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Model Systems for Activation of Nucleic Acid Encoded Prodrugs

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The development of more selective chemotherapeutic agents for benign treatments of malicious diseases is highly desirable. In recent years model systems for the release of small molecule drugs from nucleic acid conjugates by templated chemical or photochemical reactions have been designed. Common for these systems is that the stoichiometric or catalytic drug release is controlled by the highly selective hybridization between complementary strands of nucleic acids. Herein, the concepts of the new field of nucleic acid templated release reactions are outlined.

Introduction

The concept of encoded prodrugs that are activated according to the individual genetic information in the diseased cell is considered a major aspiration in the area of chemotherapy.[1] In the ideal case, treatment with such drugs would not require detailed biochemical knowledge of the diseased cell, using only the genetic information per se, such that complications due to mutations in the target, resistance, or genetic differences between individual patients could be evaded by only changing the sensing part of the encoded drug. Other approaches rely on, for example, the targeting of a gene coding for a prodrug metabolizing enzyme (GDEPT),[2–3] or activation of prodrugs by exogenous enzymes delivered to tumor cells from DNA constructs containing the corresponding gene (ADEPT).[4] Prodrugs can be defined as agents that are transformed into pharmacologically active species after administration.

One of the major problems often encountered in chemotherapy is that there is usually little biochemical difference between, for example, a normal cell and a cancerous cell. Therefore, the genetic sensing part of the encoded prodrug will have to be of a highly selective nature and the obvious basis for this is the high selectivity present in the recognition between complementary strands of nucleic acids. DNA chip technology and genomic sequencing now allow the routine determination of the genetic compositions of diseases.[5] Specifically, a unique or overexpressed nucleic acid sequence in the diseased cell may serve as a template for the activation of a nucleic acid-conjugated prodrug, such that a cytotoxic molecule is generated or released inside the cell and kills it. The target should preferentially be abundant in the cell and readily accessible for Watson–Crick base pairing with the prodrug nucleic acid-probe and any additional components employed in prodrug activation. With respect to the question of choosing either DNA or RNA for targeting, the reader is directed to recent literature on the subject.[6] The delivery of such nucleic acid-conjugated prodrugs inside cells constitutes an additional challenge. Several drug delivery systems and more conventional methods have been used to deliver oligonucleotides across the cell membrane.[7] Furthermore, the complex chemistry occurring inside the cell may render the prodrug useless because of enzymatic degradation and unwanted background reaction in healthy cells. In this paper, we will concentrate on two strategies based on either a nucleic acid-templated chemical reaction or a nucleic acid-templated light-induced event that may be used for the release or generation of small molecule cytotoxic drugs.

Prodrug activation based on nucleic acid-templated catalytic reactions

Nature's approach to controlling fundamental biological processes, for example, transcription of DNA into RNA and translation of RNA into proteins, through nucleic acid-templated synthesis has in recent years been employed to control a variety of simple chemical reactions.[8] Whereas a considerable number of different DNA-directed reactions have thus far been demonstrated to work efficiently in vitro, few of these holds any potential for the activation of a prodrug, not possessing the required bioorthogonality and compatibility allowing them to be used in vivo in the presence of a plethora of cellular components and reactions.[9]

In the context of prodrug activation, the architecture of the nucleic acid-templated reaction will typically be A + B + A′B′, where A′B′ denotes the template/target (for example, cellular RNA), and A and B are the two oligonucleotides containing the reacting groups. It is desirable if hybridization of the reactants

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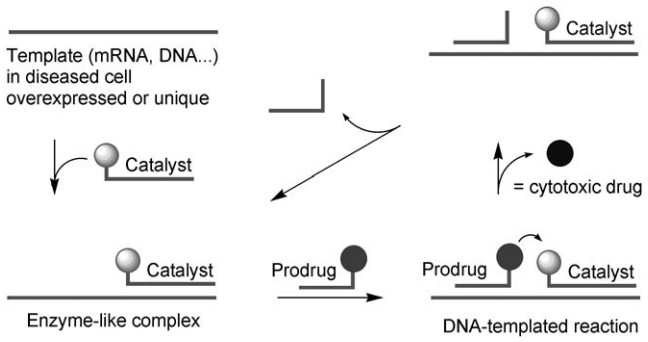
A and B to template A′B′ and the exchange of the products on the template with new reactants are both highly favored processes. In that case the template works in a catalytic fashion which is important as the cellular oligonucleotide target may be present in low numbers, although targets such as ribosomal RNA are usually more abundant. Further improvement can be obtained for a given signal output (release of a drug or fluorescence) if one of the functionalities bound to; for example, A serves a catalyst whereas sequence B contains the prodrug.

The general concept of nucleic acid-templated catalytic drug release as originally envisaged by Taylor and co-workers is outlined in Scheme 1.[10] In this concept, presence of the abundant



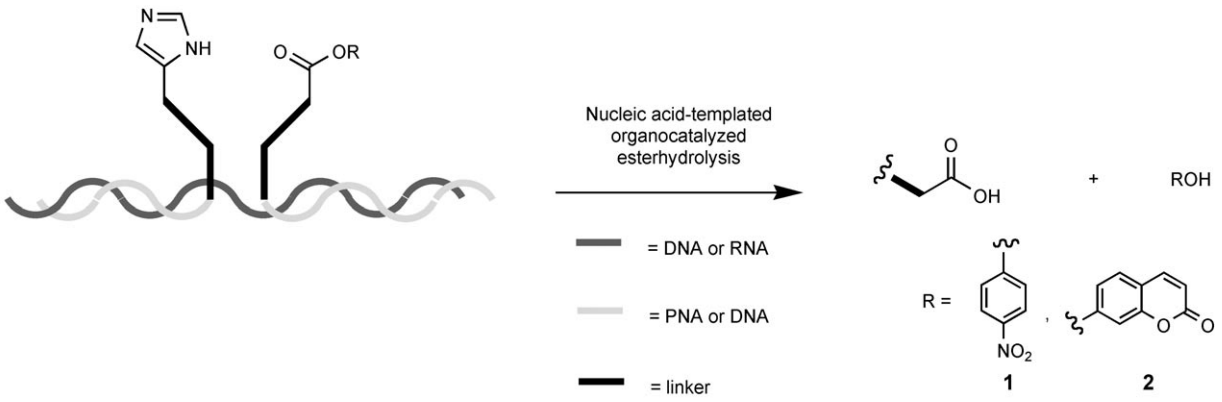
Scheme 1. Nucleic acid-templated catalytic drug release.

or unique nucleic acid template specific for the diseased cell can be used to tightly bind the catalyst component, giving rise to an enzyme-like complex. In turn, this complex can act as a template for a reversibly bound prodrug (masked drug linked to an oligonucleotide) bringing the catalyst and the reactive group of the prodrug in close proximity and thus leading to the nucleic acid-templated reaction and the release of the cytotoxic drug. As the two reactive strands are not ligated in the reaction, and the release of the prodrug should have minimal influence on the affinity of the prodrug oligonucleotide for the template, a catalytic effect with respect to the enzyme-like complex may be obtainable.

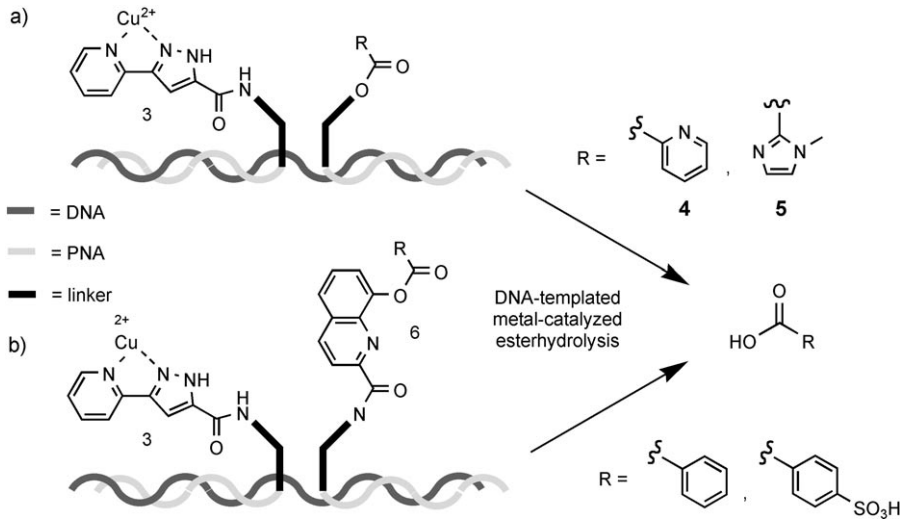
In the initial proof-of-principle, Taylor and co-workers utilized a prodrug system based on the release of a phenol by ester hydrolysis which was effectuated by an oligonucleotide-conjugated imidazole organocatalyst (Scheme 2).[10]

Two different prodrug models tested were a *p*-nitrophenyl ester (**1**) and a coumarin ester conjugated (**2**) to DNA and PNA, respectively. The release of *p*-nitrophenol or coumarin upon ester hydrolysis could be detected using UV-absorbance or fluorescence, respectively.[10,11] The release of a phenol is the key step in the activation of trimethylene lock-based prodrugs, for example in using Taxol derived prodrugs.[12] It was demonstrated that the catalyst-template complex indeed behaves like an enzyme and followed Michaelis–Menten kinetics. Single mismatches in the template resulted in only up to tenfold decrease in initial rate of drug release in the first reports using DNA-conjugated prodrug and catalyst components.[10] An improved model system using a PNA-conjugated prodrug with a modified prodrug linker and a PNA-conjugated catalyst resulted in a 23- and 30-fold decrease in initial rate with mismatch in either catalytic or prodrug binding site of the template, respectively. Kinetic data supported the view that the turnover rate was limited by the dissociation of the hydrolyzed prodrug component from the template. Therefore, optimization of prodrug sequence length may increase the overall catalytic efficiency of the system. As such, ester hydrolysis is not a bioorthogonal reaction, and the hydrolysis of the ester linkage in various prodrug components attached by different linkers was also found to proceed at a significant rate in human serum with a maximum half-life of 3 h. Hence, the system is expected to be of limited use in vivo, unless ester linkages considerably more resistant to hydrolysis by endogenous enzymes can be found.

Another related system that features catalytic DNA-templated ester hydrolysis using a Cu^{II}-complex as the catalyst has been reported by Kraemer and Mokhir.[13] Their previous work on copper-catalyzed carboxylic and phosphate ester cleavage led them to design a strongly Cu^{II}-chelating pyridylpyrazolyl moiety conjugated with PNA (Scheme 3).[14] In fact, the Cu^{2+}-PNA complex **3** could be detected by MALDI-TOF mass spectrometry. Common for the various ester PNA-conjugates tested in the reaction were the presence of a Cu^{2+}-anchoring site (for example, the pyridyl donor in **4**) which greatly facilitates hydrolysis compared to simple esters.



Scheme 2. Nucleic acid-templated organocatalyzed ester hydrolysis



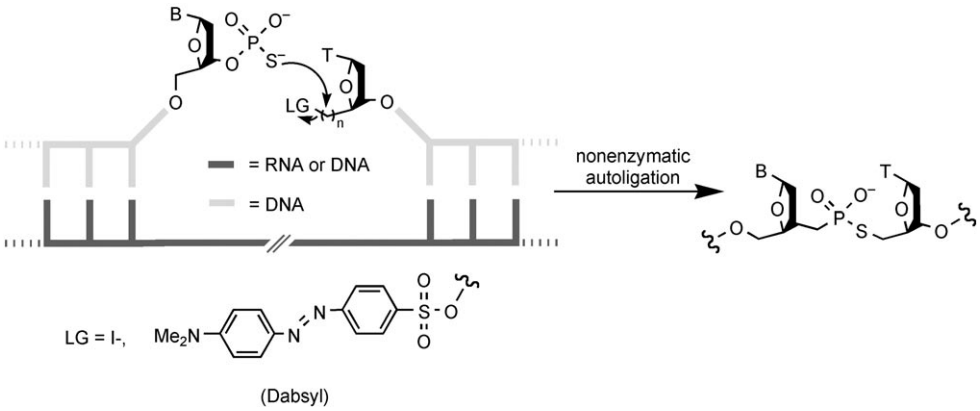
Scheme 3. DNA-templated metal-catalyzed esterhydrolysis (Kraemer and Mokhir).

Preliminary results using picolinic ester **4** afforded an initial rate 150-times higher in the presence of the template than in its absence, and 7- to 15-fold decrease in initial rate with single mismatches in the template (Scheme 3a). Background hydrolysis rates where generally high however, they could be reduced by optimizing the linker between the picolinic ester and PNA leading to up to 112-fold single mismatch discrimination. Substituting the 2-pyridyl group for the *N*-methyl-2-imidazoyl group in the ester (**5**) did not alter the reaction rate significantly. Notably, it was demonstrated that the DNA-templated hydrolysis is compatible with buffers used for PCR and a physiological buffer containing species that may compete with the Cu^{2+}-ion for binding to the *N*,*N*-ligand and inhibit catalysis. This proves the particular strong affinity of the Cu^{2+}-ion for the *N*,*N*-ligand. However, Cu^{II} is not available in cells. This limits application of the reaction in vivo.

A further development involved the use of a metal-cleavable linker based on a quinoloxy-2-carboxylic ester containing the ester group and attached to PNA as in **6** (Scheme 3b). The advantage of this system is that the anchoring effect in ester substituent R is no longer required, thus, the structural constrains on the ester group are decreased. In this system, approximately ten turnovers in the reaction could be detected and a single mismatch in the ester-template duplex decreases the initial rate fourfold, although the system possesses a rather high background rate of reaction.

Evidently, improved systems for prodrug activation through nucleic acid-templated reactions are desirable, as their use in vivo may be hampered by issues such as the high rate of background reaction and low stability of substrates.

Letsinger,[15] Seitz,[16] and Kool[17] have explored self-ligations (or "autoligations") of oligonucleotides, in which the reactive ends of two oligonucleotides are joined by a nucleic acid-templated reaction. All these self-ligations are based on displacement reactions which could potentially be used for drug release or activation as well. Some encouragement for this idea comes from the work by Kool which demonstrates the viability of self-ligations in living cells targeting rRNA.[17a–c] Kool has extensively explored the self-ligation reaction of terminal functionalized phosphothioate oligonucleotides with other terminal 5′-iodide or 5′-dabsyl functionalized oligonucleotide (Scheme 4). This has led to the development of the quenched autoligation probes (QUAL).[17f] In this setup, one oligonucleotide strand (probe) contains a terminally attached dabsyl quencher as leaving group together with a nearby fluorophore, and the fluorescence of the probe is therefore suppressed. Another probe contains the phosphothioate nucleophile. By hybridization to neighboring sites on the target (for example, rRNA), the displacement reaction ensues ligating the two oligonucleotide strands and causing the fluorescent probe to light up. Obviously, the fluorophore could be omitted and the quencher possibly substituted for a suitable drug, hence, resulting in drug release.



Scheme 4. Release of a dabsyl dye by self-ligation.

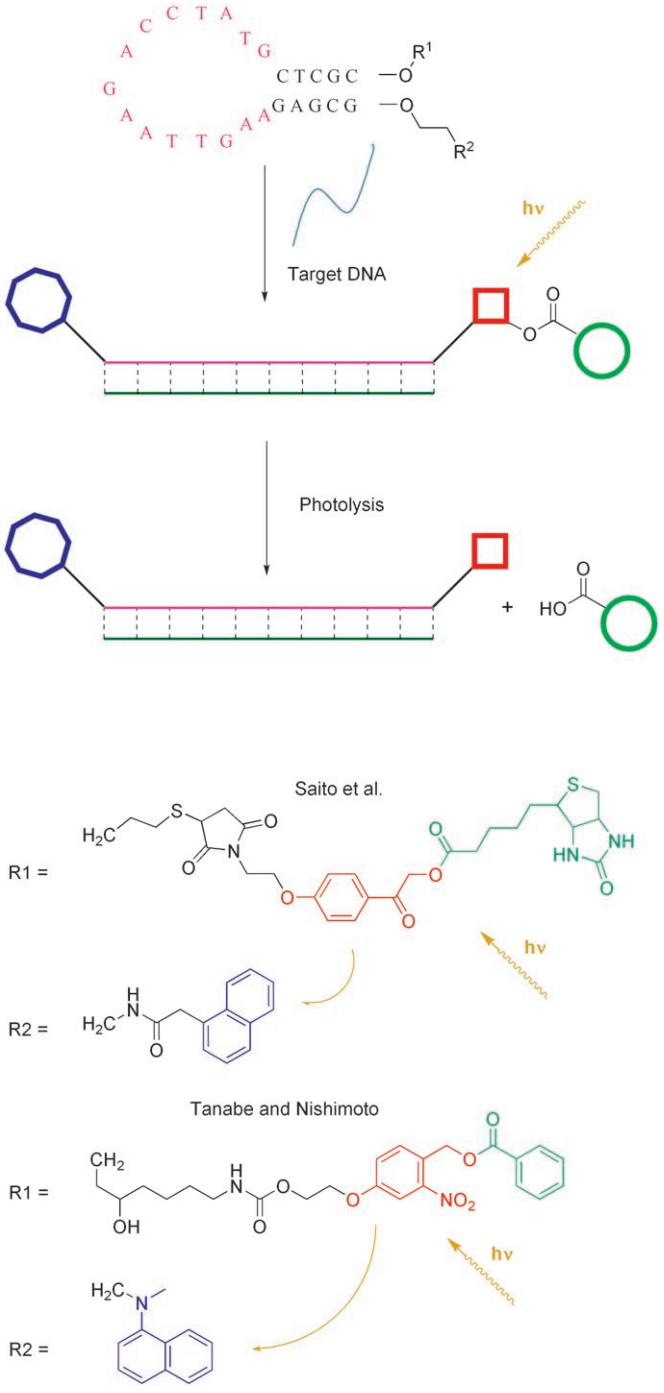
In addition to the already described nucleic-acid templated model drug release systems, several others could possibly be converted into systems capable of activating prodrugs.[6]

Photoinduced DNA templated prodrug activation

In this section we outline two types of light-responsive nucleic acid-based molecular architectures to pursue the controlled release of cytotoxic agents. The first relies upon the target-dependent photolysis of prodrugs whereas the second deals with the activation (that is, photosensitization) of molecular oxygen (prodrug) generating singlet oxygen (drug).

Photoremovable protecting groups (cage compounds) displace an appended molecular cargo in response to a specific light irradiation.[18] Saito and co-workers devised an active drug release system by combining the use of photoremovable protecting groups caging a model drug with oligonucleotide-based stem-loop type probes.[19] Similar oligonucleotide hairpin shaped probes, commonly addressed as molecular beacons,[20] are widely used fluorogenic reporters for gene analysis and consist of a short oligonucleotide strand (20–40 nt) with mutually complementary terminal domains (4–8 nt) each equipped with either a fluorophore or a dark quencher. In solution, in the absence of the strand complementary (that is, target) to the region bridging the termini (that is, probe domain) the molecular beacon acquires the stem-loop (hairpin) conformation holding the fluorophore/quencher pair in close proximity, hence greatly attenuating the fluorophores emission because of FRET and/or contact interactions between the two chromophores. In the presence of the target strand, the longer intermolecular target/probe hybrid forms, thus imposing conformational change to the stem-loop structure, which keeps fluorophore and quenchers apart from each other, and most importantly, allowing the fluorophore emission signal to be revived. Saito exploited a similar strategy to achieve a target-dependent photoinduced release of functional molecules (for example, a model drug). To this end, the model drug, namely biotin, was conjugated by an ester linkage to a phenacyl caging unit appended at the 3′ end of a molecular beacon (25 nt with a stem domain comprising 5 bp) whereas a naphthalene unit was selected as a triplet quencher and it was conjugated at the 5′ end, thereby creating a photoactive probe oligonucleotide (Scheme 5). Quantification of the biotin released by photolysis from the photoactive probe upon light irradiation at 312 nm revealed that the simultaneous presence of the complementary target strand gave rise to 84% yield of uncaged biotin in solution, as opposed to 12% yield obtained by the photoactive probe in a target-free solution. The authors ascribe the suppression of the biotin phototriggered release in the absence of the target strand to the efficient intramolecular quenching of the phenacyl ester triplet excited state operated by the naphthalene moiety in the probe stem-loop conformation, which accordingly prevents the photochemistry needed for the displacement of biotin.

In the same vein, Tanabe and Nishimoto later tested the phototriggered drug release efficiency of the same photoactive probe oligonucleotide containing a different cage/quencher



Scheme 5. Structures and mode of action of photoactive probe oligonucleotide (Saito, and Tanabe and Nishimoto).

pair.[21] In this instance the chosen caging moiety was an *o*-nitrobenzyl chromophore, which released benzoic acid as the model drug, whereas an aminonaphthalene derivative served as quencher (Scheme 5). However, the phototriggered release of the drug turned out to be much less selective in comparison to Saito's work. In fact, only a twofold increase of the concentration of uncaged benzoic acid was recorded after irradiation of the double stranded target/probe hybrid as opposed to the probe in the absence of the target. On the other hand,

testing carried out in the presence of target strand with a single mismatch or a fully noncomplementary strand did not reveal any significant enhancement of the photolysis compared to the probe in the stem-loop form.

The molecular hairpin-based strategies for phototriggered drug release presented above are based in a single component, and as such they are set structurally apart from the systems presented in the previous section, which require the presence of both prodrug and catalyst oligonucleotides. Accordingly, their internalization within a cellular environment could be potentially more straightforward as only one molecular adduct is involved in the process. Nevertheless, they lack a true catalytic nature; there is only a 1:1 stoichiometric ratio between the photoactive probe and the drug being released. Furthermore, any hydrolysis of the ester linkages in vivo by endogenous enzymes will add to the amount of drug release. Finally, it should be also considered that the wavelength of the light applied to trigger the drug release is in the UV region. Hence, it will not allow tissue penetration in the human body which prevents the proficient utilization of the photoactive probes inside organs of recondite location.

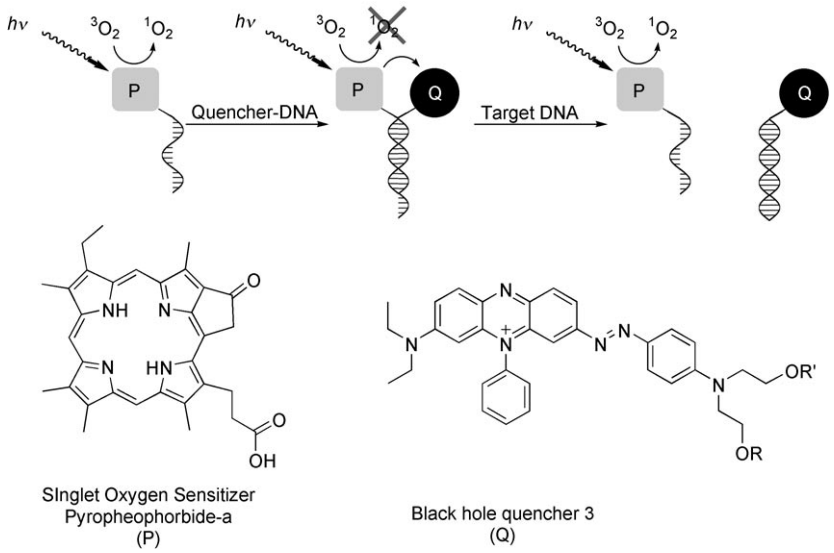
In a recent work by Gothelf et al., it was demonstrated for the first time that nucleic acid hybridization may serve to tightly control the photosensitized production of singlet oxygen ($^1O_2$) allowing for a specific nucleic acid domain (target) to serve as a switch for the generation of $^1O_2$ (Scheme 6).[22] A great deal of research activity has focused on the role that singlet oxygen plays in mechanisms of photoinduced cell death.[23] Photoinduced singlet oxygen mediated cell death is the foundation upon which photodynamic therapy (PDT) is based. PDT is a procedure whereby undesired tissue can be destroyed as a consequence of the combined action of light, oxygen, and a photosensitizer.[24] PDT is now an acknowledged treatment for a number of maladies including some cancers and macular degeneration.[25]

In their proof-of-principle study,[22] the chromophore pyropheophorbide-*a* (P), known to proficiently photosensitize the production of singlet oxygen upon energy transfer from its triple excited state,[26] was conjugated to the 5′ terminal of a 15-mer DNA sequence (Scheme 6). On another complementary 21-mer DNA sequence the so-called "Black Hole Quencher 3" (Q) was attached at the 3′ terminal. Whereas the P-DNA conjugate alone was found to efficiently photosensitize the production of singlet oxygen, formation of the hybrid between P-DNA and Q-DNA virtually switched off the photoproduction of singlet oxygen. Annealing of the conjugates force P and Q into close proximity leading to efficient quenching of the singlet oxygen production.

The singlet oxygen production could be switched on again in the presence of a DNA target containing a 21-mer sequence complimentary to the Q-DNA sequence. By competitive DNA hybridization the P-DNA is released and up to 85% of the original singlet oxygen production was restored. This approach is pertinent in the context of this concept for two main reasons, 1) because singlet oxygen can indeed be considered as a cytotoxic drug when present above a certain threshold in the cell,[24a,27] and 2) ground state molecular oxygen ($^3O_2$), which serves as a prodrug for singlet oxygen, is ubiquitous inside any living organism hence holding the promise for a general applicability of this strategy. Contrary to the previously described drug release systems, the present system involves no covalent chemical changes, merely the photosensitized production of singlet oxygen. In particular this system has a higher turnover. For example, in a simplified aqueous system containing 4 nmol pyropheophorbide-*a* (P) the total amount of singlet oxygen generated by photosensitization would be in the order of 10 μmol.[28]

Conclusions

We have presented some novel approaches to the development of model systems for DNA-encoded chemotherapeutic agents which are released or generated upon recognition of tailored short nucleic acid strands with complementary templates. Whereas it remains to be demonstrated whether such approaches will work in vivo, they are expected to possess some distinct advantages compared to available chemotherapeutic agents, such as the high selectivity inherent in hybridization between complementary nucleic strands and the relative ease with which the agent can be tailored according to individual genetic information. Two of the systems described feature a DNA-templated catalytic ester-hydrolysis employing either an organocatalyst (Taylor) or metal catalyst (Kraemer and Mokhir), and although both systems were demonstrated to exhibit



Scheme 6. DNA-templated phototriggered singlet oxygen production.

high turnover numbers with respect to the template, the development and use of more bioorthogonal chemical reactions are desirable. Furthermore, a molecular beacon strategy in which the model drug could be released by using a photocleavable group in the presence of a target template as developed by Saito and Nishimoto was described. Whereas Saito's system exhibited a sevenfold increase in the release of a model drug in the presence of target template as opposed to in the absence of the target, Nishimoto's system yielded only a twofold increase.

The field of DNA encoded molecular prodrugs is still in its infancy and faces several challenges that have to be overcome before the concept can be realized and converted into new and more selective drug systems that would be suitable for clinical trials. Such challenges include: 1) Delivery of oligonucleotide conjugates to diseased tissue. This is a challenge that all oligonucleotide (and analogues) based drug strategies such as for example, antisense- and siRNA-based treatments have in common and massive efforts are dedicated worldwide to solve this problem and we have chosen not to focus on that aspect here. As described above, there are examples on the application of the method to cells in which the templated release is identified with some success. 2) Most of the release reactions studied involve cleavage of esters, which will eventually also be cleaved by esterases in biological systems. One of the major challenges is to develop truly bioorthogonal reactions which will release only the active drug in the templated reaction. Furthermore, the reagent/catalyst that induces the release reaction should also be stable and nontoxic. 3) In almost all cases model systems have been studied in which a chemical signaling probe is released instead of a real drug and extension to real drugs is an obvious goal for the next generation. The example described by us on template induced control of photogenerated singlet oxygen is an exception in regard to both 2) and 3) as the quenching is truly bioortogonal and as singlet oxygen is a real drug that can induce apoptosis. The limitation of this method is that it can only be applied for generation of reactive oxygen species.

If the major challenges are faced and eventually overcome, the concept described here will offer a unique possibility to encode prodrugs for release/activation specifically in diseased cells. This would provide much more benign treatments and reduce unwanted side effects compared to conventional drug treatments. The method would complement antisense- and siRNA-based treatments, which are limited to inhibition of the expression of proteins. If realized the DNA-encoded molecular prodrugs would enable targeting of a much broader range of the cellular functions.

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Keywords: drug discovery · nucleic acid · photochemistry · prodrug · singlet oxygen

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[28] The system's volume was considered to be 1 cm^3, homogenously irradiated at 400 nm (where ε of P was considered to be approximately 76 000 $cm^{-1}\,M^{-1}$) and delivering a total energy of 10 J. Furthermore, it was assumed that the singlet oxygen formed did not diffuse out of the irradiated volume, that the power of the irradiating light was not saturating the system, and that no photobleaching of the sensitizer occurred during the process.

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