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Phenylethynylpyrene-labeled oligonucleotide probes for excimer fluorescence SNP analysis of 23S rRNA gene in clarithromycin-resistant *Helicobacter pylori* strains

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Abstract

The use of phenylethynylpyrene excimer forming pair in the design of specific fluorescent probes for determination of A2144G (A2143G and/or A2143C) mutations in 23S rRNA gene of *Helicobacter pylori* is described. Analysis of fluorescence spectra of model duplexes revealed optimal positions of fluorophore residues in the probe sequences for maximum efficiency of SNP detection. Application of excimer forming probes for analysis of DNA samples isolated from natural bacterial strains of *H. pylori* was demonstrated.

Keywords: Phenylethynylpyrene; DNA probes; Excimer fluorescence; SNP detection; *Helicobacter pylori*

1. Introduction

Single nucleotide polymorphism (SNP) analysis is an important part of modern DNA diagnostics, as SNPs are often utilized to determine hereditary susceptibility to various diseases and individual sensitivity to drugs [1]. The fluorescence based strategies at present take the leading position among genetic analysis techniques [2–5]. Methods using two dyes are of particular interest. If both dyes are fluorescent and there is an overlap between an emission band of one dye and an absorbance band of a second dye, fluorescence resonance energy transfer (FRET) can occur. The efficiency of FRET is dependent on the distance between the dyes, and this property was used in several DNA detection assays. The most popular in analysis, however, become methods where the acceptor dye is a non-fluorescent quencher. The first DNA detection format based on fluorophore–quencher interactions, molecular beacons, was published in 1996 [6] and many other effective techniques have been developed since [2–5]. Among these, the FRET version of TaqMan analysis proved especially suitable for SNP detection [7].

Hybridization probes based on fluorescent dyes sensitive to microenvironment could be successfully used for the detection of enzymatically amplified DNA. The probe must be capable of distinguishing a single nucleotide alteration in the target or matched/mismatched probe–target duplexes. Conjugates of peptide nucleic acids (PNA) oligomers and thiazole orange (a dye for double stranded DNA staining)

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bound to N-terminus displayed emission enhancement upon binding to DNA [8]. Recently, PNA probes containing internal thiazole orange derivative as a base surrogate have been designed. These show a high sensitivity to the nearest mismatches, along with low susceptibility to fluorescence quenching by adjacent nucleobases [9].

Fluorescent tetracyclic aromatic hydrocarbon pyrene and its derivatives have a long excited state lifetime, so their emission is sensitive to microenvironment [10]. The difference in pyrene interaction with single stranded DNA, and with perfect and mismatched DNA duplexes, has been used in several protocols for SNP detection. In the MagiProbe system, pyrene and fluorescein in the middle part of a DNA conjugate constitute a mismatch-sensitive energy transfer pair: the fluorescein emission is quenched by pyrene in a free probe and remains quenched upon hybridization with mismatched target, but it is kindling in the case of a perfect match [11]. Oligonucleotide probes containing pyrene modified nucleosides 5-[3-(1-pyrenecarboxamido)propynyl]-2′-deoxyuridine [12] and 5-(pyren-1-ylethynyl)-2′-deoxyuridine [13] as single labels allow the detection of SNP.

The excimer emission from two pyrenes placed in close proximity (λ_{max} around 470 nm) is easily distinguishable from the emission of a single pyrene label (λ_{max} 370 and 400 nm). Because of this interesting property, two oligonucleotide probes bearing pyrene residues on their 3′- and on the 5′-termini can be hybridized to a single stranded target in tandem for the mismatch detection [14–17].

Another approach is to label the probe with two pyrene residues. Several pyrene bichromophores have been designed and their oligonucleotide conjugates showed excimer-to-monomer emission ratio changes upon hybridization [18–22], which made them suitable for SNP analysis [23]. Pyrene bichromophore containing probes can be prepared using two condensation steps of a single-pyrene non-nucleotide reagent [24–26].

1-Phenylethynylpyrene (PEPy), a derivative with extended π-conjugation compared to pyrene, also easily forms excimers but possesses brighter and red shifted emission [27]. A phosphoroamidite reagent for the solid phase synthesis of PEPy labeled oligonucleotides was prepared and PEPy conjugates were used for excimer formation and energy transfer studies [28,29]. Very recently, Filichev and Pedersen also observed PEPy excimer emission on oligonucleotides [30]. The potential of PEPy as a highly fluorescent label for biomolecules has been recognized [31]. In this paper we propose a new detection system comprising two proximal PEPy fluorophores acting as a molecular switch for homogeneous analysis of known SNPs A2143G, A2143C, A2144G in the gene of 23S rRNA *H. pylori*. These mutations determine clarithromycin resistance of *H. pylori* strains due to inhibition of clarithromycin binding to the 23S rRNA [32–34].

2. Materials and methods

2.1. Probe synthesis

The synthesis of the modified PEPy phosphoramidite reagent has been described previously [29]. All oligonucleotides were synthesized on an automated ASM-102U DNA/RNA synthesizer (BIOSSET Ltd., Russia) using a phosphoramidite protocol on a 0.2 μmol scale according manufacturer's guidelines with standard reagents (Cruachem). The PEPy amidite was used as a 0.1 M solution in a 1:1 (v/v) mixture of dry MeCN and CH_2Cl_2. After synthesis and removal from the solid support with concentrated aqueous ammonia, the oligonucleotides were deprotected for 6 h at 55 °C. Resulting solutions were evaporated, redissolved in 300 μl 1.0 M $LiClO_4$ and precipitated with acetone (1.5 ml). Oligonucleotides were purified by denaturing 20% PAGE with 7 M urea. The bands containing target products were visualized at 260 nm, removed from the gel, and eluted with 0.5 M $LiClO_4$. This was then concentrated and desalted on Sephadex G-25 columns.

The structure of oligonucleotides was confirmed by MALDI-TOF analysis on a Voyager-DE BioSpectrometry Workstation (PerSeptive Biosystems, positive ion mode) with a mixture of 2,5-dihydroxyacetophenone and diammonium hydrogen citrate as the ionization matrix. All the probes showed correct masses ±0.05%. Probe concentrations were calculated based on their room-temperature absorbance at 260 nm measured on a UVIKON 810 spectrometer (KONTRON Instruments, Switzerland).

2.2. Asymmetric PCR amplification

DNA samples with a known point mutation in 23S rRNA gene *H. pylori* were provided by Prof. F. Megraud (Bordeaux, France). Direct primer Cl1 (5′-GCGTTGAATTGAAGCCCG-AGTAAAC-3′) and reverse primer Ap23r8 (5′-AGTAAA-GGTCCACGGGGTCT-3′) were used for the amplification of a 200 bp fragment of the 23S rRNA gene. Each amplification reaction mixture was made up of 5 μl of DNA solution (2 ng/μl), 4 μl of a 2.5 mM solution of dNTPs, 5 μl of 10× PCR buffer, 2 μl of a 5 μM solution of each primer, 0.2 μl Taq-polymerase (5 U) and water, making a final volume of 50 μl. After denaturation at 94 °C for 3 min, 30 cycles of amplification (94 °C for 1 min, 55 °C for 20 s and 72 °C for 30 s) were performed in a TerCyc cycler (DNA-Technology, Russia). Obtained PCR products were used as templates in an asymmetric PCR. The asymmetric PCR mixture usually contained 5 μl of a reaction mixture from the previous PCR, 4 μl of a 2.5 mM solution of dNTPs, 5 μl of 10× PCR buffer, 20 μl of a 5 μM solution of the reverse primer Ap23r8, 0.2 μl Taq-polymerase (5 U) and water, making a final volume of 50 μl. The amplifica-