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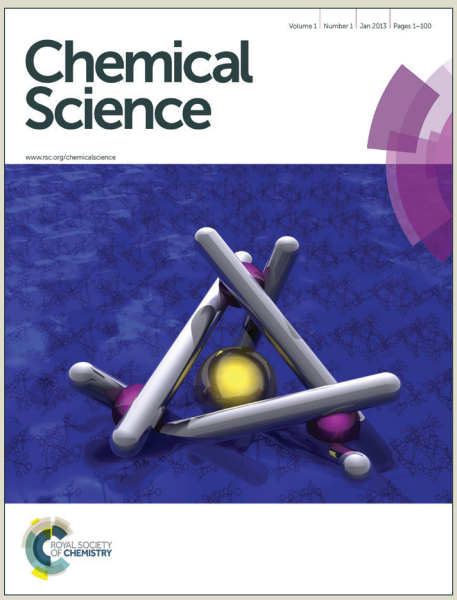

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Cytotoxic peptide-PNA conjugates by RNA-programmed peptidyl transfer with turnover

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We describe a RNA-programmed peptidyl transfer reaction, which triggers the formation of a cytotoxic, cell permeant 14-mer peptide-PNA conjugate from inactive fragments. Turnover in the RNA template is required to evoke the bioactivity. HeLa cells enabled the read-out of the reaction, which proceeded rapidly when it is performed on matched RNA templates.

Introduction

According to a fascinating proposal, RNA molecules overexpressed in diseased cells could be hijacked and used as templates in the RNA-directed synthesis of bioactive molecules.[1-4] For example, it is envisioned that RNA-templated reactions could lead to cell toxic molecules, which would be formed in and, therefore, act only on those cells characterized by a disease-type gene expression pattern. This proposal relies on the availability of nucleic acid-programmed chemical reactions that proceed in complex environments. A diverse set of potentially useful templated chemistries has been developed for potential applications in nucleic acid diagnosis and reaction/drug discovery.[5-29]

In a few reports, nucleic acid templated chemical reactions have been tested with the aim to explore options for the *in situ* perturbation of biological processes. Taylor *et al.* developed a model system, in which a DNA-triggered hydrolysis reaction led to the release of p-nitrophenol.[1] Template triggered reactions were used to unleash metal complexes[30] upon hydrolysis or to liberate coumarin[31] or estradiol[32] through Staudinger reduction. Recently, Abe *et al.* demonstrated that templated Staudinger reactions allowed the activation of gene expression by means of rRNA-triggered liberation of isopropyl-β-D-thiogalactoside (IPTG).[33] In addition, hybridization triggered strand exchange promoted the unquenching of DNA-linked photosensitizers, which were capable of releasing biotin[34] or producing singlet oxygen in living cells[35]. We assumed that nucleic acid-triggered bond forming reactions would eliminate the possibility for undesired release caused by biodegradation and therefore provide high target specificity.

Our model reaction system involved the DNA-triggered transfer of an α-aminoacyl group onto a tripeptide-PNA conjugate.[4, 36] The reaction proceeded in cell lysate and the tetrapeptide conjugate formed was capable of rescuing inhibited caspase-9 in cell lysates. The study proved the feasibility of DNA-templated aminoacyl transfer in complex environments. However, while we took it for granted that peptide bond formation would proceed also on RNA templates, it remained unclear whether the reaction extends to the transfer of larger peptidyl units. In the previous reports, DNA was used as a trigger of the synthesis of rather small peptides (≤ 6 aa).[4, 36-38] Yet, peptidyl transfer rather than aminoacyl transfer would be required to access peptides of a length (> 10 aa) sufficient to enable high affinity interactions with biological targets. Another difficulty arises from the difference in concentrations of the RNA template and the protein molecules targeted by a drug or drug-like compound. Typically, proteins are expressed in higher copy numbers than mRNA. In this study, we considered the necessity for the development of a catalytic reaction system, wherein one RNA molecule would promote the formation of many product molecules. To provide unambiguous evidence for template catalysis, we developed a reaction system, in which turnover in an exogeneously added RNA template is required to evoke the bioactivity of the reaction product. We demonstrate the catalytic effectiveness of the exogeneously added RNA template, which instructed the synthesis of a cell penetrating, cationic peptide-PNA conjugate. We show that HeLa cells enable a turnover-specific read-out.

Results and discussion

Our reaction design involved the RNA-triggered transfer of a peptidyl fragment from a thioester-linked PNA conjugate to the N-terminal cysteine of a peptide-PNA acceptor (Fig. 1).

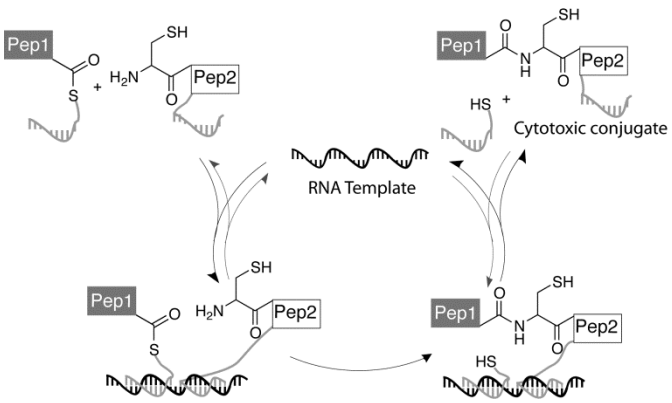

Fig. 1 RNA-promoted synthesis of a cytotoxic peptide-PNA conjugate.

According to the mechanism of native chemical ligation,[39] the proximity-enhanced thiol exchange will be followed by an S→N acyl shift, which will provide the bioactive peptide. The peptide product formed upon the RNA-triggered reaction was envisaged to serve as an inhibitor of cancer cell proliferation. We selected the 14-mer cationic amphipathic $[KLAKLAK]_2$ peptide. Once inside the cells, this peptide displays cytotoxic properties *in vitro* as well as *in vivo* by disrupting the mitochondrial membrane and triggering apoptosis.[40-43] In order to reach its target, the peptide requires the aid of a cell penetrating agent such as oligoarginine, penetratin or Tat-derived peptides. However, the RNA template added will mask the positive charge required to confer the bioactivity. Therefore, only those reaction systems that enable turnover in template will show cytotoxic effects. Because we avoided potential problems caused by differential cellular uptake and/or RNA accessibility the cytotoxic effects observed in this model study will directly reflect the specificity and the catalytic effect of the RNA-programmed peptide synthesis.

The synthetic approach relied upon the native chemical ligation and required the mutation of a residue of the $[KLAKLAK]_2$ sequence to a cysteine. We performed a cysteine scan in order to identify suitable positions for cysteine placements in the oligoargine-modified KLAKLAK peptide **1**. Cell proliferation inhibition assays performed with two different cell lines, HeLa (Fig. 2) and HepG2 (Fig. S46), revealed a high cytotoxic activity for the A→C and K→C mutants **3**, **4**, **6**, **7** and **8**. Control experiments showed that both the $[KLAKLAK]_2$ segment and the cell penetrating oligoarginine unit were required for cytotoxicity (Fig. S47A). We next evaluated the cysteinyl fragments **10**-**17**. Amongst the peptides tested **13**, **15** and **17** were least active in preventing cell proliferation. The cell proliferation assays suggested that the reactions **13**→**5** and **15**→**7** would offer the highest gain of cytotoxicity upon transfer of four (KLAK) and six (KLAKLA) residues, respectively.

We next explored the RNA-controlled peptidyl transfer reactions. The RNA target corresponded to a segment of mRNA coding for the X-linked inhibitor of apoptosis protein (XIAP), which is overexpressed in the case of prostate, pancreatic, gastric, ovarian, lung, breast cancers.[44] The

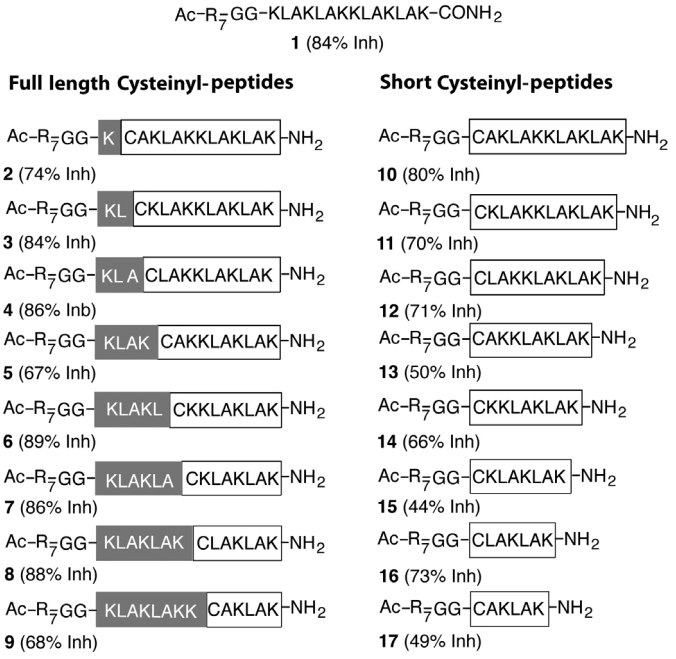

Fig. 2 Native sequence of the peptide $[KLAKLAK]_2$ and the cysteine scan. The values in brackets specify to which extent proliferation of HeLa cells is inhibited after 16 h incubation with 100 μM peptide in DMEM buffer. Cell proliferation inhibition was assessed by means of the MTS assay (see Suppl. Inf.)

thioester-linked tetrapeptide- and hexapeptide-donor conjugates **22** and **24** were allowed to react with the decapeptide and octapeptide acceptors **23** and **25**, respectively (Fig. 3). For comparison, we also studied the transfer of a single lysine residue to the tridecapeptide acceptor **21**. The reactions were performed in absence or on stoichiometric RNA template and were analyzed by means of HPLC/MS and MALDI-TOF-MS

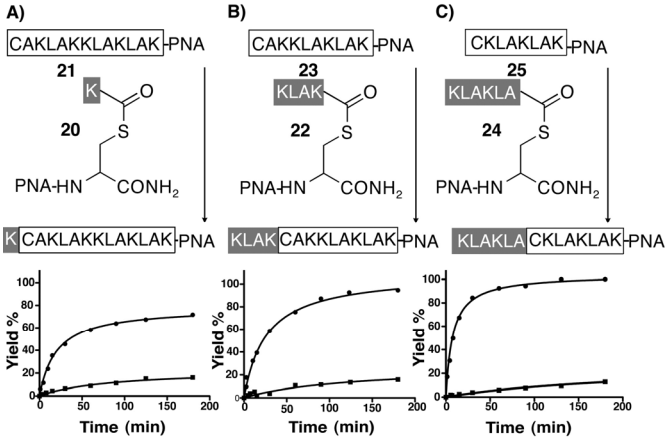

Fig. 3. Time course of product formation in transfer reactions in absence (square) or in presence of 1 equiv template (circle). Conditions: 10 μM thioester peptide-PNA conjugate (**20**, **22** or **24**), 5 μM Cys-peptide-PNA conjugate (**21**, **23** or **24**) in buffer (10 mM MOPS, 200 mM NaCl, 0.2 mM TCEP, 0.2 mg·L^{-1} RiboLock, pH 7.0, 37 ºC. XIAP RNA: 5'-GAUAGAUGGC AAUAUGGAGAC-UCAGCAGUUGGAA -3'. PNA sequence for **20**, **22** and **24** = gctgagt and PNA sequence for **21**, **23** and **25** = catattgccatc-Gly.

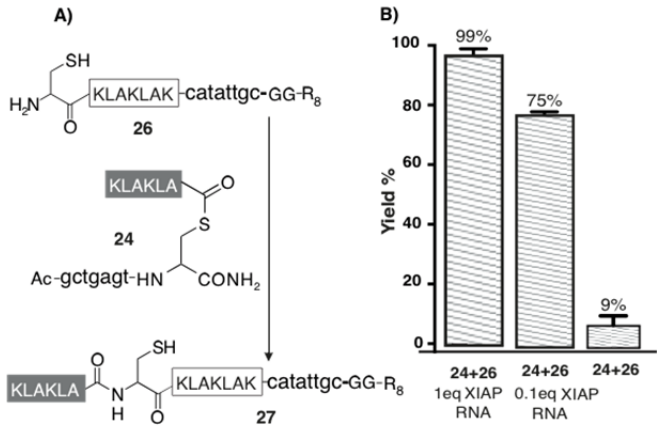

Fig. 4 A) RNA-triggered peptidyl transfer reaction between **24** and **26** under turnover conditions. B) Yield of transfer product **27** after 3 h reaction at varied template loads. Conditions: see Fig. 3, 10 μM **24**, 5 μM **26**.

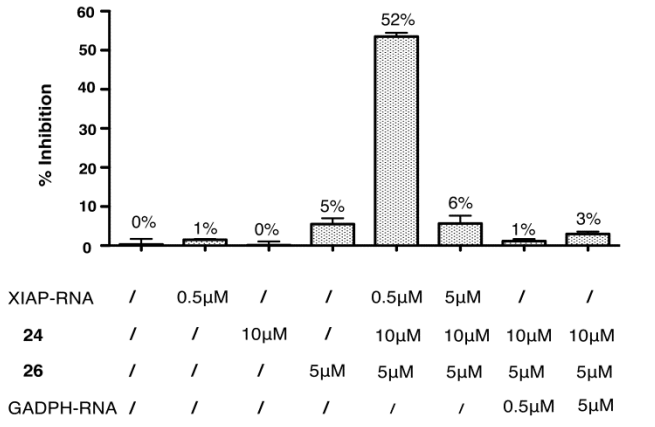

Fig. 5 Inhibition of cell proliferation after conducting the peptidyl transfer reaction at varied loads of RNA template and control experiments. Conditions: Reaction of 70 μM **24** or/and 35 μM **26** in 70 mM MOPS, 200 mM NaCl, 0.4 mM TCEP, 0.2 mg·L^{-1} RiboLock, pH 7.0, 37 °C for 3 h on 35 μM or 3.5 μM template (XIAP RNA: 5'-GAUAGAUGGCAAUAUGGAGACUCAG-CAGUUGGA-A-3'; GAPDH RNA : 5'-CCGUCAAGGCUGAG-AACGGGAAGCUUGUCAUCAA-3'), then dilution (7-fold) in DMEM cell media and 16 h incubation with HeLa cells. Cell viability was determined by the MTS assay.

measurements. As expected, low product yields were obtained in absence of template. The addition of RNA template conferred significant rate enhancements and the products were formed in 55-92 % yield after only one hour. Of note, the efficiency of the reaction increased with increasing length of the transferred peptidyl fragment (30 min: K transfer, 45 %; KLAK transfer, 58 %; KLAKLA, 84 %). The data suggested that the C-terminal amino acid of the thioester and the length of the acceptor influence the rates to a larger extent than the length of the transferred peptide. These results are in line with previous reports on native chemical ligation in protein synthesis,[45] in which alanine (**24**) showed higher thioester reactivity than lysine (**22** and **20**) and acylated amino acids (**20**) higher reactivity than non-acylated aminoacids (**22**). Furthermore, the high flexibility provided by long acceptor peptides such as **21** may affect the template-induced enhancements of the effective concentration. We concluded that the length of the transferred peptidyl fragment is not decisive in the search for suitable ligation sites used in RNA-directed peptide synthesis.

The most reactive donor/acceptor pair **24**/**25** was selected for the development of a reaction system that allowed for turnover in template. The oligoarginine was appended to the PNA conjugate bearing the full length peptide after reaction. To enable strand exchange at the reaction conditions (37 °C) the length of the PNA arms was reduced in acceptor **26** (Fig. 4). The reaction between **24** and **26** on stoichiometric amounts of RNA proceeded in analogy to the reaction with reactive conjugates **24** and **25** lacking the cell penetrating oligoarginine unit. Product **27** was formed in 99 % yield within 60 minutes upon addition of template. Importantly, the reaction system provided turnover; 75 % product was obtained after 180 min reaction on 0.1 equivalent template. Thus, the concentration of product molecules exceeded the concentration of the RNA template by a factor of 7.

We next examined whether the RNA-induced formation of the cytotoxic peptide-PNA conjugate **27** allowed inhibition of cell proliferation. The reaction was performed for 180 min and the mixture added to the cell culture media without intermediary purification steps. The data from the cytotoxicity assays showed that a 2.7 μM concentration of transfer product inhibits proliferation of HeLa cells by 62 % (Fig. S48). Yet, little cytotoxicity was observed when the cells were exposed to a mixture from the reaction of precursors **24** and **26** (at 10 and 5 μM final concentration, respectively) on 1 equivalent RNA template (Fig. 5). Control experiments revealed a low cytotoxicity for 1:1 product-RNA complexes (Fig. S48). To avoid capture of the cytotoxic peptide by RNA template the reaction must proceed with turnover in template. Indeed, cell proliferation was inhibited by more than 50 % when the reaction was performed on 0.1 equivalent RNA template. The RNA alone showed no cytotoxicity. Of note, RNA-promoted peptidyl transfer proved sequence specific (Fig. 5). Cell proliferation was virtually unaffected if RNA corresponding to housekeeping GAPDH was added to precursors **24** and **26**.

Conclusions

In summary, we have demonstrated the RNA-programmed synthesis of a cell penetrating $(KLAKLAK)_2$-PNA conjugate. The reaction was performed outside cells. The reaction mixture proved able to inhibit the proliferation of cancer cells and the cytotoxic product was formed under the condition that a specific RNA molecule was present. At this stage of research we explored an exogenously added RNA target. Although the discovery of free circulating nucleic acids (CNAPS) as tumor biomarkers in plasma, blood and serum suggests the significance of extracellular RNA templates,[46, 47] it is the ambition to use cell endogenous RNA as template. Prior to the envisaged use of intracellular targets, several challenges need to be addressed. In this study, we focussed on the concentration issue. Unless ribosomal RNA would be targeted, the concentrations of the RNA template would be significantly lower than concentration of the target of the drug-like molecule

formed upon the RNA-templated reaction. We showed that turnover in template provides a means for product amplification so that the concentration of the product molecules exceeds the concentration of the RNA instructor. In addition to the concentration issue, cellular delivery and template accessibility present major hurdles. The previous reports on RNA imaging[48-50] and RNA directed therapy[51] with peptide-PNA conjugates encourage future work aimed at RNA-promoted peptidyl transfer in live cells.

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Notes and references

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