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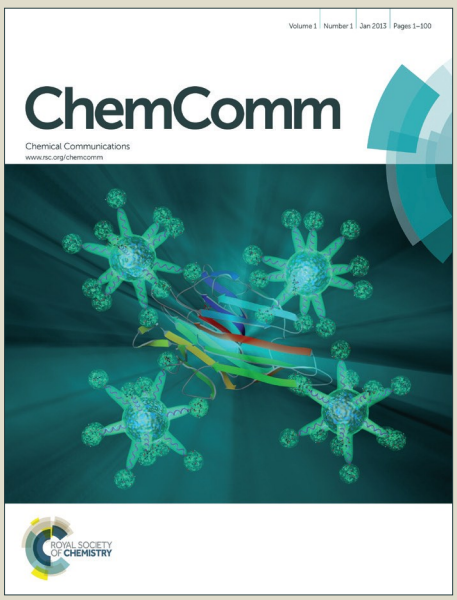

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Single Point Mutation Detection in Living Cancer Cells by Far-red Emitting PNA-FIT Probes

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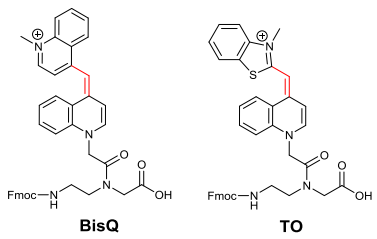
N. Kolevzon ,‡[a] D. Hashoul,‡[a] S. Naik,[a] A. Rubinstein[a] and E. Yavin[*a]

Peptide Nucleic Acid-Bis Quinoline conjugates are reported as attractive far-red emmiting porbes that detect mutated mRNA in living cells at SNP resolution.

In the past decades much effort has been put forth in the discovery of biomarkers in cancer as means for designing novel drug targets and for diagnostic purposes[1-3].

Using RNA as a biomarker has the advantage that detection could, in principle, be based not only on the over-expression of a given RNA molecule in malignant cells but also on the discrimination between mutated and non-mutated transcripts that are manifested in many types of malignancies[4, 5]. In this regard, the KRAS oncogene is an important biomarker since it is activated in many types of adenocarcinomas and has frequent single base mutations associated with early stages of tumorogenesis[6]. For example, 90% of the activating mutations in pancriatic cancer are found in codon 12 (wild-type- GGT) and the most frequently observed types of mutations are G to A transitions[7]. Therefore KRAS was selected in this study as a model gene for detection of mRNA in malignant cell lines. Seitz and co-workers introduced the idea of using a PNA molecule containing the cyanine dye thiazole orange (TO) as a replacement of a canonical nucleobase[8-11]. These forced intercalation probes (FIT-probes) exhibit a remarkable fluorescence enhancement upon hybridization to their target DNA/RNA sequence, making them suitable for sequence specific detection of RNA and DNA. These PNA probes are based on a well-known property of monomethine cyanine dyes, containing a flexible methine bond, which contributes to a non-planar conformation and non-radiative decay of the dye molecule[12]. Upon intercalation within dsDNA, TO adopts a planar conformation and therefore becomes strongly fluorescent. Additionally, it was shown, that the enhancement of fluorescence by TO-based FIT probes is highly sensitive to localized perturbations of the duplex structure such as those imposed by an adjacent base mismatch, allowing the detection of SNP[8]. We and others have previously used this approach as means of detecting a cancer biomarker by targeting mutant KRAS[13, 14] and showed that this mRNA transcript can be detected and discriminated at a single nucleotide resolution in living cells. Nonetheless, TO and other related cyanine dyes absorb light in the visible region from ca. 500 nm (TO) to lower wavelengths[15]. These spectral properties are not suitable for *in-vivo* or *in-situ* imaging because tissue (e.g. hemoglobin) and cells auto-fluorescence at these wavelengths. Herein we present the design and synthesis of a new mono-methine red shifted dye (denoted **BisQ**, Abs_{max}=588 nm, Em_{max}=609 nm) that is incorporated into PNAs targeting the KRAS oncogene.

We have used the following criteria for designing a surrogate base that could be introduced into the PNA backbone while preserving the light-up properties of FIT-PNA probes: (1) the surrogate base should not be too bulky to allow RNA hybridization and, (2) a mono-methine bond should connect both parts of the molecule (Scheme 1). Two quinolines connected via a mono-methine bond were reported to emit light in the far-red region[16]. Thus, we designed and synthesized such a probe molecule on a PNA backbone (Scheme S1, Fig. S1, ESI†).



Scheme 1 Chemical structures of far-red emitting surrogate base (**BisQ**) compared to thiazole orange (**TO**). Mono-methine bond is shown in red.

Two BisQ-FIT-PNA probes targeting the KRAS oncogene (Table 1) were designed and prepared by solid phase synthesis (Fig. S2-S5, ESI†). One PNA (**PNA 1**) has the **BisQ** opposite a U/T base while the other (**PNA 2**) has the surrogate base (**BisQ**) opposite a G. Generally, conjugation of a variety of short cell penetrating peptides (CPPs) have been shown to be a simple and useful route to allow PNA cellular uptake into living cells[17-20]. Thus, a sequence of four D-lysines was introduced on the PNA's C-terminus providing solubility and increased cellular internalization to the designed BisQ-FIT-PNA probes.

[a] The Institute for Drug Research, The School of Pharmacy, The Hebrew University of Jerusalem, Hadassah Ein-Kerem, Jerusalem 91120, Israel. E-mail: eylony@ekmd.huji.ac.il
†Electronic Supplementary Information (ESI) available: Details on synthesis and spectroscopic properties of ***BisQ*** and ***PNA1*** and ***PNA2*** as well as ***PNA1*** stability in human serum. See DOI:
‡Contributed equally to this work

Table 1 PNA and DNA sequences. Underlined sequence complementary to PNA. In red – mutation site in KRAS.

Name	Description	Construct
PNA-1	BisQ paired with **G**	(D-Lys)$_4$-CCTCGA[**BisQ**]TACCGCATCC-NH$_2$
PNA-2	BisQ paired with **T**	(D-Lys)$_4$-CCTCGACT[**BisQ**]CCGCATCC-NH$_2$
DNA	mismatch in adjacent nucleotide	5'-GTAGTTGGAGCTGGTGGCGTAGGCAAGAGT
RNA	mismatch in adjacent nucleotide	5'-GUUGGAGCUGGUGGCGUAGGCAAG
Mutated DNA	full complementarity	5'-GTAGTTGGAGCTGATGGCGTAGGCAAGAGT
Mutated RNA	full complementarity	5'-GUUGGAGCUGAUGGCGUAGGCAAG

Table 2 Photophysical properties of **BisQ** and PNAs.

Compound	λ_{max}abs (nm)	ε_{max} [M^{-1}cm^{-1}]	λ_{max}em (nm)	ϕ
BisQ	591	41,370	n.a.	n.a.
TO	505	43,000	n.a.	n.a.
PNA1	593	82,219	n.a.	n.a.
PNA1+DNA	593	94,535	610	0.25
PNA1+RNA	593	96,182	613	0.26
PNA2	588	83,000	n.a.	n.a.
PNA2+DNA	587	112,309	609	0.22
PNA2+RNA	588	112,937	612	0.23

The UV-visible spectrum of compounds **BisQ** and **TO** (as PNA monomers) were measured in MeOH due to their poor water solubility (Fig. S6, ESI†). **BisQ** shows a broad peak in the visible range with an absorbance maximum at 591nm; an 86 nm bathochromic shift relative to the TO-PNA monomer. Both molecules exhibit a similar ε_{max} value of approx. 40,000 M^{-1}cm^{-1} (Table 2).

The absorbance spectrum of the **PNA2** as well as **PNA2**-DNA duplex (Fig. S7-S8, ESI†) show a slight hypsochromic shift (3-4 nm) in absorbance maxima relative to free **BisQ**-monomer from 591 to 587-588 nm (Table 2). **PNA1** shows a slight bathochromic shift (2 nm, Fig. S9-S10, ESI†). Surprisingly, incorporation of **BisQ** into **PNA1** and **PNA2** resulted in a dramatic increase in absorbance at 588 and 593 nm, respectively (Table 2, two-fold). Indeed, such an increase in ε_{max} is partially derived from the solvent effect (the UV-visible spectrum of **PNA1** and **PNA2** were recorded in water), but may also depend on the flanking bases of the PNA as demonstrated for TO-FIT-PNAs relative to the TO monomer[21]. Additionally, **PNA2** (and to a lesser extent **PNA1**) shows remarkable brightness after DNA/RNA hybridization (ε_{max} = 112,300 and 112,937 cm^{-1} M^{-1}, respectively, Table 2), which is brighter than the best performing TO-based FIT probe reported to date[22].

The designed 17-mer BisQ-FIT-PNAs are fully complementary to the mutated mRNA found in pancreatic cancer cells (Panc-1) and have a single mismatch adjacent to the **BisQ** dye (wild type KRAS) as found in the pancreatic cancer cell line (Bxpc-3) as well as in colon cancer cells (HT-29). As depicted in Table 1, both **PNA1** and **PNA2** vary only in pairing partners to **BisQ** (G and U, respectively). In order to explore the potential of the new FIT-PNA probes as a diagnostic tool for the detection of SNPs, we studied single mismatch discrimination by hybridization of PNA conjugates (**PNA 1** and **PNA2**) with fully complementary (mutated KRAS) DNA and RNA strands versus single mismatched (wild type KRAS) DNA/RNA strands (Table 1).

Upon hybridization with fully complementary DNA, **PNA1** showed a 5-fold fluorescence enhancement, relative to PNA emission (in single strand form, Fig. 1A). Hybridization with **PNA2** resulted in an almost 25-fold enhancement, as demonstrated in Fig. 1B.

In the case of RNA, similar fluorescence enhancement was found for **PNA1** (Fig. 1C). However, **PNA2** showed improved mismatch discrimination (mmRNA) and a ca. 40 fold fluorescence enhancement after the addition of fully complementary RNA (Fig. 1D). However, **PNA1** was more sensitive than **PNA2** in discriminating a GU mismatch as designed for cell experiments (*vide post*, Panc-1).

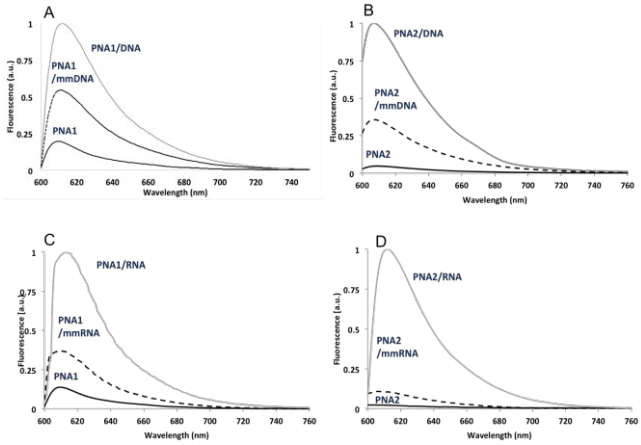


Fig. 1 Fluorescence enhancement of PNA FIT probes after the addition of DNA (A, B) and RNA (C, D). Fluorescence of **PNA1** and **PNA2** were recorded at 1.5 μM in buffered solution (black curve), with single mmDNA or single mmRNA (2 μM, dotted black curve), and with complementary (biomarker) DNA or RNA (2 μM, gray curve).

To follow KRAS mRNA detection in living cells, **PNA1** and **PNA2** probes were incubated with three types of cancer cell lines (Panc-1, BxPC-3, and HT-29). Panc-1 is a cell line derived from pancreatic cancer cells; these cells express the mutated form of KRAS (G to A transition) and are therefore expected to provide a positive fluorescent signal with the designed PNA probes. BxPC-3 and HT-29 are cell lines originating from pancreatic and colon cancer cells, respectively. Both cell lines are not mutated in KRAS and therefore are expected to exert a very low background fluorescent signal; assuming SNP detection by the designed PNA-FIT probes (**PNA1** and **PNA2**).

Incubation of and **PNA1** (1 μM, PNA with BisQ opposite G) for 3 hours with cells resulted in a clear fluorescent signal in the far-red, exclusively in Panc-1 cells (Fig. 2). A negligible fluorescent signal was seen in the other two cell lines (Fig. 2). **PNA2** (with BisQ opposite T) gave a weak fluorescent signal in all cases (data not shown).

We have previously reported the use of a PNA-FIT probe with TO as a surrogate base[13]. In this study, the same principal design was used where TO is opposite a G in the mRNA sequence. It is not clear why **PNA2** showed little response in living cells; a result that does not

correlate with the fluorescence spectroscopy of this PNA with synthetic DNA and RNA (Fig. 1). There might be factors in cells that do not exist in the test tube (e.g. crowdedness, RNA concentration).

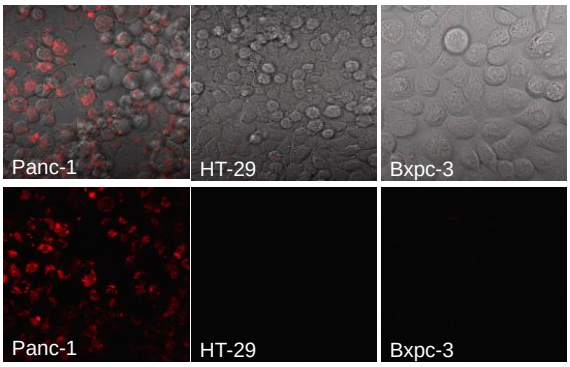


Fig. 2 Fluorescence microscopy images of Panc-1, HT-29, and Bxpc-3 cells incubated for 3 hours at 37^0C with 1 μM **PNA1**. Lower panel shows the red emission solely in Panc-1 cells.

In conclusion, we have presented PNA-FIT probes designed with a novel surrogate base (**BisQ**) that upon hybridization to target RNA (or DNA) fluoresce in the far-red region. One of these probes (**PNA1**) was found to fluoresce only in living cells with mutated KRAS (Panc-1) and not so in living cells that are not mutated in KRAS (Bxpc-3 and HT-29). Given the known bio-stability [23] (see also **PNA1** stability in human serum, Fig. S11, ESI†)and low toxicity of PNAs [18], together with the simple means of providing these PNA-FIT probes with water solubility and cellular uptake activity (i.e. 4 D-lysines), we believe that such probes could be attractive molecules for cancer diagnostics *in-situ* and *in-vivo*.

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Notes and references

1. K. Maebert, M. Cojoc, C. Peitzsch, I. Kurth, S. Souchelnytskyi and A. Dubrovska, *Int. J. Radiation Biol.*, 2014, **90**, 659-677.
2. J. Zheng, R. Yang, M. Shi, C. Wu, X. Fang, Y. Li, J. Li and W. Tan, *Chem. Soc. Rev.*, 2015, **44**, 3036-3055.
3. C. Wu, S. Cansiz, L. Zhang, I. T. Teng, L. Qiu, J. Li, Y. Liu, C. Zhou, R. Hu, T. Zhang, C. Cui, L. Cui and W. Tan, *J. Am. Chem. Soc.*, 2015, **137**, 4900-4903.
4. M. Brink, A. F. P. M. De Goeij, M. P. Weijenberg, G. M. J. M. Roemen, M. H. F. M. Lentjes, M. M. M. Pachen, K. M. Smits, A. P. De Bruine, R. A. Goldbohm and P. A. Van Den Brandt, *Carcinogenesis*, 2003, **24**, 703-710.
5. L. Dahan, L. Huang, R. Kedmi, M. A. Behlke and D. Peer, *PLoS One*, 2013, **8**, e72389.
6. N. S. Pellegata, F. Sessa, B. Renault, M. Bonato, B. E. Leone, E. Solcia and G. N. Ranzani, *Cancer Res.*, 1994, **54**, 1556-1560.
7. A. Yilmaz, N. Mohamed, K. A. Patterson, Y. Tang, K. Shilo, M. A. Villalona-Calero, M. E. Davis, X.-P. Zhou, W. Frankel, G. A. Otterson and W. Zhao, *Inl. J. Environmen. Res. Public Health*, 2014, **11**, 8645-8660.
8. O. Koehler, D. V. Jarikote and O. Seitz, *ChemBioChem*, 2005, **6**, 69-77.
9. S. Kummer, A. Knoll, E. Socher, L. Bethge, A. Herrmann and O. Seitz, *Angew. Chem. Int. Ed.*, 2011, **50**, 1931-1934.
10. S. Kummer, A. Knoll, E. Sucher, L. Bethge, A. Herrmann and O. Seitz, *Bioconjugate Chem.*, 2012, **23**, 2051-2060.
11. E. Socher, L. Bethge, A. Knoll, N. Jungnick, A. Herrmann and O. Seitz, *Angew. Chem., Int. Ed.*, 2008, **47**, 9555-9559.
12. G. L. Silva, V. Ediz, D. Yaron and B. A. Armitage, *J. Am. Chem. Soc.*, 2007, **129**, 5710-5718.
13. Y. Kam, A. Rubinstein, A. Nissan, D. Halle and E. Yavin, *Molecular Pharmaceutics*, 2012, **9**, 685-693.
14. M. V. Sonar, M. E. Wampole, Y.-Y. Jin, C.-P. Chen, M. L. Thakur and E. Wickstrom, *Bioconjugate Chem.*, 2014, **25**, 1697-1708.
15. L. Bethge, D. V. Jarikote and O. Seitz, *Bioorg. Med. Chem.*, 2008, **16**, 114-125.
16. R. Lartia and U. Asseline, *Chem. - Eur. J.*, 2006, **12**, 2270-2281.
17. C. Cordier, F. Boutimah, M. Bourdeloux, F. Dupuy, E. Met, P. Alberti, F. Loll, G. Chassaing, F. Burlina and T. E. Saison-Behmoaras, *Plos One*, 2014, **9**, e104999.
18. M. M. Fabani, C. Abreu-Goodger, D. Williams, P. A. Lyons, A. G. Torres, K. G. C. Smith, A. J. Enright, M. J. Gait and E. Vigorito, *Nucl. Acids Res.*, 2010, **38**, 4466-4475.
19. B. Lebleu, H. M. Moulton, R. Abes, G. D. Ivanova, S. Abes, D. A. Stein, P. L. Iversen, A. A. Arzumanov and M. J. Gait, *Adv. Drug Delivery Rev.*, 2008, **60**, 517-529.
20. P. Sazani, S. H. Kang, M. A. Maier, C. F. Wei, J. Dillman, J. Summerton, M. Manoharan and R. Kole, *Nucl. Acids Res.*, 2001, **29**, 3965-3974.
21. D. V. Jarikote, N. Krebs, S. Tannert, B. Roder and O. Seitz, *Chemistry*, 2007, **13**, 300-310.
22. F. Hoevelmann, I. Gaspar, S. Loibl, E. A. Ermilov, B. Roeder, J. Wengel, A. Ephrussi and O. Seitz, *Angew. Chem. Int. Ed.*, 2014, **53**, 11370-11375.
23. V. V. Demidov, V. N. Potaman, M. D. Frankkamenetskii, M. Egholm, O. Buchard, S. H. Sonnichsen and P. E. Nielsen, *Biochem. Pharmacol.*, 1994, **48**, 1310-1313.