Anti-gene padlocks eliminate *Escherichia coli* based on their genotype

Chanjuan Shi¹†, Antony R. Parker¹†, Li Hua¹, Craig N. Morrell¹,², Soo Chin Lee³‡, Viswanath Bandaru⁴, J. Stephen Dumler¹, T. C. Wu¹,³ and James R. Eshleman¹,³*

1Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD 21231, USA; 2Department of Comparative Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21231, USA; 3Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, MD 21231, USA; 4Department of Microbiology and Molecular Genetics, The University of Vermont, 224 Stafford Hall, Burlington, VT 05405, USA

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**Objectives:** Several therapeutic strategies that target nucleic acids exist; however, most approaches target messenger RNA, rather than genomic DNA. We describe a novel oligonucleotide-based strategy, called anti-gene padlocks (AGPs), which eliminate *Escherichia coli* based on their genotype.

**Methods:** The strategy employs an oligonucleotide with a double hairpin structure where both strands of the AGP are complementary to both strands of a target gene. We tested AGPs for *in vitro* binding and inhibition of DNA polymerization. AGPs were electroporated into bacterial cells with and without gene targets along with an ampicillin resistance plasmid, and cell survival was measured.

**Results:** In *in vitro*, AGPs bound the DNA target in a sequence-dependent fashion and inhibited DNA synthesis. When transformed into bacterial cells containing 10, 20 or 30 bp lacZ or 20 bp proA DNA targets in their genomes, AGPs selectively killed or otherwise inhibited growth of these cells, while those lacking the target demonstrated little, if any, toxicity. A single transformation resulted in ~30% to 40% loss of target-bearing cells. Structure–function experiments were performed to define essential AGP requirements.

**Conclusions:** These results suggest that AGPs may be a useful tool to eliminate specific cell populations.

**Keywords:** novel therapeutics, oligonucleotide, gene targeting, antimicrobial agents, antibiotics, padlock probes

**Introduction**

With many diseases, patients exist as cell chimeras, in that they have acquired a second cell population (e.g. malignant cells, bacterial cells, HIV-infected cells). In each case, this second cell population contains additional genes, which not only define these cells as unique, but also can in theory provide distinctive genetic targets for cell elimination. We asked whether we could design a general strategy to kill cells based on their unique genetic composition.

Several oligonucleotide-based therapeutic approaches have been developed to manipulate disease-related gene function inside cells. These include antisense RNA,¹–³ triplex DNA,⁴–⁶ ribozymes⁷,⁸ and suicide gene therapy.⁹,¹⁰ Antisense is the most mature of these, and the first agent for treating CMV retinitis, Vitravene, has been cleared by the FDA.¹¹–¹³ In the antisense approach, oligonucleotides are used to block specific gene expression by targeting and destroying specific mRNA sequences, mainly via RNase H-dependent mechanisms.¹⁴ However, its efficacy and reliability can be somewhat variable.¹⁴,¹⁵ In addition, target selection of antisense molecules can be problematic, since many designed antisense molecules are incapable of accessing the target mRNA for efficient hybridization because of unique mRNA secondary structure features.¹²,¹⁶ Despite those issues, antisense technology is promising with several additional agents in advanced phase clinical trials.¹²–¹⁴,¹⁷ Bacteria, and more specifically, antibiotic drug resistance genes, have been targeted...
using both RNA oligonucleotides that act through an RNase P mechanism and antisense oligodeoxynucleotides.18–20

An exciting new molecular targeting strategy is small interfering RNA (siRNA). siRNAs are double-strand RNA molecules of 21–23 bp.21 They specifically destroy complementary mRNA molecules following association with a protein–enzyme complex, the RNA-induced silencing complex.22,23 In tissue culture, siRNAs have been shown to kill cancer cells24 and inhibit viral infection.25 In an animal model of Fas-mediated liver damage, siRNAs targeting Fas protect mice from fulminant hepatitis.26 However, for all of these approaches to delete a specific cell population, they require that the targeted transcript be an essential gene for the cell to survive.

In this paper, we present an alternative approach that is directed at genomic DNA. This strategy is based on ‘padlock probes’, large oligonucleotides (70–92 bases) that can detect single-base mutations using a variety of different in vitro reporter assays.27–29 Both arms are complementary to one strand of a target DNA, and they wrap around this strand in an end-to-end orientation, and are ligated only if a perfect match exists between the arms and target (Figure 1a). Since the arms are typically about 20 bases each, together they are envisioned to wrap around a DNA target approximately four times (one turn per ~10 bases) before being ‘locked’ through ligation. In this way, they are inextricably bound to the DNA target (hence ‘padlock’). Their backbone is, however, non-complementary to the target DNA.

We hypothesized that diagnostic padlock probes could be redesigned for use as a novel gene targeting approach that would kill or otherwise inhibit cells by irreversibly binding to unique gene targets thereby preventing DNA replication and/or transcription. In this report, we demonstrated that AGPs bound in a sequence-specific manner and inhibited DNA synthesis in vitro. We also demonstrated that if a target was present in the genomic DNA of bacteria, AGPs of various lengths selectively killed, or inhibited growth of, these cells in a dose-dependent fashion. Finally, we defined essential structural requirements for AGPs. We propose that AGPs may be an effective tool for selectively killing cells, or otherwise inhibiting their growth, based on their genotype.

Materials and methods

**Primers and AGP oligonucleotides**

All oligonucleotides were purchased from Invitrogen (Carlsbad, CA, USA). PCR primers were cartridge purified, whereas AGP oligonucleotides were gel or HPLC purified. AGPs used in the in vitro studies were phosphorylated at the 5’ terminal using [γ-32P]ATP or dATP as previously published.30 Control AGPs were synthesized for each gene-specific AGP by counting the number of each nucleotide in the arms and randomizing them. All other structural features were maintained by making the backbones complementary except for the mispairs corresponding to the terminal bases of the arms. Hinges always consisted of three thymidines.

**Plasmids**

pUC19 was purchased from Life Technologies (Rockville, MD, USA) and pSG5 from Stratagene (La Jolla, CA, USA). pUC19-ΔPL was constructed by digesting pUC19 with SacI and HindIII restriction enzymes to remove the polylinker. Primers containing an underlined NotI site, 5’-AGCTAGCAGGCAGCGGAGCACAGCT-3’ and 5’-TGTCGCGGCGGCTGCGCT-3’, were mixed with the digested plasmid, incubated with T4 DNA ligase and polylinker removal was confirmed by sequencing.

**Electrophoretic mobility shift assay (EMSA)**

32P-labelled pUC19 AGPs, sequence-specific and control, (1.9 nM) were incubated with the various plasmids (1 μM) overnight at 37°C in a reaction buffer containing 7 mM Tris–HCl, pH 7.6, 7 mM MgCl2 and 50 mM NaCl in the presence and absence of 400 U of T4 DNA Ligase (New England Biolabs Inc., Beverly, MA, USA). Products were electrophoresed on a 1.5% agarose gel in 1× TBE buffer.

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**Figure 1.** Structure of a traditional padlock probe, proposed AGP conformations and target mechanism of AGP target binding. (a) Traditional padlock (blue) bound to DNA (red). Note that the backbone is non-complementary to the target and contains biotin groups to facilitate detection in subsequent steps. Note that both arms are bound to the target but have not been ligated. Adapted from ref. 27. (b) AGPs (blue) are shown in the native closed conformation where the terminal bases of both arms are mispaired with the backbone. (c) This structure may be in equilibrium between closed (b) and open forms (c) where the number of bases of the arms, hinges and backbone are designated for a 46 base AGP. (d and e) The gene target may also be in equilibrium between double-stranded (d) and locally denatured (e) forms. (g) The AGP is predicted to interact with the denatured target. Note the perfect homology between the arms (including the terminal bases) and the target, while the mispairing between the AGP and the target is in the backbone. (f) The proposed structure of a bound and ligated AGP is shown. Since the backbone and combined arm lengths are 20 bases each, an AGP of 46 bases should be intertwined two times with each strand of the target DNA before being ligated.
Cycle sequencing of AGP-bound pUC19

The pUC19 sequence-specific AGP (1.9 nM) was mixed with pUC19 (1 μM), denatured at 95°C for 2 min, in a reaction buffer containing 7 mM Tris–HCl, pH 7.6, 7 mM MgCl2 and 50 mM NaCl, and allowed to cool to room temperature. The resulting reactants were then incubated in the presence and absence of 400 U of T4 DNA ligase. The ligated and unligated samples were subjected to cycle sequencing using either primer A (5′-TACCCAGATCTCGTAAACG-3′) or primer B (5′-ATGCA GCTGGCAGCACAGGT-3′) and BigDye terminators (Applied Biosystems, Foster City, CA, USA) according to manufacturer’s instructions and analysed on an ABI 3700.

Bacterial strains used

E. coli HB101 [supE44, hsdR2(rk-mK), recA13, ara-14, proA2, lacY1, galK2, rpsL20, leuB6, thi-1, xyl-5, mtl-1], E. coli lac– 8036 (ara, thi, trpE9777, Δpro-lac, F′) and E. coli lac+ C600 (supE44, hsdR, thi-1, thr-1, leuB6, lacY1, tonA21) were used in the chromosome targeting experiments. E. coli HB101 was purchased from Invitrogen and E. coli C600 was provided by the E. coli genetic stock centre, Yale University (New Haven, CT, USA). E. coli 8036 was originally constructed by Dr Barry Shi et al. kindly provided by Drs David Sedwick and Martina Veigl (CWRU, Cleveland, OH, USA).

Transformation of bacteria with AGPs

Electrocompetent bacteria were co-transformed with the AGPs (molar ratio 10,000:1 unless otherwise indicated) and the ampicillin-resistant plasmid pSK5 (which does not contain lacZ) using a Bio-Rad Micropulser™, program EC2 according to manufacturer’s instructions, washed with VB salts and immediately plated onto ampicillin-containing plates. Minimal media plates containing proline (50 μg/mL), glucose (0.2%), IPTG (24 μg/mL), X-gal (50 μg/mL), tryptophan (55 μg/mL), thiamine (5 μg/mL), leucine (50 μg/mL), threonine (50 μg/mL) and biotin (50.7 ng/mL) were routinely used in the presence or absence of ampicillin (100 μg/mL). Percentage colony reduction of HB101 cells or C600 cells after transformation with specific or control AGPs was calculated as follows: 1 − (observed blue colonies/no. expected, where no. expected = (no. white colonies, no AGP)/[(no. white colonies, AGP treated) × (no. blue colonies, no AGP)].

Determination of potential targets

A C program (Codewarrior Version 4.2.5, MetroWerks, Austin, TX, USA) was written to compare two DNA sequences. It first creates an array of size 4^t, where t is the size of the target, and fills it with all of the potential targets (e.g. for t = 3, AAA, AAC, AAG...TTT). It then scans the two DNA sequences base-by-base (e.g. bases 1–3, 2–4 etc.) in both directions, counting the number of each potential target. It then reports the number of targets present in the DNA of the cell to be eliminated (e.g. the lacZ-positive cells or the O157 cells) and absent in the non-targeted cells (e.g. normal E. coli cells). The files examined were lacZ (GenBank number: AE000141), E. coli K12 (GenBank number: U00096) and E. coli O157 (GenBank number: BA000007).

Intelligent designation of 26 base AGPs

A total of 52 potential targets (10 bp targets) that were identified in the lacZ gene and absent in E. coli K-12 were analysed further. From this set, intelligently designed AGPs were chosen that had the least potential for cross-reactivity by eliminating those that would be reactive if either the first or last base was altered. The computer program was also employed to choose control AGP oligonucleotides for 26 base AGPs. To do so, all possible 10 base targets using the same number of each nucleotide from their AGPs were generated, which were subsequently compared with the E. coli genome. Only oligonucleotides that were not present in the E. coli genome were selected.

Establishment of a ΔlacZ C600

ΔlacZ C600 was established by bacteriophage P1-mediated transduction using E. coli 8036 (in which lacZ was deleted) as donor and C600 (with wild-type lacZ gene) as recipient. A colony of E. coli 8036 was first grown in 5 mL of LB medium with 10 mM CaCl2 until turbidity was evident. Wild-type bacteriophage P1 (5 μM) was then added to infect and lyse 8036 bacteria. The phage supernatant was collected and used to infect lacZ+ C600 cells. The P1 infection was terminated by adding one drop of 1 M sodium citrate. The infected cells were plated in the X-gal/IPTG minimal plates containing thiamine, leucine and threonine. To confirm the deletion of the lacZ gene in white colonies, bacterial DNA was extracted from C600 and the white colonies using DNeasy tissue kit (Qiagen, Valencia, CA, USA) and was subject to PCR amplification of the lacZ gene and surrounding genes.

Direct live/dead bacterial quantification

Live and dead cells were stained using a LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Eugene, OR, USA) according to manufacturer’s instructions and examined 0, 1, 2, 3, 4 and 5 h following electroporation, where dead cells were stained with propidium iodide (red), and live cells with Syto9 green (green). The red and green cells were counted using an Olympus BX41 fluorescent microscope and the percentage of dead cells was calculated.

Results

Design of the gene-targeting AGPs

To test our hypothesis, we empirically made two major design changes to the diagnostic padlock probes (Figure 1a) for use as gene-targeting molecules. First, rather than the non-homologous backbone of the padlock probes, we made both the backbone and the arms complementary to both strands of the DNA target, and therefore to each other (Figure 1b). In the cell, we hypothesized that an equilibrium would exist between this closed inactive form and an active open form in which the AGP arms are denatured (Figure 1c). In the denatured form, the backbone and arms should, in theory, have the ability to bind to both strands of locally denatured target DNA (Figure 1e), creating two DNA duplexes, using Watson and Crick pairing (Figure 1g). Since DNA ‘turns’ every 10 bases, the AGPs and the target should be intertwined. Figure 1(f) shows the intertwined form after ligase, where it seems likely that the target DNA and the AGP...
should be inextricably bound. If so, the target DNA would likely be unable to denature as is required during both transcription and replication.

The second design change was required since we wanted to make both the arms and backbone complementary to both strands of the target; they are therefore also complementary to each other. We imagined that as such, the unbound lock with the two arms juxtaposed would be ligated to each other and thereby inactivated prior to any interaction with the target. To prevent inactivation in this way, we therefore created mispairs between the terminal bases of the arms and the backbone (Figure 1b). This was accomplished by making the base changes in the backbone rather than in the arms so that the arms would retain full complementarity to the target gene (Figure 1g). Both of these empirical design changes ultimately proved important, but we first tested the AGPs for binding activity and DNA synthesis inhibition in vitro.

AGPs bound in a sequence-specific fashion and could be ‘locked’
To initially determine if these novel AGPs would bind specifically to the target DNA, we synthesized an AGP complementary to the pUC19 polylinker (Figure 2a). The 32P end-labelled AGP was incubated with a pUC19 plasmid containing the polylinker target at 37°C overnight without prior heat denaturation of either
the plasmid or AGP. Under these conditions, the radioactively
labelled AGP was shifted to a higher molecular mass, consistent
with plasmid binding (Figure 2b, lane 1). Specificity was
demonstrated by the lack of a gel-shifted band when the pUC19
AGP was incubated without the plasmid (lane 2), with a plasmid
that contained a different polylinker (pSG5, lane 3) or with a
pUC19-derived plasmid in which the target polylinker had been
deleted (pUC19-ΔPL, lane 4).

AGPs were designed with the hypothesis that ligation of the
5′ and 3′ termini would be required for these structures to func-
tion as inhibitors in the cell. With the addition of T4 DNA
ligase to the in vitro reaction, at the end of the 37°C overnight
incubation with the plasmid, the AGP remained shifted and the
amount of shifted form increased (Figure 2c, lanes 2 and 3).

It is tempting to conclude that AGPs bound to their target in a
mechanism similar to the cartoon shown in Figure 1(f). Alternatively, this binding may well be due to some alternative
mechanism such as Hoogsteen binding leading to triplex for-
meration as inhibitors in the cell. With the addition of T4 DNA
ligase to the in vitro reaction, at the end of the 37°C overnight
incubation with the plasmid, the AGP remained shifted and the
amount of shifted form increased (Figure 2c, lanes 2 and 3).

AGPs inhibited in vitro DNA synthesis

Having determined that the AGP bound in a sequence-dependent
manner, we hypothesized that if bound and ligated as shown in
Figure 1(f), then the two target DNA strands would not be
capable of undergoing the normal strand denaturation required
for DNA synthesis. Since we wished to maximize the percentage
of plasmid with bound AGP, the pUC19 plasmid was mixed
with the AGP, heat denatured at 95°C and cooled. Half of this
reaction was then treated with T4 DNA ligase. These samples
were subjected to cycle sequencing to determine whether the
presence of the ligated AGP would influence the ability of the
DNA sequencing polymerase to synthesize DNA through the
‘locked’ region.

As shown in Figure 2(d), using either of the two sequencing
primers (A and B) from opposite directions, sequence products
terminated at the approximate position where the polymerase
was predicted to encounter the ligated AGP. With the portion of
the reaction not treated by DNA ligase, as expected, the DNA
polymerase was able to sequence through this region, presum-
ably by melting the unligated AGP from the target DNA strands
during the denaturation phase of the cycle sequencing. These
data indicate that specific binding and ligation of the AGPs
resulted in successful inhibition of DNA synthesis in vitro.
However, while ligation was clearly required to inhibit cycle
sequencing-based DNA synthesis in vitro, we do not interpret
this to necessarily indicate that ligation will be required for
AGPs to be active inside cells. Further, successful inhibition of
DNA PolII used in DNA sequencing reactions in vitro does not
necessarily imply that PolIII holoenzyme will be successfully
inhibited when AGPs are introduced into cells.

**Table 1. Anti-gene padlocks**

<table>
<thead>
<tr>
<th>Target length</th>
<th>Target site</th>
<th>AGP sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>92 bases (pUC19)</td>
<td>40</td>
<td>5′-AGTCGACCTGCAAGGATGCTGCA-TTT-CTTGCAGTGGCC TGCAAGGTCGACATAGCTGTCCCGGGATCGCA-TTT-TCGGTACCCGGGGATCGTCGTAG-3′</td>
</tr>
<tr>
<td>lacZ 66 base AGP</td>
<td>30</td>
<td>5′-GTGACTGGAAAAACC-TTT-GGTGTTCACGTACGTCTGCTGCTAGTAAACGAT-TTT-TGCAGTTTACAGCTAC-3′</td>
</tr>
<tr>
<td>lacZ 46 base AGP</td>
<td>20</td>
<td>5′-GTGACTGGA-TTT-TGCCAGTCAAGCATGTGTA-TTT-TTACAGGGAAGTAT-3′</td>
</tr>
<tr>
<td>lacZ ID1 AGP</td>
<td>10</td>
<td>5′-AGGTAGACTTTTACTGCCGATAGCTGCA-TTT-GTGACCAGTTT-3′</td>
</tr>
<tr>
<td>lacZ ID2 AGP</td>
<td>10</td>
<td>5′-CTCCCT-TTT-AGGACCATGTT-TTT-ACGAG-3′</td>
</tr>
<tr>
<td>lacZ ID3 AGP</td>
<td>10</td>
<td>5′-GGCCCT-TTT-AGGCGAGGT-TTT-ACCTA-3′</td>
</tr>
<tr>
<td>lacZ ID4 AGP</td>
<td>10</td>
<td>5′-CTCGTTTTACAACGTC-3′</td>
</tr>
<tr>
<td>proA 46 AGP</td>
<td>20</td>
<td>5′-TGCGCAGGCA-TTT-TGCGAGGT-3′</td>
</tr>
<tr>
<td>proA 46 control</td>
<td>20</td>
<td>5′-AGGTAGACTTTTACTGCCGATAGCTGCA-TTT-GTGACCAGTTT-3′</td>
</tr>
<tr>
<td>lacZ AGPs selectively killed lacZ target-bearing E. coli cells</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Having demonstrated that AGPs inhibited DNA synthesis in vitro, we then asked whether AGPs could be used to target
specific genes in living bacteria. AGPs targeting the lacZ gene
in the E. coli genome were designed (Table 1). The bacterial
strain E. coli HB101 was chosen since its chromosome contains
a lacZ gene. For these experiments, we mixed two bacterial cell
populations together, E. coli HB101 (lacZ wild-type E. coli,

 nc, non-coding strand; c, coding strand.
Triple thymines (separated by hyphens) represent the two hinges. Unpaired bases and backbone mispairs are underlined.

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Gene-specific cell elimination

![Figure 3](https://academic.oup.com/jac/article-abstract/61/2/262/767480)

**Figure 3.** AGPs of various lengths selectively eliminated target-bearing *E. coli* cells. (a) and (b) Mixtures of *E. coli* HB101 (containing the wild-type lac operon in the chromosome) and *E. coli* 8036 (with lacZ gene deletion) were transformed with AGPs and an ampicillin-resistant pSG5 plasmid, and plated on X-gal plates with ampicillin and IPTG. When exposed to AGPs specific for lacZ, a selective loss of β-galactosidase-positive blue colonies was seen (b). This was not observed with the control randomized AGPs (white bars). The data represent the means of 4–8 independent experiments and error bars represent SEM. *P* values were calculated using Student’s *t*-test (*P* < 0.01 for all AGPs compared with controls). (c) Bar graph comparing the specific cell elimination of the HB101/8036 cell mixture using lacZ AGPs of 26 (ID1), 46 or 66 bases (black bars) with their control randomized AGPs (white bars). The data represent the means of 4–8 independent experiments and error bars represent SEM. (d) Bar graph reporting blue colony reduction using the 46 base proA AGP (black bar) versus randomized control (white bar) in C600/8036 cell mixtures. The data represent the means of four independent experiments and error bars represent SEM (*P* < 0.01). A colour version of this figure is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

Phenotypically blue on X-gal plates and *E. coli* 8036 (containing a lacZ deletion, white on X-gal plates), since the ratio of two different cell types (blue to white colonies) is relatively consistent when plated on replicate plates (data not shown). Because the majority of cells during electroporation are not transformed, we co-transformed these cells with an ampicillin resistance encoding plasmid (pSG5) so that only cells that have taken up DNA are analysed. The cell mixtures were transformed with pSG5 alone, pSG5 plus control AGP or pSG5 plus lacZ AGP, washed and immediately plated on X-gal plates containing ampicillin. Control AGPs were designed by counting the number of each nucleotide in the specific AGP and randomizing them while maintaining the overall AGP structure (see the Materials and methods section). Figure 3(b) (lacZ AGP) shows selective loss of target-containing blue colonies following transformation of the cell mixture with a 46 base AGP designed to interact with a 20 bp target of the lacZ gene. The lacZ AGP did not significantly reduce the number of white cell colonies (lacking the lacZ target) and the control AGP (Figure 3a).

**proA AGPs selectively eliminate proA target-bearing E. coli cells**

Because we were assaying the number of blue colonies from β-galactosidase activity and the lacZ gene was targeted in the previous experiments, it was possible that the loss of blue colonies was not due to cell death or growth inhibition, but rather through some other mechanism such as loss of expression or mutation of the lacZ gene. We therefore designed AGPs towards a second target, the proline synthesis gene, proA (Table 1). The proA gene, located near the lacZ gene, is present in the C600 chromosome and lacking in 8036. When transformed into bacterial cell mixtures and plated on proline-containing plates, we detected 40% to 50% loss of proA target-bearing cells (Figure 3d). In these experiments, β-galactosidase was simply a marker of the cells being targeted since the lacZ gene is reduced neither cell population, indicating relatively selective cell killing of the target-bearing cells by the AGP. Since the bacteria are quickly plated after electroporation, we consider it unlikely that altered ratios reflect population shifts based on differential growth rates, however, the lack of blue colonies could certainly reflect bacteriostatic rather than bacteriocidal activity.

To examine potential length requirements for AGPs, we tested effects of 26, 46 and 66 base AGPs on the *E. coli* HB101/8036 mixture, which targeted 10, 20 and 30 bases of the lacZ gene in HB101, respectively (Table 1). These AGPs selectively reduced the number of blue colonies by ~50% to 40% (Figure 3c). Although there was a trend that longer locks were more effective, this effect did not achieve statistical significance. In contrast, the randomly designed control AGPs exhibited little or no reduction in target-bearing blue colonies (Figure 3c, consistently <5% reduction). The reduction in blue colonies by each AGP relative to its randomized control was significant (*P* < 0.01).

The absolute number of target-lacking white colonies remained relatively constant. The combination of the *in vitro* results that demonstrated specific binding of AGPs to the target DNA and the *in vivo* results that demonstrated that AGPs selectively eliminated target-containing bacteria suggests that their cell toxicity may be mediated via a specific AGP and DNA interaction at target sites.

To test whether toxicity would be increased if the target gene was being actively transcribed, we grew the bacteria in IPTG prior to electroporation. Somewhat to our surprise, no increase in toxicity was seen under these conditions (data not shown).

As an independent assay for cell death, we counted cells after transformation using fluorescence microscopy after staining with a green dye (Syto9) that stains viable bacteria and a red dye (propidium iodide) that stains dead organisms that have lost membrane integrity. In bacterial transformation, the majority of bacteria are not transformed, and these experiments therefore required omitting ampicillin to keep the background number of dead cells sufficiently low. At 2, 3, 4 and 5 h, 6% to 7% of the cells were dead after transformation of C600 cells (containing lacZ in their chromosome) with the lacZ AGP, whereas only 3% to 4% dead cells resulted after transformation with the control scrambled padlock. Since we typically counted 500–600 bacteria for these experiments, these were statistically significant (*χ*², *P* < 0.01 for 3 and 4 h time points).
Number of potential targets that are present in the lacZ gene but absent in E. coli K12 (with lacZ deletion) as a function of target size. (b) Number of potential targets that are present in E. coli O157 but are absent in E. coli K12 as a function of target size. (c) Selective cell elimination of lacZ+ HB101 using intelligently designed 26 base lacZ AGPs in comparison with their intelligently designed control AGPs in the HB101 and 8036 cell mixture. (d) Selective elimination of target-bearing C600 cells by 66, 46 and 26 base (ID1) AGPs in the C600/lacZ C600 cell mixture. The data represent the means of 4–10 independent experiments and error bars represent SEM. Black bars, AGPs; white bars, controls. P values were calculated using Student’s t-test and were P < 0.01 for all AGP versus control comparisons.

Isogenic E. coli cells showed differential cell elimination

Since the above work uses two bacterial strains that differ in many genes in addition to the lacZ gene, we wanted to exclude the possibility that the selective cell toxicity observed was due to an idiosyncratic response of these bacterial strains. We therefore constructed an isogenic E. coli C600 strain in which the lacZ gene had been deleted from the chromosome (designated ΔlacZ C600) using bacteriophage P1 transduction (see the Materials and methods section). lacZ AGPs were tested in a cell mixture of C600 and the ΔlacZ C600 as described above.

Transformation of AGPs into this cell mixture also selectively and significantly reduced the number of lacZ+ C600 cells, compared with their control AGPs (P < 0.01). The 66, 46 and 26 base AGPs reduced the number of blue colonies by 30% to 40% (Figure 4d). In contrast, control AGPs had relatively little toxicity (2% to 8%) in C600 cells. No significant reduction in the number of white colonies was observed in the AGP-transformed cell mixtures, this further supporting the notion that the cell elimination by AGPs likely results from target-specific interactions.

Dose–response experiments

To determine if AGPs selectively kill or inhibit bacteria in a dose-dependent manner, we transformed the cell mixture with the 46 base AGP and the ampicillin resistance plasmid at molar ratios of 10:1, 100:1, 1000:1 and 10 000:1 (AGP/pSG5). Although a ratio of 10:1 showed essentially no effect, significant cell elimination was observed at a ratio of 100:1, albeit reduced in comparison with 10 000:1 (Figure 5a, 12%, P < 0.01). Doses of 1000:1 and 10 000:1 were similar and eliminated ~30% of target-bearing cells. These results were obtained with electroperoration and might vary with the method used to transform the cells.

Structure–function experiments

To determine the minimal essential features for AGP activity, several 46 base AGP variants were created by mixing and matching the prototypic 46 base AGP (Figure 5b, bar 1) and the control randomized AGP (bar 2) in the C600/ΔlacZ C600 cell mixture. Two variants are chimeras, composed of half AGP and half control AGP, and they showed no toxicity towards the
target-bearing blue colonies (bars 3 and 4). Another variant contained arms complementary to the target, but a backbone that was random, and this showed little if any activity (bar 5). Interestingly, this inactive AGP resembles the original padlock structure designed for in vitro diagnostic strategy (Figure 1a).27 In addition, a variant AGP with 100% complementarity between the arms and backbone (i.e. lacking terminal base mispairs) did not produce any specific cell killing, presumably because it was rapidly ligated upon entering the cell, before it could react with the target (bar 6). A variant with mispairs in the terminal bases of the arms compared with the target (instead of in the backbone), also showed no specific cell toxicity, consistent with the hypothesis that ligation may be required for AGP activity (bar 7). Finally, as an initial test to determine whether ligation was required for AGP activity in vivo, we constructed a variant AGP where the normal terminal cytosine base was replaced with a di-deoxy cytosine, since this should prevent ligation.32 This variant had no activity in the target-bearing cells (bar 8), suggesting that ligation was important for in vivo activity. These results indicated that the empirical design features originally made were functionally important and provide support for, but do not insure, the basic structures envisioned in Figure 1(b–f).

**Discussion**

In this study, we reported a novel potential cell killing technique, AGPs, and demonstrated that they bound in a sequence-dependent manner to their target genes and inhibited DNA synthesis in vitro. AGPs selectively killed or inhibited the growth of *E. coli* bacteria with two different chromosomal targets, and in a dose-dependent manner. Targeting at least 10 bp of chromosomal DNA was required for AGPs to selectively kill target-containing bacteria.

Since our primary endpoint is inhibition of colony growth, the lack of blue colonies could be due to bacteriostatic rather than bacteriocidal activity. We did document an increase in the percentage of dead cells by direct staining, however, this needed to be done in the absence of ampicillin, and when the entire population of bacteria (transformed and non-transformed) was analysed, the increase in dead cells was relatively modest as expected. Due to the fact that anti-gene padlocks only enter a minor subpopulation of the bacterial cells, we are not reporting conventional MIC or MBC data. It is also conceivable that alterations in colony number could be due to population shifts, although this is somewhat unlikely since transformed bacteria were plated quickly after electroporation to avoid this potential problem. The lack of blue colonies could also theoretically be due to lack of expression of the targeted genes, although this seems unlikely given that these genes are non-essential under these plating conditions (in the presence of glucose and proline).

Although the purpose of these experiments was primarily to demonstrate proof-of-principle of this novel cell killing approach, it is tempting to speculate about their future potential as novel therapeutics. In this regard, a relatively low percentage of cells was killed or inhibited using the AGPs in a single transformation (typically 30% to 40%); however, use of standard DNA oligonucleotides probably reflects the worst-case scenario for activity. Standard DNA oligonucleotides are susceptible to exonuclease activity and backbone modification (such as with phosphorothioate) could be used to circumvent this problem, although this is somewhat unlikely since transformed bacteria were plated quickly after electroporation to avoid this potential problem. The lack of blue colonies could also theoretically be due to lack of expression of the targeted genes, although this seems unlikely given that these genes are non-essential under these plating conditions (in the presence of glucose and proline).
does not seem to be an issue (Figure 5a), AGPs must gain proximity to their DNA target. Moreover, the arms of the AGP must presumably denature from the backbone to interact with the target DNA, and pseudocomplementary bases might facilitate this denaturation while maintaining the ability to interact with both strands of the target. The DNA target also needs to locally denature for these molecules to interact, or the AGPs must invade the DNA duplex. In this regard, substitution of the standard DNA backbone with higher affinity backbones (e.g. peptide nucleic acids or locking nucleic acids) may facilitate this step in the process. Finally, the cell must be incapable of repairing these structures when bound to the target, though this seems likely given that these structures should be relatively foreign to evolutionarily selected DNA repair machinery. Any of these steps individually or in combination might improve killing efficiency.

In addition to the above considerations after entry into the cell, there are other general issues that will complicate delivery of oligonucleotides in whole animals. First, delivery of AGPs to cells may be difficult since AGPs are extremely large and anionic. Second, a large amount of oligonucleotide will likely be needed due to their high molecular weight. Third, standard DNA oligonucleotides are degraded by serum. In addition, the efficiency of oligonucleotide uptake by bacterial cells is estimated to be <10%. Furthermore, some studies suggest that bacterial cells possess poorly elucidated mechanisms to expel intracellular oligonucleotides, further decreasing their intracellular concentration. Although the peptide backbone modification of oligonucleotides might be used to increase their resistance to enzyme degradation and their affinity to the target DNA, uptake into bacterial cells can be limited by the outer-membrane LPS layer of Gram-negative bacteria. The combination of these issues results in significant impediments to clinical use of the agents and it is certainly likely that this strategy would be restricted to infections that cannot be treated using conventional antimicrobial agents.

Delivery to bacterial cells in vivo might be accomplished either by packaging AGPs into a bacteriophage or by covalent attachment to peptides capable of spontaneous membrane translocation. Wu et al. have identified unique DNA sequences that are taken up by eukaryotic cells from pools of random DNA sequences, and analogous experiments might work in bacterial cells and might even be species-specific. Activity may be further increased by targeting genes (polymerases etc.) that are essential to cell survival or by using multiple treatments. Alternatively, one might target pathogenesis-associated genes that are conserved between different bacterial species, such as toxin genes, pathogenicity islands or type III secretion mechanisms.

Non-specific toxicity is another important issue for oligonucleotide-based techniques, especially for antisense therapy, where relatively high concentrations are required to achieve good inhibition of gene expression due to the potential thousands of mRNA targets existing in the cell. For AGPs, however, it is possible that lower concentrations will be required, since only one to a few targets are present per cell. Our data show that non-specific toxicity of the AGPs in these experiments is generally low.

One important question is how are AGPs working in cells? We hypothesized that the arms and backbone of the AGPs each inextricably intertwine with one strand of an unwound, open DNA target using Watson and Crick binding (Figure 1f). Such a process may involve a single-strand invasion process possibly triggered by the initial accessibility of the gene target, similar to D-loop formation. In the structure–function experiments, we demonstrated that both halves of the molecule were required. Furthermore, one empirical design change from the original in situ padlock probes was to make both strands complementary to the target, and the variant with the non-complementary backbone (Figure 5b, bar 5) confirmed this design requirement. The result from the experiment using the AGP with two mismatches between the arms and the target (Figure 5b, bar 7) suggested that one may be able to use this strategy against cells that contain mutations of only 1–2 bases. The requirement of perfect complementarity of the arms suggested that ligation might be a key step for their activity. In the in vitro study, we demonstrated that the ligated AGPs are not removed from the target even during high temperature denaturation, causing a DNA polymerase to stop DNA synthesis (Figure 2d).

In vivo, the di-deoxy cytosine variant demonstrated no activity (Figure 5b, bar 8), suggesting that ligation may be required for activity also inside the cells. Although translesion replication is known to be capable of bypassing some lesions in DNA, an AGP bound to its target is substantially larger than most of the DNA adducts previously studied. Further, while the AGPs were designed to intertwine with both DNA strands, we demonstrated DNA synthesis inhibition in vitro only, and it remains to be demonstrated in transformed cells directly.

It is possible that gene-targeting AGPs act through a more complicated mechanism involving either homologous recombination or some other DNA repair process. Homologous recombination is unlikely to be a critical element of bacterial killing since one of the E. coli strains (HB101) we have successfully targeted is recA+. Although speculative, it is possible that bound AGPs initiate a repair process, which is stalled because the cell is incapable of recognizing and/or repairing these unusual bound structures. Transcriptional inhibition seems an unlikely mechanism for AGP induced cell death since these experiments were specifically designed to target genes that are non-essential under these growth conditions. The AGPs we report are somewhat reminiscent to the RNA: DNA hairpins presented by Cole-Strauss et al. as a potential strategy for gene correction.

If in further experiments we are able to increase activity, it is easy to imagine many potential gene targets for AGPs. In theory, one could target any unique gene region in pathogenic organisms, many of whose genomes have now been sequenced (e.g. E. coli O157 and Vibrio cholerae). Most significantly, once further optimized, AGPs may be a new tool to combat infectious diseases, and one intriguing application would be against multidrug-resistant or extensively drug-resistant Mycobacterium tuberculosis strains. In this regard, we have demonstrated the existence of over 1 million different 12 base targets that are unique to the O157 organism (compared with wild-type E. coli, Figure 4b). Although bacteria have a genome size in the order of several million bp, many mammalian species have genomes ~3 orders of magnitude larger. Therefore, eliminating eukaryotic cell populations using AGPs will likely be more difficult. However, in mammalian cells, extensive evidence has shown that oligonucleotides such as triplex-forming oligonucleotides can gain access to and bind to their DNA targets. We consider it likely that AGPs could also be used in mammalian cells. Targets in human cells might include viral genes...
important in malignancy (e.g. HPV genes in cervical cancer and HBV genes in hepatocellular carcinoma) or infectious diseases (e.g. HIV proviral DNA in latent cell reservoirs). With respect to delivery, a novel expression vector was recently developed to express single-strand DNA in mammalian cells that might provide an effective tool for delivering AGPs. Although sequence-based anti-gene approaches have been relatively limited to date, we are hopeful that the AGPs we report here will prove to be a valuable tool to genetically eliminate or inhibit specific cell populations.

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Transparency declarations

J. R. E. and A. R. P. have a pending patent application filed with the US-PTO. Other authors: none to declare.

Supplementary data

Colour versions of Figures 2 and 3 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References

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