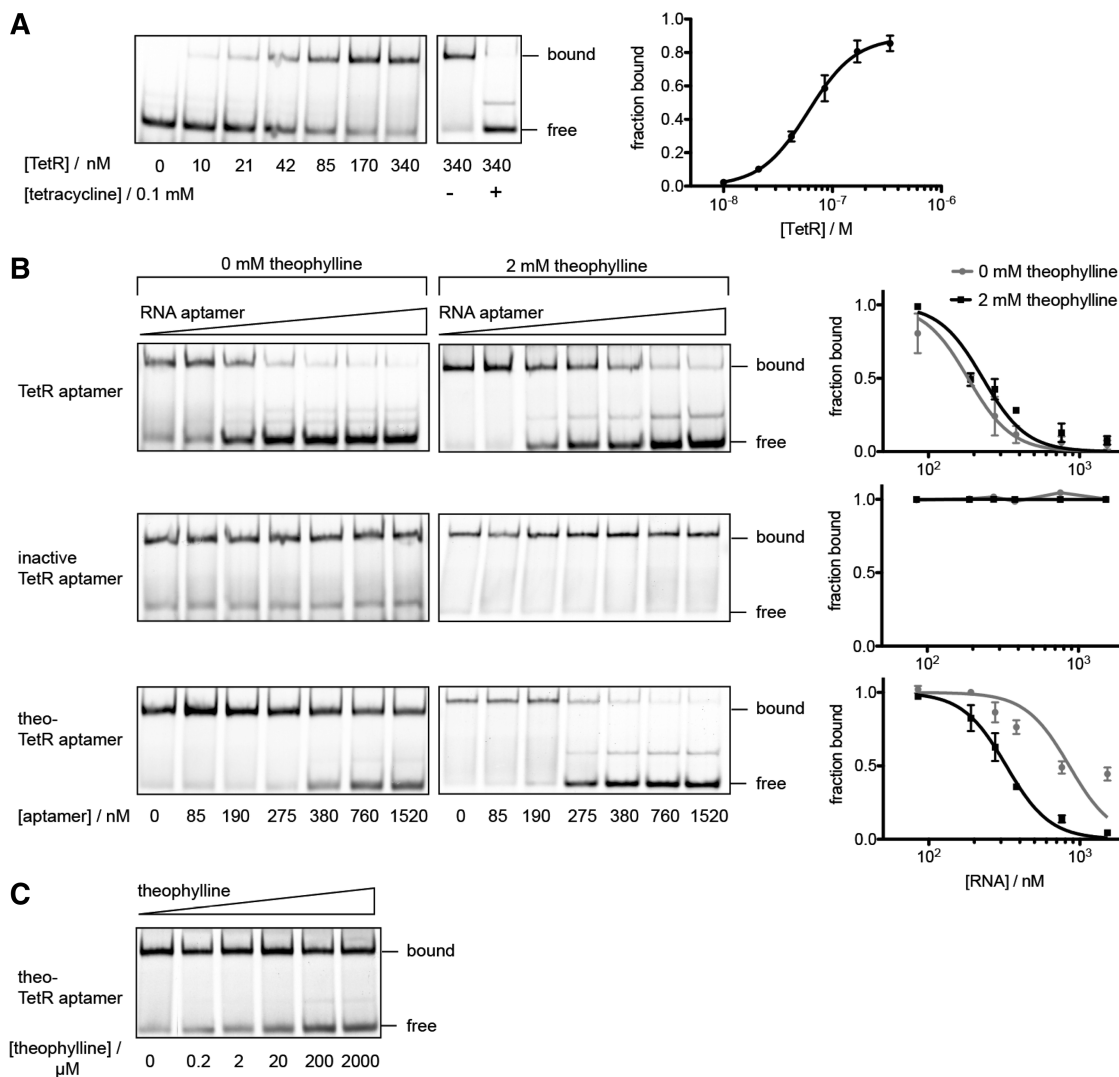
**Figure 5.** *In vitro* competition assays of RNA aptamers withCy5-labeled dsDNA *tetO* for TetR binding. (**A**) Quantification of concentration-dependent binding of purified TetR protein to *tetO* resulted in an apparent $K_d = 60\,nM$. TetR was released from DNA upon addition of tetracycline. (**B**) While the inactive TetR aptamer has no influence, the active variant competes with *tetO* for TetR binding independent of theophylline ($K_{c,no\ theo} = 58\,nM$ and $K_{c,\ 2\ mM\ theo} = 76\,nM$). Binding of the theophylline-responsive variant to TetR, however, is strongly improved in the presence of 2 mM theophylline ($K_{c,no\ theo} = 276\,nM$ and $K_{c,\ 2\ mM\ theo} = 106\,nM$). (**C**) Theophylline-dependent competition of theo-TetR intramer with *tetO* for TetR binding. If not stated separately, TetR concentration was kept constant at 340 nM, *tetO* at 190 nM and theo-TetR intramer at 275 nM.

Intriguingly, this rationally designed RNA part operated as expected *in vivo*. In the absence of theophylline, the theo-TetR intramer does not inhibit tTA which therefore mediates maximum transgene expression, almost comparable to levels reached when using the genetically inactivated control intramer. However, after the addition of theophylline, SEAP expression is dose-dependently reduced and reaches a repression level typically achieved by using the native TetR intramer (Figures 3B and 4A). The specificity was demonstrated by the addition of the theophylline homologue caffeine, which did not modulate aptamer function (Figure 3C). The importance of the connection sequence was demonstrated by further mutational analysis: destabilization of the active fold by removing one base pair of the connection sequence resulted in increased SEAP levels. The opposite effect was observed

by introducing an additional base pair, thus following a predictable rational in which the connector stability directly correlates with the formation of the active TetR aptamer structure, see Figure 3B.

Also, comparable switching rates were achieved following co-transfection of the constructs into different host cell lines (HEK293-T and CHO-K1 cells; Figure 3B). Noteworthy, we observed theophylline-dependent cytotoxicity in both cell types resulting in an unspecific 25% reduction of reporter gene expression (Figure 3C). This is in accordance with previous reports using theophylline in mammalian cells (11,18,21), thus highlighting the need for novel aptamers responsive to non-toxic compounds.

A cross-titration of the theo-TetR intramer with theophylline and doxycycline, which represses gene expression in the tTA system, revealed both effectors to be

compatible and complementary, see Figure 4B. This should allow the application of the newly constructed intramer in numerous synthetic gene networks based on the tTA system (44).

### *In vitro* characterization of the theo-TetR intramer

We further characterized the theo-TetR intramer by performing *in vitro*-binding studies. Since the original TetR aptamer binds specifically to the DNA-binding domain of TetR (33), we also used the latter protein for the *in vitro* studies. Using EMSA, His-tagged TetR protein that was purified from *E. coli* was shown to bind to a Cy5-labeled dsDNA harboring the *tetO* sequence in a tetracycline-responsive manner. Compared to the wt-TetR ($K_d$ = 5.6 nM) (46), the His-tagged variant exhibited a slightly lower binding affinity ($K_d$ = 60 nM; Figure 5A). As shown before (33), TetR binding to the *tetO* dsDNA can be competed with increasing concentrations of wt-TetR aptamer. However, addition of the inactive TetR aptamer variant had no influence on TetR-*tetO* binding. *In vitro* competition assays supported the *in vivo* observations, that the presence of theophylline had no influence on the TetR-binding characteristics of both, active and inactive aptamer variants (Figure 5B). Although the theo-TetR aptamer showed lower target affinity ($K_c$ = 276 nM) compared to the wt-TetR aptamer ($K_c$ = 58 nM), its TetR-binding capacity could be adjusted by theophylline to $K_{c,theo}$ = 106 nM. By adding increasing concentrations of the ligand the aptamer could compete with *tetO* for Tet repressor protein binding (Figure 5B and C).

In summary, we designed and constructed a ligand-responsive intramer for adjustable transgene expression in mammalian cells. By rationally replacing a stabilizing stem-loop structure of the TetR intramer with the theophylline aptamer, we achieved an RNA construct that allows regulation of TetR/tTA protein function by an external ligand. The resulting artificial RNA switch shows a 5-fold inhibition of gene expression, which is in the same range of other types of RNA switches constructed for mammalian cells (11,21). Since the tTA system is an essential part of many synthetic genetic networks (44,47,48), the presented theo-TetR intramer offers a simple but promising way to introduce a new layer of regulation. The possibility to control the same gene output by two different molecule inputs will facilitate the construction of more complex synthetic gene networks in future. This is from greatest interest for cellular Boolean calculations but could also be used as a safeguard in microencapsulated cell implants where one input allows a concentration-dependent fine-tuning of therapeutic protein expression while the second input resets the system.

Also, the basic principle of building a small molecule-responsive regulator of distinct protein functions via rational design could be valuable for the fields of Synthetic and Chemical Biology. In future experiments, we will try to generalize this principle by using intramers targeting other proteins not exclusively involved in gene regulation.

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