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Few constraints limit the design of quinone methide-oligonucleotide self-adducts for directing DNA alkylation†

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Abstract

Nucleotide sequences minimally containing adenosine, cytosine or guanosine are sufficient to form intrastrand oligonucleotide quinone methide self-adducts reversibly for subsequent alkylation of complementary DNA. The general lack of sequence restrictions should now allow for alkylation of most any target of interest although reaction is most efficient when the self-adducts contain guanine residues and do not form hairpin structures.

The ability of certain quinone methide (QM) derivatives to sustain reversible alkylation offers numerous opportunities in organic, medicinal and biological chemistry.^{1–5} For example, reversible and intramolecular trapping of QM-oligonucleotide conjugates to form self-adducts provides an attractive strategy for sequence selective alkylation of DNA (Scheme 1).^{6–8} Reaction selectivity is supported in part by the efficiency of reversible self-trapping relative to irreversible and competing reactions with non-targeted and often very strong nucleophiles represented by 2-mercaptoethanol. Effective intermolecular transfer of QM is only observed after pre-association with the intended acceptor such as complementary DNA. Consequently, the covalent chemistry of a highly reactive intermediate can be directed to and promoted by a target sequence without need of an external trigger such as UV irradiation or redox chemistry for activation.

Our ultimate goal is to create QM-based self-adducts for controlling gene expression in research and therapeutic applications. However, success of this method depends on its adaptability for any target of interest. Both theoretical and experimental systems have previously identified key parameters affecting the reversibility of alkylation by model QMs,^{9–13} but only a very limited number of self-adducts have been tested to date, and design guidelines for such species remain to be established.^{6–8,14} Initial self-adducts were alternatively derived from a monofunctional quinone methide precursor (QMP) anchored through an irreversible link or from a bisfunctional derivative (bisQMP) captured by two reversible linkages.^{7,8} Both support selective QM transfer to complementary DNA, but irreversible attachment prevents potential migration of the QM derivative away from its intended target.¹⁴ The general sequence and structure dependence of the monofunctional QM system described below is now shown to function with few limitations.

[†]Electronic supplementary information (ESI) available: general preparation, characterization and reaction of oligonucleotide conjugates, conversion of **QMP-OD2** to its self-adduct and alkylation of its target as monitored by reverse-phase chromatography, target alkylation by hairpin and linear self-adducts and competition studies as monitored by gel electrophoresis. See DOI: 10.1039/c0cc03317k

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The original oligonucleotide used to direct QM reactivity contained a mixed sequence with a 5'-terminal adenosine (A), an aminolinker and an attached QMP (**QMP-OD1**, Table 1). This derivative is convenient for storage and converts quantitatively to its self-adduct as monitored by reverse-phase C-18 HPLC after addition of fluoride to remove the silyl protecting group.⁷ Water addition to the QM does not compete with intramolecular trapping unless all of the nucleophilic nitrogens are removed by conjugating QMP to an oligonucleotide containing only thymines (T), a residue that has not been observed to react with the simple *ortho* QM of this conjugate.^{7,12} In contrast, substitution of individual nucleotides within **OD1** by T did not suppress self-adduct formation nor subsequent target alkylation. Consequently, complete substitution of each type of nucleoside was necessary to assess its role in self-adduct formation.

To identify the requirement for A in this system, conjugate (**QMP-OD2**) was prepared in which all As of **QMP-OD1** were replaced with Ts. The N1 of A was one of the most likely candidates for intrastrand trapping of the nascent QM since it acts as a strong and reversible nucleophile^{9,12} and represents one of the nearest nitrogens although still 25-atoms from the electrophilic site of reaction. Additionally, A had already demonstrated an ability to prolong the lifetime of a bisQMP equivalent by effectively competing against water for reaction.⁸ Despite the absence of As, **QMP-OD2** still formed its self-adduct, and this adduct still alkylated its complementary target (**OD2c**, Table 1) as detected by HPLC in analogy to earlier analysis of **QMP-OD1**.^{7,15} These results indicate that target directed alkylation can function independent of A.

The alternative candidates, G and C, were next considered as possible sites for self-adduct formation. Both of these nucleobases also share an ability to generate reversible self-adducts with their strong nucleophiles (N3 of C and N7 of G).¹² QMP conjugates in which all Cs were replaced with Ts (**QMP-OD3**) or all Gs were replaced with Ts (**QMP-OD4**) again supported formation of self-adducts.¹⁵ These adducts similarly alkylated their corresponding targets (**OD3c** and **OD4c**) (Table 1).¹⁵ Such results demonstrate that no particular nucleobase among A, C and G is required at any specific site although the presence of G in the self-adduct enhances the ultimate yield of target alkylation. The observed plasticity of self-adduct formation suggested a potential weakness in the design of the conjugates. The same dynamic and heterogeneous population of self-adducts that supports the broad tolerance of sequences might also cause target affinity and QM transfer kinetics to vary widely within the mixture. Only a limited number of species at low steady-state concentration can be expected to alkylate a target efficiently.

The consequences of a heterogeneous population of self-adducts was investigated with a second set of derivatives. These were inspired by the general design of molecular beacons that maintain proximity between a fluorophore and quencher by their conjugation to opposite termini of a hairpin stem.^{16,17} This proximity is subsequently lost when the hairpin loop binds to a complementary sequence and promotes denaturation of the stem. Equivalent use of a hairpin forming sequence in a QMP conjugate should then guide intrastrand trapping of the QM to a small number of sites and minimize the heterogeneity of self-adduct formation. This approach also disentangles the nucleotide sequence required for initial target recognition and the sequence required for self-adduct formation. The proximity of reactive partners enforced by hairpin structures has previously been applied to a covalent conjugate designed to release a drug model under the combined influence of UV irradiation and a target sequence of DNA.¹⁸

To test the hairpin design for QM self-adducts, QMP was attached to an appropriate oligonucleotide for targeting the *c-erbB-2* oncogene.¹⁹ The loop region and 5'-terminus containing the QMP were complementary to the oncogene to maximize proximity for QM

transfer (Scheme 2). The 3'-terminus was not complementary to the target and instead complementary to the 5'-terminus of the QMP conjugate to establish the desired hairpin-forming self-adduct. Due to the greater reactivity of QM for single- *versus* double-stranded DNA,²⁰ the 3'-terminus of the conjugate was extended with the three unpaired residues (CAG) capable of forming reversible self-adducts with QM. Addition of fluoride to initiate QM formation produced the expected self-adduct in 24 h similar to the samples above (Table 1). A target (**erb1-CAG**) for this self-adduct was designed to contain the same CAG extension of the hairpin so that QM transfer would not be driven merely by a preference for a particular nucleophilic partner.²¹ Incubation of the self-adduct (1.1 μ M) and target (1.0 μ M) yielded the expected high molecular weight product of interstrand alkylation as detected by its slow mobility during denaturing gel electrophoresis (Fig. 1). Thus, covalent linkage within the hairpin self-adduct can be overcome by addition of a complementary sequence. However, the yield of target alkylation was low (16%) and the kinetics of its formation was very slow (96 h).

The initial choice of a CAG extension to capture the QM in both the self-adduct and target strands was selected for its inclusion of all possible sites that react reversibly with QM since no optimum site was apparent from the experiments above. To evaluate the role of individual nucleobases in the 3'-extension, a related set of hairpin forming self-adducts was next examined. The potential dependence on G was characterized by the hairpin conjugate (**QMP- Ω 2-GGG**) equivalent to **QMP- Ω 1-CAG** except for replacement of the CAG extension with a GGG sequence and addition of an A-T base pair at the terminus of the hairpin stem to separate the single- and double-stranded Gs.¹⁵ Again, the QM self-adduct formed after deprotection of its precursor, and again this adduct transferred its tethered QM to a corresponding target containing an equivalent GGG acceptor (**erb2-GGG**).¹⁵ Still, the yield and kinetics of target alkylation were poor (~7% alkylation after 72 h) despite the general effectiveness of G N7 to alkylate and release QM.^{12,22} To confirm that these surprising results were the consequence of hairpin formation, the melting temperature of **QMP- Ω 2-GGG** was measured over a concentration range of 1.4–5.9 μ M and found to be relatively invariant at 52 ± 1 °C as expected for a unimolecular hairpin system.

The efficacy of A and C relative to G was also tested by preparing corresponding self-adducts of **QMP- Ω 2-AAA** and **QMP- Ω 2-CCC**.¹⁵ For these, a common CAG extension was used in the target strand (**erb2-CAG**). Neither self-adduct was very competent at target alkylation although the AAA extension generated about twice the yield (17% in 96 h) of target alkylation than that observed for the CCC extension (9% in 96 h).¹⁵ The lower yield generated by **QMP- Ω 2-CCC** likely reflected the instability of its self-adduct which lost half of its activity during the 96 h incubation.¹⁵ In contrast, the self-adduct of **QMP- Ω 2-AAA** lost only 15% of its activity under comparable conditions.¹⁵

The various unpaired nucleotides extending from the 3'-terminus of the hairpins did little for target alkylation except lower the yields relative to the unstructured series in Table 1. For context, the parent hairpin (**QMP- Ω 2**) without an extension and even a derivative (**QMP-OD5**) lacking the 3'-stem region and unable to form a hairpin were tested against target DNA. In each case, self-adducts were generated *in situ* by deprotection of the QMP prior to addition of the same target as above (**erb2-CAG**). Surprisingly, these two self-adducts were more effective than the initial hairpin forming derivatives and alkylated the target with yields of *ca.* 30%.¹⁵ Thus, the single-stranded extensions were detrimental, not beneficial to the design of self-adducts. Consequently, attempts to limit the heterogeneity of self-adducts by forming hairpin intermediates did not improve the efficiency of target alkylation as illustrated by **QMP- Ω 2** and **QMP-OD5**.

A conjugate system based on the hairpin design did at least achieve very good selectivity as demonstrated in a pair of competition studies (Scheme 3). Alkylation of a radiolabeled and complementary target ([³²P]-**erb2-CAG** 1.0 μM) by **QMP-Ω2** (1.1 μM) was not greatly suppressed by the presence of an alternative target **erb3-CAG** that contained three mispaired bases.¹⁵ Even 25-fold excess **erb3-CAG** inhibited alkylation of [³²P]-**erb2-CAG** by only *ca.* 50%. In contrast, one equivalent of the fully matched target **erb2-CAG** was sufficient to inhibit alkylation of [³²P]-**erb3-CAG** by 90%. These initial results bode well for future applications *in vivo* for which selectivity will be crucial.

Formation of reversible self-adducts by QM-oligonucleotide conjugates appears to be quite general and can be designed to alkylate most any desired nucleotide sequence available for recognition by Watson–Crick base pairing. Self-adducts can also be envisioned for targeting duplex DNA in future applications *in vivo* based on triplex assembly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Time dependent alkylation of target DNA. The quinone methide (QM) self-adduct of 5'-QMP-d(CGCTCTCCTCCTCCACCTCCGAGCGCAG) (**QMP-Ω1-CAG**, 1.1 μM) in 100 mM MES pH 7 was generated *in situ* by addition of 100 mM KF and incubated under ambient conditions for 24 h. The target strand 5'-[³²P]-d(AGGAGGAGGTGGAGGAGGAGAGCGCAG) ([³²P]-**erb1-CAG**, 1.0 μM, 90 nCi) was added at 0 h and incubation was continued under ambient conditions. At the indicated times, samples were analyzed by denaturing 20% (7 M urea) polyacrylamide gel electrophoresis. Product yields were estimated by the conversion (%) of target strand to its alkylated derivative using phosphoimager and ImageQuant software.

Scheme 1.

Preparation and application of a quinone methide oligonucleotide self-adduct. (A) Deprotection of a quinone methide precursor (QMP), (B) spontaneous quinone methide (QM) formation and reversible intrastrand trapping to form self-adduct(s), (C) interstrand QM transfer competes with self-adduct formation only after target association.

Scheme 2.

Spontaneous quinone methide regeneration in a hairpin-forming self-adduct allows for alkylation of complementary DNA.

Scheme 3.

Competition between matched and mismatched DNA for alkylation by interstrand transfer of a quinone methide.

Table 1

Target Alkylation by Conjugates Alternatively Lacking A, C and G

Sequence ^a		ESI-MS (<i>m/z</i>)		
		Calc	Obs	Interstrand alkylation
5'-QMP-d(ACGTCAGGTGGCACT)	QMP-OD1	1701.4 ^b	1700.8 ^b	50% ^d
3'-d(GAATCTGCAGTCCACCGTGA)-φ ₂	OD1c	1618 ^{c,d}	1618 ^{c,d}	
5'-QMP-d(TCGTCTGGTGGCTCT)	QMP-OD2	1692.0 ^b	1691.7 ^b	85%
3'-d(GAATCAGCAGACCACCGAGA)-φ ₂	OD2c	1624.5 ^c	1624.3 ^c	
5'-QMP-d(ATGTTAGGTGGTATT)	QMP-OD3	1721.0 ^b	1720.9 ^b	85%
3'-d(GAATCTACAATCCACCATAA)-φ ₂	OD3c	1601.7 ^c	1601.4 ^c	
5'-QMP-d(ACTTCATTTTTCACCT)	QMP-OD4	1659.3 ^b	1659.0 ^b	33%
3'-d(GAATCTGAAGTAAAAAGTGA)-φ ₂	OD4c	1647.8 ^c	n/d	

^aSee Supplementary Information for experimental details. Diphenylacetyl groups (φ₂) were appended to aminolinkers at the 5' termini of the complementary strands to improve chromatographic resolution.⁷

^bESI-MS (M-3H⁺)³⁻.

^cESI-MS (M-4H⁺)⁴⁻.

^dDetermined previously.⁷