Basic peptide-morpholino oligomer conjugate that is very effective in killing bacteria by gene-specific and nonspecific modes

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Basic peptides covalently linked to nucleic acids, or chemically modified nucleic acids, enable the insertion of such a conjugate into bacteria grown in liquid medium and mammalian cells in tissue culture. A unique peptide, derived from human T cells, has been employed in a chemical synthesis to make a conjugate with a morpholino oligonucleotide. This new conjugate is at least 10- to 100-fold more effective than previous peptides used in altering the phenotype of host bacteria if the external guide sequence methodology is employed in these experiments. Bacteria with target genes expressing chloramphenicol resistance, penicillin resistance, or gyrase A function can effectively be reduced in their expression and the host cells killed. Several bacteria are susceptible to this treatment, which has a broad range of potency. The loss in viability of bacteria is not due only to complementarity with a target RNA and the action of RNase P, but also to a non-gene-specific tight binding of the complexed nontargeted RNA to the basic polypeptide-morpholino oligonucleotide.

pathogenic bacteria | gene expression

Several methods that involve RNA, or some chemically modified form of RNA or DNA (siRNA, hammerhead RNA, antisense RNA, LNA (locked nucleic acid), or PNA (peptide nucleic acid); refs. 1–3 for LNA and PNA) are currently employed in attempts to inhibit the expression of genes in vivo. Among these are the use of basic peptide-morpholino oligonucleotide conjugates. Some practical success has been observed, in particular, in terms of curing mice infected with bacteria and prevention of infection of mammalian tissue by dangerous viruses (4–9). A previously undescribed basic peptide (cell-penetrating peptide: CPP), derived from a protein found in human T cells, Tyr-Ala-Arg-Val-Arg-Arg-Arg-Gly-Pro-Arg-Gly-Tyr-Ala-Arg-Val-Arg-Arg-Arg-Gly-Pro-Arg-Arg, which has no biological activity on its own (10), has been employed to make a previously undescribed conjugate with phosphorodiamidate morpholino oligonucleotides (PMOs) by the chemical method described by Abes et al. (11). This conjugate has been used to change gene expression in bacteria, and the results described here indicate that it is successful in this regard. The conjugate provides a method of transporting the sequence of bases in the PMO into bacteria where the external guide sequence (EGS) methodology allows the breakage of the target RNA by the resident RNase P (12). This combination of chemical and biological methods allows the killing of drug resistant bacteria and of bacteria in which the gene for gyrase A is targeted at concentrations of conjugate at least tenfold lower than previously used (13). Others have also attacked bacterial drug resistance in a similar manner (7–9) without the seemingly practical success now reported here for bacterial cells in liquid culture.

Results

Synthesis of Conjugate. The peptide was covalently bound to a primary amine at the 3′ end of the PMO (Fig. 1). No difference in biological activity between linkage at the 5′ or 3′ ends of the PMO was previously shown with other peptides (13). Several deletion versions of the peptide reported here (11, 14, 15, and 19 residues; Table S1) were linked to chloramphenicol transacetylase (CAT) or gyr PMOs, but they had no biological effect at all. Some PMOs had different numbers of nt (nucleotides) compared to a full-length conjugate, and these are indicated by the last numbers in the name of the conjugate (Table S1) and their efficiency is as measured in the text.

Location of Conjugates Inside Bacterial Cells. Fluorescein-labeled conjugate (AB2-FLgyr241) at a concentration of 1 μM was mixed with growing bacteria, incubated for a further 3 h (see legend to Fig. S1), and then samples were taken and examined in a fluorescent microscope (14). Because the cells were washed extensively after the fluorescent conjugate was added, any fluorescence in the cells likely indicates that the conjugate was internal and not located on the surface of the cells. The number of cells with fluorescein-conjugate in vivo was about 90% of total *Escherichia coli* cells, as it was with *Bacillus subtilis*. For *Klebsiella* the value of cell integrated fluorescence was about 17%, and for *Enterococcus* and *Staphylococcus aureus* it was close to zero with respect to the average background in the control cells (15). The viability assays shown below reflect these numbers. However, with *S. aureus* at 5 μM, the conjugate did inactivate the bacteria.

General Method for Testing Efficiency of Different Conjugates. Various base sequences were found to be effective as EGSs (13, 16) in part by assaying them in vitro as guides of RNase P to the target RNA. After conjugation of the PMO with the relevant base sequence to the peptide to make the compound, the resultant conjugate was added to bacteria growing in liquid culture to test whether the gyrase A target RNA conjugate was effective in killing bacteria and whether drug resistance [chloramphenicol (Cm) or penicillin (bla)] cells had also been killed by targeting those genes (see *Materials and Methods*) as described below.

The Effect of Conjugates Is Governed by the Action of RNase P. To ascertain that the results were mediated by the RNase P cleavage mechanism of target RNAs, a typical reaction was analyzed in a RNase P mutant strain of *E. coli* BW (17). In 10 mM arabinose, the strain made RNase P and it functioned normally. If arabinose was washed out of the growth medium and 0.5% glucose was added, no RNase P was made. Thus, cells were grown overnight in arabinose and were then diluted into fresh medium in two

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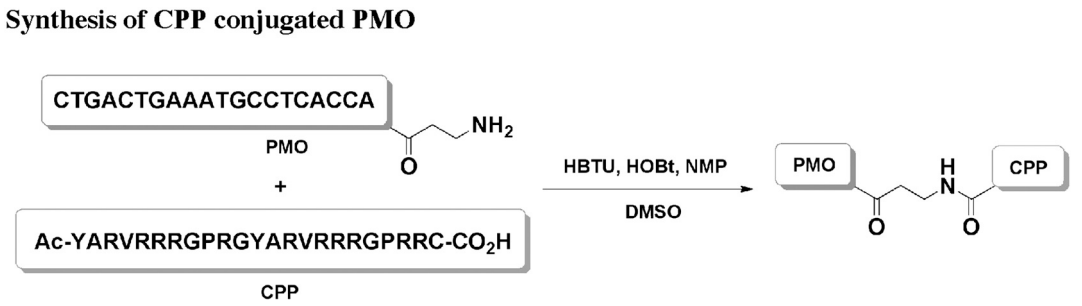

Fig. 1. A scheme for synthesis of the CPP-PMO conjugate (see *Materials and Methods*).

aliquots. One contained glucose and the other arabinose. After 6 h, the cells in glucose showed no effect on viability of two conjugates (500 nM) that contained either gyr313 or a scrambled sequence (Table 1; the characteristics of scrambled sequences are discussed below). However, the strain grown in arabinose showed a viability of approximately 10^{-2} after 6 h and a lower viability at 7 h. The data indicate clearly that RNase P does function to cleave whatever sequences are complexed with the specific and partially specific sequences of target RNA.

The Gyrase A Target Gene. The gyrase A target sequence was examined extensively by database analysis. A conserved region of the *gyrase A* gene was identified in bacteria and this sequence formed the basis for the EGS in *E. coli* and was useful in testing other bacteria (13). Data on the effectiveness of the low concentration of the conjugate that targeted the gyrase A gene are shown in Table 2. The viability of bacteria after 6 h of mixing was 7.7×10^{-5} for two separate conjugates tested at 5 μM (only one is reported in Table 4, gyr 313). The phenotypic change was from live to dead bacteria. Bacteria that differed from the *E. coli gyrase A* sequence by only a few nts (Table S2) could be tested with the same conjugate because a mismatch of 3 nt out of 12 had little effect on the success of the EGS sequence (18) if the 3 nt are not contiguous or are not located close to each other. Table 4, described below, illustrates the results of a screen of different bacteria with even lower concentrations of the conjugate.

Scrambled Sequences. Conjugates with scrambled sequences were effective as controls with the original PPMOs (peptide phosphorodiamiate morpholino oligonucleotides) used (13) in that they showed no decrease in viability of test strains under the conditions where gene-targeted PPMOs did. However, with the CPP in the conjugates used here, scrambled sequences in the morpholino oligonucleotides had a significant effect on viability (Table S4). In fact, the scrambled sequences had partial complementarity to various RNAs as judged by genomic searches in *E. coli*. The previously undescribed CPP-PMO conjugates reported here, which have more favorable binding to target RNAs and stabilized the complexes made with conjugates (see below), yielded the result in Table S4. These data indicate that the power of the technique reported here to decrease cell viability extends well beyond the extended matches made to specific gene sequences. Sequences with only 5 or 9 nt of complementarity also decrease cell viability. This would be expected only if the CPP in the conjugates had extremely strong binding to a region of target RNA (or DNA) in vivo to which a small sequence, of e.g., 5 nts, has complementarity. In fact, SCR9 was less effective compared to the other scrambled sequences in Table S4 in decreasing cell viability at lower conjugate concentrations. The effectiveness of SCR 9 was also about 70-fold less than AB2-gyr313-14 as a function of concentration from 50 nM to 1 μM. AB2-T11, which has a sequence of 11 consecutive Ts, had no effect at concentrations up to 500 nM but did have a 20% decrease in viability at 1 μM. This sequence would be a suitable control for most reactions reported here.

When AB2 synthesized by biopeptides was used as the CPP, the effective concentration of the resulting conjugate was severalfold lower than that of AB1.

Drug Resistance. Conjugates were added to growing bacteria to test whether cells harboring drug resistance [chloramphenicol (cat) or penicillin (bla)] had resulted in the cells killed by inactivation of expression of the *cat* and *bla* genes.

An examination of databases showed that a sequence in the gene for Cm resistance that was used successfully for an EGS (19) was in fact completely homologous (100% sequence identity) to a 12-nt sequence of several different genes in *E. coli* and several other bacteria. As the stringency was lowered in this test, many more sequences in different genes showed homology to partial sequences of the *cat* gene. In fact, a test of this sequence in our conjugate showed a loss of viability on several bacteria in the presence or absence of Cm in LB broth we used for assays. In fact, the gene for Cm resistance did function well for assays in our system as shown in Table 3. The viability of *E. coli* after administration of the conjugate dropped to about 5×10^{-4} when the assay was done at 4 h after administration, but this compound was still effective at lower concentrations, e.g., 500 nM, rather than the 5 μM used in most other experiments. Although the results with more than one concentration of CPP-PMO are given here, it is useful to note that using a lower concentration (500 nM), which may not be as effective as an agent at higher concentration (5 μM), might still be practical in experiments with animals. It should also be noted that when the conjugate

Table 1. RNase P controls the change of phenotypic response

6 h	10 mM arabinose	0.5%, wt/vol, glucose
gyr 313	1.7×10^{-2}	0.5
SCR	1.3×10^{-2}	0.27
7 h		
gyr313	1.8×10^{-3}	2.2
SCR	2.6×10^{-4}	0.68

E. coli BW was assayed under different conditions as indicated in the text. The assay was done twice at 6 h and once at 7 h. The conjugates used were AB1gyr313 and AB1SCR, both at 500 nM, and viability, measured as colony-forming units on agar plates, is indicated in the table

Table 2. Loss of viability of bacterial phenotype after mixing with the CPP-PMO conjugate

Assays for killing of bacteria by the gyrase A conjugate.

Conjugate	Concentration, μM	Viability
AB1 gyr313	5	7.7×10^{-5}
AB1 gyr313	0.5	1.4×10^{-2}

Cells were grown in LB broth as described in *Materials and Methods* and mixed with the conjugate with the relevant base sequence after dilution from an overnight culture. Viability was assayed after 6 h. At least two experiments were done in each case. The peptide used was abbreviated as AB1 (see *Introduction*).

Table 3. Loss of viability of bacterial phenotype after mixing with the CPP-PMO conjugate

Assays for killing of bacteria that contained genes targeted to chloramphenicol (Cm) and ampicillin (bla) resistance.

Conjugate	Concentration, μM	Viability
AB1 CAT	9	4.4×10^{-4}
AB1 CAT	5	3.4×10^{-4}
AB1 CAT	0.5	9.7×10^{-3}
AB1 bla	5	4.3×10^{-4}
AB1 bla	1	5×10^{-4}

Cells were grown in LB broth as described in *Materials and Methods* and mixed with a conjugate with the relevant base sequence after dilution from an overnight culture. The host cells were *E. coli BL21/pACYC* and *SM105* for Cm at 0. 5 μM at 4 h. *E. coli BL21/pUC19* was used for the bla experiment and was assayed at 6 h after conjugate addition. At least two experiments were done in each case. The peptide used was abbreviated as AB1 (see *Introduction*).

targeted to Cm resistance is added in conjunction with a conjugate targeted to the gyrA mRNA, each at 100 nM, the viability of cells decreased to about 10^{-2} from about 0.5 when the conjugates were added separately as has been noted before with a different experimental system (17).

As a quick assay to determine if cells were susceptible to the peptide, either the peptide alone was added to a growing culture or the peptide was added simultaneously with the chloramphenicol (CAT) PMO alone; viability was examined at 4 h of culture growth. The results of such assays (nine samples) showed the viability with CPP does drop in *E. coli* to about 0.7, or 0.63 for cells with plasmids, compared to control cells when nothing is added for concentrations of the peptide of 5 μM. Viability goes up as the peptide concentration is lowered. However, when the PMO is added noncovalently with the CPP, viability decreases about 30% compared to when CPP was present alone, an indication that the bacteria tested are susceptible to the CPP-PMO treatment. No decrease in viability was seen when PMO was added by itself.

There is more than one mode of resistance to penicillin in many bacteria as judged by database searches. An EGS was used with success against the penicillin resistance gene in *E. coli* (19).

Table 4. Summary of different bacteria tested for sensitivity to different conjugates

Bacterium	Conjugate	Conc., μM	Viability
E. coli	AB2-gyr313-14	0.5	10^{-6}
S. typhimurium	gyr313-14	0.5	10^{-3}
B. subtilis	gyr313-14	0.5	6×10^{-6}
P. syringae	gyr313-14	2	6×10^{-2}
Acinetobacter	gyr313-11	0.5	3×10^{-3}
K. pneumoniae	gyr313-14	0.5	4×10^{-5}
S. aureus	Sagyr313-14	5	7.5×10^{-4}
Enterobacter	AB1-CAT	5	0.02
Enterobacter	AB2-gyr313-11	5	0.08
S. aureus	AB2-gyr313-14	1	0.30
E. faecalis	AB2-gyr313-11	2	0.03
M. marinum	AB1-mmbla	1	0.27
M. marinum	AB1-gyr313	1	0.12

M. marinum was assayed after two days of inclusion of the conjugates at 30 °C and was also incubated for one day from an overnight culture. The full-length conjugates had a morpholino oligonucleotide of 17 nt plus ACCA at their 3′ termini. All assays in the top part of the table were at 6 h after administration of the conjugate. Assays in the bottom of the table for AB1 conjugates were assayed after 4 h. The last two digits at the end of the AB2 designation indicate the number of nts in the PMO. The one conjugate preceded by Sa was designed with specific complementarity to *S. aureus gyrase* gene. Tests were done as described in the legend to Table 1. Viability of CPP alone in the top part of the table is greater than 0.5 at 1 μM or higher. All the conjugates in the top part of the table are AB2

When possible, the same conjugate was used for other bacteria. In other cases, new conjugates will have to be made. For the conjugate with the previously successful EGS sequence (17), the viability of bacteria in which the *bla* gene was located on a multicopy plasmid was 4.3×10^{-4} at 5 μM, somewhat higher at 1 μM (Table 3).

The effective concentration of the conjugate in the experiments in Tables 2–4 was between 500 nM and 5 μM. This is at least 10- to 100-fold more effective than previous measurements with CPPs in conjugates made by AVI Biopharma (13). In particular, the lowest values achieved were for *B. subtilis*, discussed below, as we determined earlier with an AVI Biopharma CPP.

Various *E. coli* strains grown with a conjugate that targeted the *bla* gene in the absence of ampicillin were also depleted in viability. These data, as was the case for the *Cm* gene noted above, indicated that partial homologies with nontargeted sequences are an important fact with conjugates that have the 23 amino acid residue sequence. There are two exceptions: One was the strain BL21/pUC19 that contains a plasmid coding for *bla* resistance and the other was strain BL21/pACYC that contains a plasmid coding for the Cm resistance gene. In the presence of ampicillin, BL21/pUC19 decreased in viability compared to strains with the *bla* gene on the chomosome by a factor of about 10. BL21/pACYC was decreased in viability about 100-fold more than strains that were either Cm resistant or nonresistant in the presence of chloramphenicol. We also note that the mmbla conjugate had an effect on the viability of *E. coli* but virtually none (10-fold lower on *E. coli*) on *Mycobacterium marinarum*. These data indicate that the primary, but certainly not complete, cause of loss of viability results from the toxic effects of the conjugate on the bacteria used and is discussed below.

Time of Administration of Conjugate. To explore the possibility that an additional mixture of the conjugate would decrease the viability of cells in our experiments, a second administration of conjugate was added 4 h after the first dose. In these experiments, cells were assayed at 6, 8, and 10 h after the first conjugate administration. The results are shown in Table S3 and indicate that the second administration of gyrase A CPP-PMO apparently killed more than 99% of bacteria in cultures of *E. coli* or *B. subtilis*, i.e., about 10^{-6} or a lower fraction of cells survived. Furthermore, the results showed that the time from the first administration of conjugate was optimal at 6 h.

Effect on Other Pathogenic Bacteria. A survey of the effects of different conjugates on other bacteria is presented in Table 4, which shows viability results with particular conjugates used that are perfectly matched in base sequence complementarity with *E. coli* genes. However, a new conjugate was also made that was perfectly matched in base sequence complementarity to the *S. aureus gyrase* gene. This conjugate was successful in reducing the viability of *S. aureus* to 7×10^{-3} at 5 μM and lower by a factor of roughly two at 10 μM. The addition of the conjugates was bacteriocidal and obviously reduced viability, e.g., for *Acinetobacter* and *Klebsiella*. Results of assays of other bacteria are shown in Table 4. In this case, the effects on *E. coli* and *B. subtilis* W168 are remarkable, as observed previously (13), for their effectiveness at low concentrations of the conjugate. *Pseudomonas syringae* and *Acinetobacter* were also severely affected by the gyrase CPP-PMO. Apparently an *Enterobacter* strain that is not identical to its most pathogenic relatives is decreased somewhat in viability at 5 μM, but not enough to justify its use as a therapeutic agent with the *E. coli*–specific gyrase A conjugate. *M. marinum* did show a slight decrease in viability in the presence of a conjugate for penicillin resistance. The other bacteria listed had to be investigated further for their potency as a function of concentration. Note that the CPP-PMO used was specific for the *E. coli gyrase A* sequence. We previously had determined that various

naked RNA EGSs, administered by a biological process in which synthetic genes coding for certain functions were transcribed, were also effective in decreasing the expression of the genes that controlled virulent functions in dangerous, pathogenic bacteria (16).

The minimum inhibitory concentration (MIC_{50}) for *E. coli* is 0.4 μM with AB2gyr313 as determined by standard procedures. The MIC_{50} for *S. aureus*, with a conjugate made specifically for that bacterium against gyr313, is 6 μM. No MIC_{50} was measured with the other bacteria that lack perfect homologies in sequence between the PMOs designed against *E. coli* genes and the corresponding genes for the other bacteria except for *mmbla* for *M. marinarum*, which was not inhibitory.

Many of the tests listed in Table 4 were performed with AB2-T11 (1 μM) as a control, which had a viability of 0.5 on average.

Complexes of Conjugates with Target RNA Analyzed by Electrophoresis in Gels. Intact, double-stranded RNA complexes can be distinguished from complexes made with the conjugates described here by a gel retardation assay (20) as shown in Fig. 2*A*. Two conjugates, AB1gyr313 and PPMO gyr313 (13), were tested in these reactions with a ^{32}P-labeled mRNA that contained the gyr313 sequence (lanes 2–3). If the complexes were heated to 90 °C and rapidly cooled, the target RNA now migrated with the control band if the complex had been denatured (lanes 13–15). If this experiment is repeated at different temperatures, the melting temperature of the conjugate should be measured accurately. The naked RNA EGS:mRNA complex did show this behavior when added at a high concentration (Fig. 2*B*). About 10% of the mRNA was complexed with the EGS as detected quantitatively by this method and at temperatures at 65 °C or higher, the amount of complex is decreased. The RNA degraded into oligo- and mononucleotides at the high temperature for longer periods of time at 90 °C. More important are the negative controls (Fig. 2*C*) in which the CAT conjugate AB1CAT did not show any binding to the gyr313 RNA and AB1gyr313 does not show any binding to the CATmRNA. This approximate method of measuring melting energy of the complex indicated that the complex with the conjugate had a higher binding energy than one with naked RNA in the complex although their base sequences are identical. These experiments give credence to the notion that the conjugate binds more tightly to RNA than does the naked RNA EGS and supports the binding of the conjugates to partial homologous sequences in bacteriocidal action.

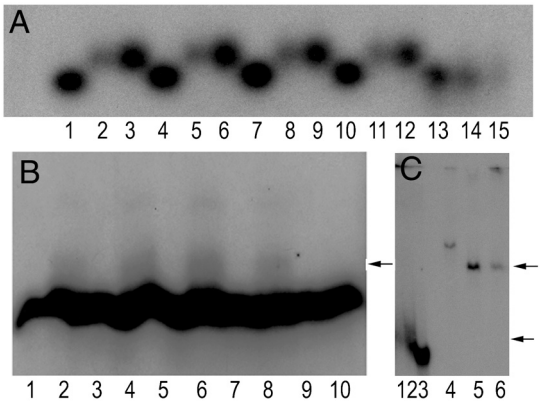

Fig. 2. Approximate determination of melting temperatures of different complexes. (*A*) Complexes with conjugate (0.5 pmol) and target RNA (0.1 pmol) were run in 1.5% agarose gels with buffers as described in Talbot and Altman (19). mRNA alone was incubated for 5 min at room temperature. Samples (10 μL) in binding buffer (19) were incubated at 37 °C for 15 min and then 10 min for the designated temperatures, placed on ice for 5 min and were loaded on a 1.5% agarose gel and run for about 1.5 h at 110–130 mAmp and 100 V. RNA: gyrA mRNA from *E. coli* (206 nt); conjugate: AB1gyr313; PPMO gyr313. Controls of each sample prepared on ice were also run and did not differ in migration from RNA alone at each temperature. Each temperature set contained the RNA alone, incubated for 5 min at 37 °C prior to addition of the rest of the sample in each case, and the conjugates AB1gyr313 and then PPMO gyr313 are in each set. Lanes 1–3: incubation at 30 °C; lanes 4–6: incubation at 37 °C; lanes 7–9: incubation at 45 °C; lanes 11–12: incubation at 55 °C; lanes 13–15: incubation at 90 °C. (*B*) Complexes with naked RNA EGS (5 pmol per reaction) and target RNA (0.1 pmol per reaction). The samples were treated as described above except that incubation of the total mixture was for 20 min at room temperature and then 20 min at the designated temperature below, and the samples were put on ice before loading on gels. Electrophoresis was in a 12% polyacrylamide gel with the following freshly made buffer for 6 h and 50–60 mAmp at 200 V: 50 mM Hepes, pH 8, 50 mM KCl, 1 mM Mg acetate. For lanes 1–10 the odd-numbered lanes were target RNA alone as above, and the even-numbered lanes were the RNA EGS-gyr313. The arrow shows the position of the complex. Lanes 1–2: 30 °C; lanes 3–4: 37 °C; lanes 5–6: 45 °C; lanes 7–8: 55 °C; lanes 9–10, 90 °C incubated for only 3 min. (*C*) Same conditions as for *B*. The top arrow shows the position of the CAT mRNA, and the bottom arrow shows the position of the complex with gyr313 mRNA. Lane 1: AB1gyr313 and RNA gyr313, 37 °C as were the following lanes; lane2: PPMO gyr313 and gyr313 RNA; lane 3: AB1CAT and gyr313 RNA; lane 4: AB1CAT and CAT RNA; lane 5: CAT RNA alone; lane 6: AB1gyr313 and CAT RNA. The results were repeated in two experiments.

Discussion

The availability of a previously undescribed basic CPP that can be conjugated to a PMO offered an opportunity to test the potency of this compound in terms of bacterial killing and drug sensitivity. Indeed, this conjugate was more effective than previous trials of this kind. The effective concentration of the conjugates we used (plural because the PMOs had different base sequences) was ten- to a hundredfold more effective than previous compounds tested. The ability of mice to withstand the administration of similar compounds (4, 5) indicates that these conjugates are worthwhile candidates for use in infected animals.

The nature of the *gyrase A* gene as a pivotal target in the viability of bacteria (21) was proven again in our experiments. This gene, in which a segment of its sequence is well conserved throughout bacteria, proved to be a valuable target for EGSs technology even though for some bacteria there were changes of a few nucleotides in comparison with our standard, *E. coli*. Although it is a simple matter to change the sequence of an EGS for *E. coli* to one appropriate for other bacteria, in fact, the same EGS works against a number of bacteria if only a few nts differ in the sequence of the conserved *gyrase A* region (18). Some discussion of the bacterial specificity is given below, although it is appropriate to indicate that some of the bacteria, even if they differ only by 3 nt compared to the *E. coli* sequence, may affect viability considerably (e.g., *Enterococcus faecalis*, *M. marinum*, and *Enterobacter cloacae*). It is necessary to make conjugates in which the PMO has the identical sequence to the genome being targeted, as has been done for *S. aureus* (Table 4).

The utility of drug resistance genes is not quite as universally useful as that of the *gyrase A* gene. Here, for chloramphenicol and ampicillin, there is a much greater variability in base sequence and, in fact, in the gene or genes that affect drug resistance of penicillin or ampicillin. In these cases, the ability of the particular conjugate to kill these cells was effective, although not quite as generally remedial as conjugates on the *gyrase A* gene (Tables 2 and 3).

Another control that is relevant includes assays with a conjugate targeted to the *lac* gene in *E. coli*. Although attacking the lac mRNA alone should have no effect on cell viability, both a wild-type *E. coli* and a strain with a deletion in the *lac* gene were reduced in viability to about 10^{-4} at 500 nM concentration of the conjugate. This is the clearest indication that gene-specific targeting, itself, is not responsible for the total effect of the conjugates used. However, in a comparison of gyr conjugates with the intact PMO sequence and a partial gyr sequence, it is apparent that targeting a particular gene does have an effect on viability (Table 4) as was also pointed out with respect to the *bla* and *cm* genes in the

absence and presence of the appropriate drugs. A full-length PMO has a greater effect on viability than does a shorter PMO that also attacks, e.g., the *gyrase* gene. The small difference in length of the PMO should not make a significant difference in reducing viability through a bacteriocidal action. The proportion of loss of viability due to bacteriocidal action and gene-specific targeting is difficult to judge, but the bacteriocidal action seems to have a greater effect. It is also clear that a contaminant in our conjugate preparations is not responsible for the bacteriocidal action because some conjugates, e.g., AB2-T11, have a much lower effect on viability than other conjugates.

The variety of bacteria affected is interesting from a pathological point of view. However, only close relatives of nonpathogenic strains have been tested to date. Rigorous viability tests on pathogenic strains, with perfectly homologous conjugates, of these bacteria under appropriate containment facilities must be carried out.

Control experiments with "scrambled" base sequences in the morpholino oligonucleotide yielded surprising results in that these conjugates also killed bacteria in a manner that did not target specific gene sequences. That is, in attempts to alter drug resistance of bacterial strains or to alter the expression of nonessential enzymes, strains were killed by scrambled sequences. In fact, scrambled sequences have considerable homology to many sequences in a bacterial genome at levels that do not necessarily include the full-length sequence that, for example, altering drug resistance requires. This conclusion concerning loss of viability is not true of conjugates that have a much shorter peptide sequence (13). This supposedly nonspecific effect of killing by scrambled sequences might require the action of RNase P. The strong binding of the unique basic peptide in the conjugates described here to any piece of nucleic acid leads to a very tightly bound complex of the conjugate with an RNA that might only have five complementary nts. In this sense, the mode of killing has little to nothing to do with targeting a specific gene, although the latter must be part of the inactivation of the expression of genes we report here. It is possible that tightly bound complexes described here may alter normal processes of translation, transcription, or replication inside cells, certainly in the latter case if the complexes also form with single-stranded DNA as a prelude to transcription or replication.

Further work on the peptide composition may be useful in terms of improving the efficiency of the conjugate for further employment, for example, by making it more efficient as a means of penetrating cell walls and membranes of different bacteria. The correlation between uptake of a fluorescent conjugate and lack of viability response to the conjugates should also be explored for further use in terms of designing appropriate basic peptides for the conjugates. Only L forms of amino acid residues were used in our CPP. D forms can be substituted at the interface with the PMO and other, nonnatural amino acid analogs can also be used. Furthermore, the size of the peptide we used is large, and it is not clear that all of it (the sequence is duplicated almost precisely twice) is necessary although the latter half alone is not sufficient for effectiveness. A rational basis for designing a CPP is still absent, but it is certain that more knowledge regarding cell wall and membrane synthesis is essential.

The future use of the conjugates reported here in human drug therapy, or similar PNA/LNA compounds (22), is especially promising in surface wound infections, where the drug can be layered or sprayed on the wound, or in internal infections by administration of oral or intravenous injection of the drug. Other RNA-like drugs, aside from those where direct injection of the drug into the affected tissue is used, as in the eye, may also be useful, although it is not clear that their utilization would be as effective as the conjugate described here. Biological delivery of drugs in humans, using the *Salmonella typhimurium* vehicle developed by Liu and coworkers (23), offers a very different mode of EGS delivery.

Materials and Methods

Bacterial Strains. *E. coli* SM105 and *E. coli* CAG5050 were obtained from the E. coli Culture Center at Yale University. *E. coli* BW was grown from our collection. *P. syringae*, grown at room temperature in trypsin soy broth (TSB), *E. cloacae* M322 and *Klebsiella pneumoniae*, grown in LB broth at 37 °C, were obtained from John Wertz (Yale University, New Haven, CT). The following strains were obtained from the American Type Culture Collection: *E. faecalis* and *M. marinum*. *Acinetobacter* ADP1 was a gift from D. Parke (Yale University, New Haven, CT) and was grown in TSB at 37 °C. *E. faecalis* was grown in brain heart infusion at 37 °C. *Staphylococcus aureus* RN4220 was a gift from A. L. Cheung (Dartmouth College, Hanover, NH) and was grown in TSB at 37 °C. *Bacillus subtilis* W168 was obtained from the B. subtilis Stock Center.

Synthesis and Column Purification of a Conjugated Peptide Phosphoroamidate Morpholino Oligonucleotide. A CPP, derived from human T cells (10) and described in the introduction, was synthesized via solid-phase chemistry at the Keck Biotechnology Resource Laboratory of Yale University, and a PMO with an appropriate base sequence was purchased from Gene Tools, LLC. The original basic polypeptide used, AB1, had an additional Cys residue at its C terminus. Subsequently, as noted below, another basic polypeptide, AB2, was used that lacked the Cys residue at its C terminus (Keck Facility or Biopeptides, Inc.). Reagents used for chemical synthesis were purchased from Sigma-Aldrich. CPP conjugated PMO was synthesized as described in ref. 11. The peptide was covalently bound to a primary amine at the 3′ end of the PMO. In all the experiments in this paper, deionized water was used.

To a solution of peptide acid (1.25 mg or lower) in 1-methyl-2-pyrrolidine (NMP, 0.5 mL) at room temperature were added *O*-(benzotriazole-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluoro phosphate (HBTU, 0.5 mg), *N,N* diisopropylethylamine (DIEA, two drops from a 20G11/2 in. needle and a 1-mL Norm-Ject syringe), and 1-hydroxybenzotriazole (HOBt, 0.3 mg). The mixture was stirred at room temperature for 10 min, and a solution of a morpholino oligonucleotide (3.1 mg or lower) dissolved in DMSO (0.5 mL) was added to the mixture. The reaction mixture was stirred at 40 °C for 3 h, cooled to 0 °C, and diluted with H_2O (5 mL).

The synthesized samples were diluted to a final volume between 10 and 15 mL and loaded onto a 30-mL CM-sepharose column prewashed with H_2O. The column was washed with H_2O (100 mL) to remove unconjugated reactants and other mixtures, and then the conjugate was eluted with 2M guanidine-HCl (50 mL). One- or two-milliliter fractions were collected. A NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies) was used to read the OD_{260} of each fraction, and fractions were run on a standard 15% (wt/vol) polyacrylamide SDS-Laemmli gel to determine which fraction contains the conjugate. The conjugate came off in the fractions just after the OD_{260} peak, and the synthesis is diagramed briefly in Fig. 1.

The fractions with the conjugate were then run over a Supel-Select HLB SPE column (6 mL, 200 mg; Supelco) to remove guanidine and to concentrate the material. The column was prepared by washing with methanol (4 mL) followed by H_2O (6 mL). The fractions with the conjugate were pooled, diluted 1:1 with H_2O, and loaded onto the column. The column was washed with H_2O (12 mL) to remove salts, and the conjugate was eluted with 50% (vol/vol) acetonitrile (6 mL). The first four eluted samples were collected in 0.5-mL fractions, and the latter four were 1 mL. These fractions were then lyophilized in a Speedvac (Savant) concentrator. Purified conjugate fractions were resuspended in H_2O, and a Nanodrop Spectrophotometer was used to read the OD_{260} of each fraction and concentrations were determined. The peak of purified conjugate was in fractions two and thee.

Calculation of concentration of conjugate in H_2O or in solutions of low molarity was achieved by measuring the optical absorbance at 260 nm in water of the PMO after that solution had been made by weighing out a standard amount from the dry material supplied by the manufacturer. The optical absorbance of conjugate was calculated using the PMO standard that was weighed out because the absorbance of the basic peptide and other chemicals in the conjugation mixture did not show high values at 260 nm. Conjugates stored in H_2O solutions at 4 °C kept their original biological activity for several months.

Fluorescein-labeled conjugates were made by covalently linking a morpholino oligonucleotide to a fluorescein-labeled basic polypeptide made by the Keck synthesis facility at Yale University. The fluorescein was linked to the N terminus of the polypeptide The procedure for conjugation was the same as the one used for unlabeled conjugates.

Assays of Effectiveness of Conjugates in Vivo and the Cleavage of Various RNAs in Vitro. The methods described in Shen et al. (13) were used with the following modifications. Briefly, growing cultures, which were diluted from overnight or stock cultures to a concentration of 10^4 cells/mL, were mixed

directly with the conjugate in liquid medium and the cells were incubated for another 4 or 6 h as listed and assayed for phenotype. Unless noted otherwise in *Materials and Methods*, cells had been suspended in 50 μL after overnight growth in LB broth in 1.5-mL Eppendorf tubes and shaken with incubation for times given in table legends. One exception to this procedure is the growth of *M. marinum*. In this case, cells were suspended in Middlebrook 7H9 medium with the ADC supplement (Difco) and grown at 30 °C in the dark for the appropriate time. *M. marinum* cells were plated on agar Middlebrook TH10 plates with the OADC supplement. *P. syringae* was grown, as noted, at room temperature.

Fluorescence of Conjugates in Bacteria. Three hundred microliters of a 1:500 dilution of the overnight culture of bacteria were incubated with 1 μM of FL-AB2-gyr241 or water (negative control). As a positive control 10 μM of the tagged PPMO conjugate (PPMO-CAT-FL) was used. The cells were incubated with the respective conjugate for 3 h at 37 °C in a shaking incubator. The cells were then washed with PBS solution three times, harvested by centrifugation at 9,000 × *g* and the pellet was resuspended in 5 μL 1× M9. The cells were placed on agar-coated slides [1% (wt/vol) agarose in 1× M9, 0.2% (vol/vol) glycerol] and covered with a glass coverslip, and immediately viewed under either a Nikon 80 I equipped with 100× phase contrast objective and a Hamamatsu Orca II ER camera or a Nikon Eclipse Ti microscope equipped with a 100× phase contrast objective and a Hamamatsu Orca ER camera. The images were analyzed using Metamorph.

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