DNA-Directed Synthesis

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DNA-Triggered Synthesis and Bioactivity of Proapoptotic Peptides**

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Diseases are frequently caused by changes in the genetic infrastructure. In such cases, the disordered state of a diseased cell is encoded in the DNA and reflected in the level and sequence of the expressed RNA molecules. The information obtained from nucleic acids may be used to direct molecular therapies only to diseased cells and tissues. In a fascinating approach, disease-specific nucleic acid sequences could be hijacked to trigger the formation or release of drug molecules.[1]

Recently, it was shown that nucleic acid hybridization processes can control the release of model drugs such as *p*-nitrophenol, coumarin,[2] biotin, or benzoic acid.[3] Gothelf, Mokhir, and co-workers introduced the DNA-programmed control of singlet oxygen production by pyropheophorbide.[4] The reported release systems relied upon ester cleavage or DNA-strand exchange. We assumed that the synthesis of bioactive agents by the creation of covalent bonds triggered by nucleic acids should provide improved specificity, because the undesired release of the active agent through esterase- or nuclease-induced cleavage reactions is avoided. Template-directed bond-forming reactions have been used in diagnostics,[5,6] as imaging tools,[7] to install DNA/RNA modifications,[8] and as a potential tool to facilitate the screening of druglike molecules.[9] In general, stoichiometric amounts of nucleic acid templates are required to drive the reaction to completion. This limits applications to templates of high abundance. However, none of the published reaction systems has, to the best of our knowledge, been used to transduce nucleic acid information into the generation of agents that—while they are being formed—inhibit or activate disease-related protein targets.

Herein we introduce a reaction system in which the sequence information of an unstructured DNA template (**Ma** in Figure 1) is used to trigger the transfer of an aminoacyl group from a donating thioester-modified peptide–nucleic acid (PNA) conjugate **1** to an acceptor peptidyl–PNA conjugate **2**. We demonstrate that the template can act as a catalyst which instructs the formation of many product molecules per template molecule. The formed peptide–PNA conjugate **3** was designed to interfere with the protein–protein interactions between caspase-9, a protease involved in the initiation of programmed cell death (apoptosis), and the X-linked inhibitor of apoptosis protein XIAP. It is shown that the nucleic acid programmed peptide synthesis allows activation of caspase-9 and a downstream caspase.

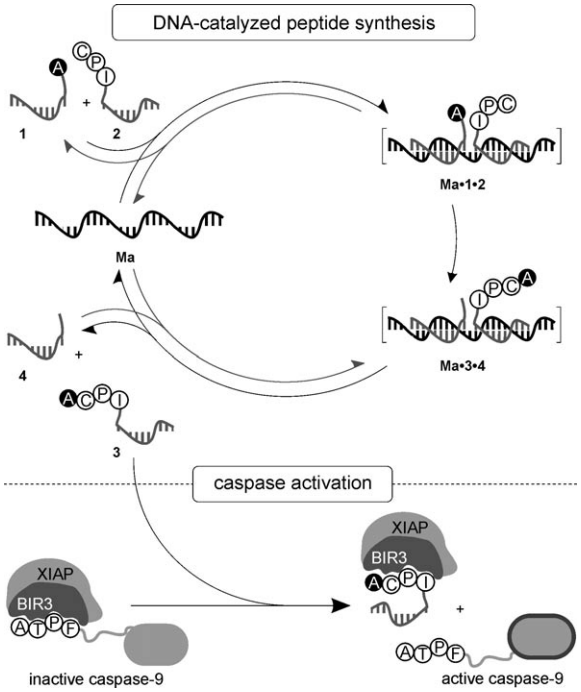

Figure 1. Top: A nucleic acid catalyzed aminoacyl transfer reaction leads to the formation of peptide–PNA conjugate **3**. Bottom: Transfer product **3** disrupts the caspase-9–XIAP interaction and activates caspase-9.

XIAP is frequently overexpressed in tumor cells and confers resistance to cancer cells against apoptotic stimuli.[10,11] The BIR3 domain of XIAP binds to the small subunit of caspase-9 and, therefore, prevents the formation of active caspase-9 (Figure 1, bottom).[12] Smac (second mitochondria-derived activator of caspases), which is released from mitochondria after proapoptotic stimuli, acts as an antagonist of XIAP. Smac shows the same interaction pattern with the BIR3 domain of XIAP as the small subunit of caspase-9 and releases the caspase by direct competition.[13] This binding of Smac is only mediated by the four N-terminal residues (Ala-Val-Pro-Ile), and it has been shown that short Smac-derived peptides disrupt the interaction of caspases with XIAP and sensitize different tumor cell lines to apoptosis induced by a variety of proapoptotic drugs in vivo and in vitro.[11,14]

We designed a template-controlled peptide-coupling reaction that provides a peptide product that harbors the N-terminal tetrapeptide motif required for recognition of the BIR3 domain of XIAP (Figure 1). This involved the amino-

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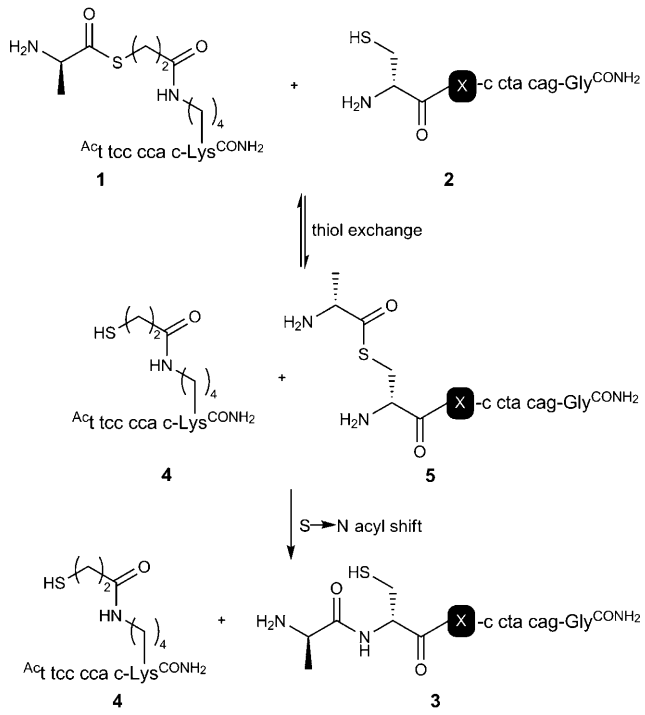
acyl donor probe **1**, in which the essential N-terminal alanine residue was attached to a PNA oligomer through a thioester linkage (Scheme 1a). The peptidyl–PNA conjugate **2** served as the acceptor in an acyl transfer reaction. The N-terminal cysteine was introduced to provide high reaction rates in a native chemical ligation-like mechanism.[15] The two short PNA oligomers **1** and **2** will bind adjacently to a complementary DNA template **Ma**. This DNA section contains the sequence information of the carcinogenic G12V point mutation of the signal transduction protein Ras. The DNA sequence of **Mi** codes for the wild-type protein (Scheme 1b). The adjacent alignment of reactive groups will accelerate a thiol exchange reaction. The formed thioester intermediate **5** undergoes a virtually irreversible S→N acyl shift, which generates the native peptide bond in the elongated peptide product **3**. The reaction products **3** and **4** offer the same number of nucleotides for template recognition as the reactants **1** and **2**. Thus, the reaction may be performed under conditions of dynamic strand exchange, whereby the template acts as a catalyst.

a)



b)

Template:

5'-GCGCTGXAGGTGTGGGGAAGAGT-3' **Ma** (X = T); **Mi** (X = G)

Accepting peptide-PNA conjugates:

2a: X = Pro-Ile-Ala-Gln-Lys

2b: X = Pro-Ile-AEEA

Transfer products:

3a: X = Pro-Ile-Ala-Gln-Lys

3b: X = Pro-Ile-AEEA

Scheme 1. a) Cysteine-mediated transfer of alanine. b) DNA templates and peptide–PNA conjugates used for DNA-catalyzed peptide synthesis. AEEA = [2-(2-aminoethoxy)ethoxy]acetyl.

The efficiency of the DNA-triggered peptide synthesis was assessed by using the peptidyl–PNA conjugates **2a** and **2b** as acceptors (Scheme 1b). The use of PNA facilitates the synthesis of reactive peptide conjugates because both the peptide and the PNA part can be assembled during a single solid-phase synthesis. The reaction products **3a** and **3b** contain the tetrapeptide motif required for binding to the BIR3 domain of XIAP. Acceptor **2a** offers the [C2V] variant of a Smac-derived hexapeptide (Smac(2–7)) for alanine transfer, while in **2b** a tripeptide hangs by a flexible AEEA tether. Both reactions succeeded in the presence of the matched template and furnished **3a** or **3b** in more than 60% yield after only 30 minutes (Figure 2a). Only trace amounts of products (0.5%) were formed in the absence of the template. The template **Ma** conferred a 250-fold increase in the initial transfer rate when conjugate **2b** was used compared to a 156-fold increase with the hexapeptidyl–PNA conjugate **2a** (Table 1). The rate acceleration is thought to be an important parameter which would critically affect the specificity of the nucleic acid encoded synthesis of drugs.

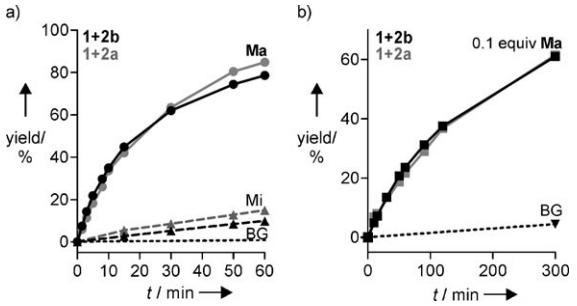


Figure 2. Time courses of the transfer reaction between **1** and **2a** or **2b** in the presence of a) stoichiometric and b) substoichiometric (0.1 equiv) concentrations of the complementary template **Ma** (solid lines), in the presence of the mismatch template **Mi** (dashed lines), and in the absence of DNA template (dotted line). Conditions: 1.5 μM **1**, 0.75 μM **2a** and **2b**, 0.75 μM or 0.075 μM template (when added) in 10 mM NaH_2PO_4, 200 mM NaCl, 0.2 mM TCEP, pH 7.0, 25 °C. BG = background, TCEP = tris(2-carboxyethyl)phosphine.

Table 1: Initial rates, rate acceleration in the presence of DNA template, selectivity towards a single nucleotide mismatch, and the corresponding product yields for the template-directed transfer of Ala to **2a** and **2b**.[a]

	1+**2a**→**3a**	**1**+**2b**→**3b**
initial rate [pM s^{-1}]		
complementary DNA (1 equiv)	406	461
background	2.6	1.9
single base mismatch DNA (1 equiv)	24.6	16.7
rate acceleration	156	250
selectivity	16.5	27.6
yield (1 h)		
complementary DNA (1 equiv)	85%	79%
complementary DNA (0.1 equiv)	22%	24%
background	<1%[b]	<1%[b]

[a] Conditions: see Figure 2. [b] Calculated from the initial rate.

We tested the sequence specificity of the DNA-programmed synthesis by investigating transfer reactions on the single mismatched DNA sequence **Mi** (Ras wild-type sequence). The transfer reactions proved inefficient with **Mi**. Interestingly, the tripeptidyl–spacer–PNA **2b** allowed more-selective alanine transfer than the hexapeptidyl–PNA conjugate **2a** (Table 1), which suggested that the tethering of the accepting cysteine residue through flexible linkers can lead to more efficient nucleic acid controlled aminoacyl transfer reactions. We challenged the transfer reaction by adding only 0.1 equivalents of template **Ma** to the aminoacyl donor **1** and acceptors **2a** or **2b**. Both reactions furnished more than 20% transfer product after 1 h (Table 1) and more than 60% transfer product after 5 h (Figure 2b). This finding and the low yields obtained in the absence of the templates proved the catalytic turnover of the reactants.

The resulting peptide–PNA conjugates **3a** or **3b** should have a similar affinity for the XIAP BIR3 domain as the mimicked Smac peptide. Thus, peptide–PNA conjugates such as **3a** or **3b** should likewise be able to relieve the apoptosis break caused by the interaction between XIAP and caspase-9 by trapping the XIAP. A homogeneous binding assay (see Figures S10 and S11 in the Supporting Information)[16] revealed IC_{50} values for **3b** (0.56 μM) and **3a** (1.52 μM) which were in the range of the IC_{50} values for the Smac peptide AVPIAQKSE (0.40 μM) and ACPI (0.65 μM).[17] We concluded that the PNA tag has little influence on the affinity of the peptides. Importantly, the PNA-bound peptide fragments **1**, **2a**, and **2b** did not show a measurable affinity for the BIR3 domain (see Figure S10 in the Supporting Information).

The results of the binding assay suggested that it should be possible to displace reference peptide **6** by in situ generated peptide–PNA conjugates **3a** or **3b**. The transfer experiments were carried out in the presence of the XIAP BIR3 protein and the reference peptide **6** (Figure 3a). The Smac-like peptide–PNA conjugates should only be formed and compete with the fluorescent probe in the presence of a complementary DNA template. Displacement of the reference peptide **6** models the dissociation of the caspase-9·XIAP complex. In the attempted reaction of **1** and **2b** in the absence of DNA **Ma**, nearly 100% of **6** remained bound to BIR3 (Figure 3b). The same result was obtained when the transfer reaction was performed in the presence of the single mismatched DNA template **Mi**. The presence of one equivalent of complementary DNA template **Ma** triggered the formation of transfer product **3b**, which liberated 45% of **6** within 30 minutes when 2 μM of nucleophile **2b**, 4 μM of thioester **1**, and 2 μM template **Ma** were added. Interestingly, the use of a substoichiometric amount of the template (1 μM) resulted in a 35% displacement of **6**. This observation and the lack of displacement in the template-only controls indicated turnover of the template. Increasing the amount of the reactive probes to 8 μM **1** and 4 μM **2b** furnished increasing concentrations of transfer product **3b** and led to 62% liberation of **6**. Further increases in the reactive probe concentration are not useful, because the template-independent reaction would result in background reactions occurring.

We next wanted to demonstrate that the DNA-catalyzed peptide synthesis also succeeds within complex biomacromolecular environments and releases the "apoptosis break" by antagonizing the interaction between the XIAP BIR3 domain and caspase-9. The proteolytic activity of caspase-9 or caspase-3 in lysate was measured by assaying the cleavage of the fluorogenic peptide substrates Ac-LEHD-AFC and Ac-DEVD-AFC (AFC, 7-amino-4-trifluoromethylcoumarin), respectively.[18] As expected, the BIR3 protein inhibited the caspase-9 activity dose-dependently (Figure S12 in the Supporting Information), despite activation by cytochrome *c* and ATP. We found that the addition of increasing amounts of transfer products **3a** or **3b** also rescued the BIR3-mediated inhibition of caspase-9 activity in a dose-dependent manner (Figure S13 in the Supporting Information). The next aim was to show that the rescue of caspase-9 activity can be achieved by means of the DNA-directed reaction. In the restoration assay, the activity of caspase-9 was inhibited by 50 nM BIR3, which was added to emulate the overexpression of XIAP in tumors (Figure 4). The reaction between **1** and **2a** as well as between **1** and **2b** was performed in the presence of matched or mismatched DNA or in the absence of the DNA template. Interestingly, 27% (20% for the reaction of **1** with **2b**) of the caspase-9 activity was restored in the reaction between **1** and **2a** when complementary DNA **Ma** was added. The caspase-9 activity in cell lysate was not increased in the absence of DNA or in the presence of mismatched DNA (Figure 4a).

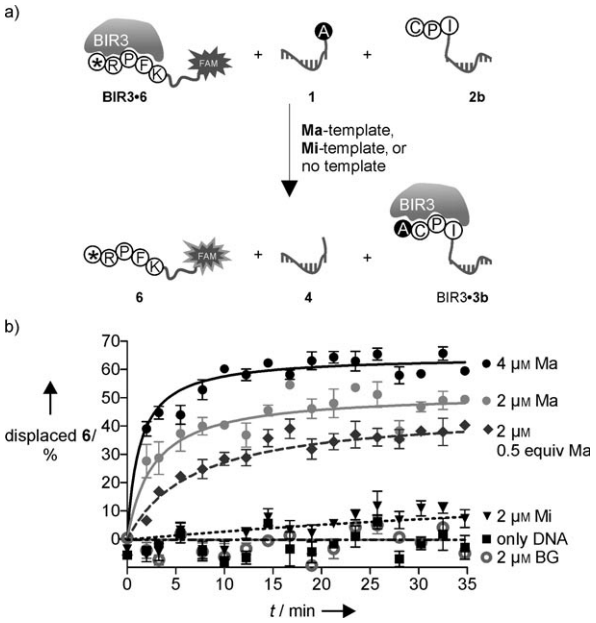


Figure 3. a) Schematic representation of the fluorescence polarization assay. The formation of **3b** leads to displacement of reference peptide **6** from the BIR3 domain and change of the fluorescence polarization. b) Displacement of reference peptide **6** during a template-catalyzed transfer reaction in the presence of BIR3 protein. Conditions: 5 nM **6** and 230 nM BIR3 in 100 mM NaH_2PO_4, 10 mM NaCl, 10 mM $MgCl_2$, 0.2 mM TCEP, 0.1 mM spermine, 0.1 mg mL^{-1} bovine serum albumin, pH 7.5, 25 °C; addition of **2b** in 2–4 μM concentration, 2 equiv **1**, 1 equiv or 0.5 equiv **Ma** and 1 equiv **Mi**, or without DNA. Normalized averages over three measurements are shown; error bars depict the standard error of the mean. * = 2-aminobutyric acid.

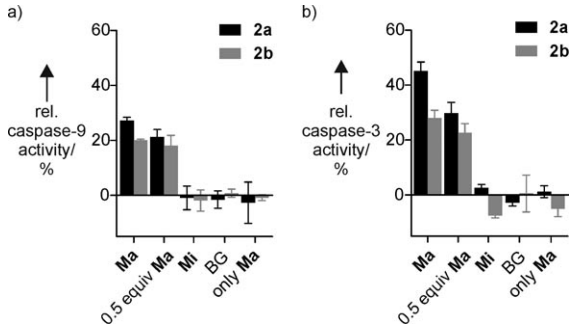


Figure 4. Reactivation of a) caspase-9 and b) caspase-3 by conducting the transfer reaction in cell lysate incubated with 50 nM BIR3. Conditions: 20 mM HEPES-KOH, 50 mM KCl, 5 mM ethylenediaminetetraacetate, 2 mM $MgCl_2$, 0.05% CHAPS, 0.2 mM TCEP, pH 7.4 with 10 μM cyctochrome *c* and 2 mM ATP, 50 nM BIR3 when added. **1** = 4 μM; **2a**,**b** = 2 μM; 1 equiv or 0.5 equiv **Ma**, and 1 equiv **Mi** or without DNA. Normalized means over four measurements are shown; error bars depict the standard error of the mean. ATP = adenosine triphosphate, CHAPS = 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, HEPES = 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid.

We next determined the activity of the downstream caspase-3. The addition of reactive conjugates **1** and **2a** or **2b** had no effect on the activity of caspase-3 (see background (BG) in Figure 4b). However, the addition of the complementary DNA **Ma** led to a 45% rescue of the caspase-3 activity in the reaction of **1** with **2a** (28% in the reaction of **1** with **2b**). The use of substoichiometric amounts of the template (0.5 equiv) led to 30% and 23% restoration. The transfer reaction proceeded sequence-specifically, as inferred from the lack of caspase-3 activation when the single mismatched template **Mi** was added (Figure 4b).

The collected data shows that DNA–PNA recognition can be used to trigger template-controlled aminoacyl transfer reactions even within a complex biomolecular environment, such as in cell lysates. The reaction system bears a resemblance to the protein biosynthesis machinery; the DNA template mimics mRNA while the PNA–peptide conjugates such as **1** mimic aminoacyl–tRNA, wherein PNA or tRNA is the adaptor that translates nucleic acid information into protein output. The in situ formed peptide conjugates acted as enzyme activators rather than inhibitors. Although small-molecule-induced activation is less frequently applied than inhibition in chemical biology, this approach provides distinct advantages because usually only small amounts of active enzyme are required to drive biological processes.[19]

Systems that are capable of a) analyzing the RNA expression of a cell and b) using this information for the control of protein function would provide fascinating opportunities for selective as well as personalized medicine. The systems proposed so far relied on nucleic acid molecules that read nucleic acid information and act, again, on nucleic acid molecules, for example, by means of antisense or RNAi effects.[20] We have introduced herein a different approach: The use of conjugates of nucleic acids and peptides enables a controlled interference with protein–protein interactions. This approach may have great potential because the majority of the available drugs act on protein rather than on nucleic acid targets. Previously, we reported that transfer of a reporter group succeeded on both DNA and RNA templates.[6] This finding and the experiments within the reducing environment of cell lysates encourage applications in living cells. However, the cellular delivery of the reactive probes will be a major challenge. Intensive research is currently being carried out to solve this problem by means of small-molecule-, protein-, lipid-, or nanotransporter-based delivery systems.[21]

In conclusion, we have demonstrated a reaction in which DNA triggers and even catalyzes the transfer of an aminoacyl group from a donating thioester-linked PNA–peptide hybrid to a peptide–PNA acceptor. The reaction proceeded sequence-specifically and enabled turnover in the template. We showed that in situ generated transfer products bind the BIR3 domain of XIAP. The PNA tag has no influence on the binding affinity. In a cell-free functional assay, which included recombinant BIR3 protein and cell lysate, the formed peptide–PNA conjugates acted as XIAP antagonists and allowed reactivation of inhibited initiator caspase-9 as well as of the executioner caspase-3. Future work will be directed to an extension of this concept to other bioactive peptides and intracellular targets.

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