Supporting information

Electrochemical primer extension for detection of single nucleotide polymorphisms inthe cardiomyopathy associated MYH7 gene

A. M. Debela[a], M. Ortiz[a], S. Thorimbert,[c] B. Hasenknopf [c]
C. K. O´Sullivan,[a,b]
[a]Departament d'Enginyeria Química, Universitat Rovira i Virgili, Avinguda Països Catalans, 26, 43007 Tarragona, Spain,
[b]ICREA, Passeig Lluis Companys 23, 08010 Barcelona, Spain
[c] Sorbonne Universités, UPMC Univ Paris 06, Institut Parisien de Chimie Moléculaire UMR CNRS 8232, 4 place Jussieu, 75005 Paris, France.

Materials and methods

All chemicals and reagents used were of analytical grade and purchased from various companies: Potasium dihydrogen phosphate (KH_2PO_4, Fluka), sodium chloride (NaCl, Probus), potassium chloride (KCl, Fluka), sodium perchlorate ($NaClO_4$, Acros Organics), mercaptohexanol (MCH, Fluka), sodium hydroxide, sulfuric acid (95-97%), Tris-HCl, boric acid (Scharlau, Barcelona, Spain), and hydrochloric acid (35%, Panreac). Phosphate-buffered saline (PBS), trisodium citrate, acetone, dimethyl sulfoxide (DMSO), perchloric acid (70%), triethyl amine, glacial acetic acid,phenothiazine, anthraquinone carboxylic acid, ferrocene carboxylic acid,N-(3-dimethylaminopropyl) N´-ethylcarbodiimide hydrochloride and N-hydroxy succinimide were all purchased from Sigma Aldrich. Mono carboxylic methylene blue is obtained from *emp*Biotech GmbH, the propargyl amino modified ddNTPs purchased from Jena Bioscience. Unless otherwise noted, all reactions were carried out under argon atmosphere with magnetic stirring. The organic solvents were redistilled before use. Thermo Sequenase polymerase was purchased from GE Healthcare.

Table S1. MYH7 Sequences used in the study

labelled ddNTP	Sequences
MB-ddUTP	thiol-5'-GGG ACT AGG GGA CTG AAG AA-3' 3'-CCC TGA TCC CCT GAC TTC TTA AAT-5'
Fc-ddGTP	thiol-5'-ACT AGG GGA CTG AAG AA-3' 3'-CCC TGA TCC CCT GAC TTC TTC AAT GTG AGG GCA TTT CTT GGT-5'
PTZ-ddCTP	thiol-5'-GTG GTG GGA CTA GTTCAC A -3' 3'-CAC CAC CCT GAT CAA GTG TGA AGC CAA AAC AGG GGC CA-5'
AQ-ddATP	thiol-5'-TAC AAG TGT TAA CAC ACA GTG AA-3' 3'-ATG TTC ACA ATT GTG TGT CAC TTT AAT TGT GTT TTA TGG AGG AGC AAT-5'

*Complementary regions are highlighted in blue colour and the red bases indicate the SNPs.

DNA sequences: The DNA sequences were designed following previous report[1] on a group of patients (Safor region, Valencia) with a mutated MYH7 gene and known to suffer from Laing

cardiomyopathy. Among the 8 SNPs reported to occur around the mutated gene sequence, only 5 (rs2277474, rs743567, rs8005199, rs11621360, rs875908) of the SNPs were used to design the sequences for electrochemical APEX. The sequences were selected using specific oligonucleotide selection and design programs (http://bioinfo.ebc.ee/apex2/). The sequences were designed in such a way to detect the SNPs using four different labels corresponding to the four bases. HPLC purified synthetic oligonucleotide sequences were purchased from Biomers.net (Germany).

Analytical details

FT-IR spectra were recorded in a Bruker Tensor 27 ATR diamond PIKE spectrophotometer. The ^{1}Hand ^{13}C NMR spectra were respectively recorded at 400and 100 MHz with a Bruker AVANCE 400 spectrometer. Chemical shifts are reported in ppm using residual ^{1}H and ^{13}C peaks of the solvent as internal reference (δ = 7.26 and 77.2 ppm, respectively, for $CDCl_3$, δ = 2.50 and 39.5 ppm for DMSO d_6, δ = 1.94 and 118.26 ppm for CD_3CN).

The mass spectrometer used to characterise the synthesized labels was Bruker micrOTOF 235 coupled to an electrospray source (ESI-MS) (ESI-MS) at the Paris Institute of Molecular Chemistry (IPCM). 10 µM of the compounds solutions in methanol were injected. The labelled ddNTPs were characterised using LTQ-Orbitrap XL (Thermo Electron Corporation, Bremen, Germany) mass spectrometer equipped with an ESI source to acquire high resolution mass spectra. All acquired data were analysed using Xcalibur 2.0.7 software (Thermo Finnigan).

Electrochemistry: All electrochemical measurements were carried out using an Autolab model PGSTAT 12 potentiostat/galvanostat controlled with the General Purpose Electrochemical System (GPES) software (Eco Chemie B.V., The Netherlands). A classical three electrode set up was used with an Ag/AgCl reference electrode, Pt wire counter electrode and gold electrode (with inner diameter of 2 mm), which were purchased from CHI Instruments, Inc. All the potentials are recorded with respect to the reference electrode.

Electrode preparation: Gold electrodes were polished with 0.3 and 0.05 µm alumina for ca. 5 min for each size; followed by sonication for 5 min in water. Then electrochemical cleaning was performed with 0.5 M H_2SO_4, sweeping for 40 cycles between 0.2 and 1.6 V. The real surface area (Ar) of the electrochemically activated gold electrode could be calculated by integrating the current beneath the cathodic wave of the cyclic voltammograms of H_2SO_4 solution on the basis of the quantity of consumed charge 482 µC cm^{-2}. According to the ratio of the real surface area (A_r) to the geometrical area (A_g), the high roughness factor (rf = A_r / A_g) of 2.54 in average was obtained, which implied the adequate activation of the bare gold electrode.[2]This step was performed to check the similarity of the electrodes used for functionalisation and analysis.

The cleaned gold electrodes were immersed in a solution of thiol/thioctic acid modified ssDNA (5 µM in 1.0 M KH_2PO_4 buffer solutions) for immobilisation following reported methods[3]. After 3 h of incubation at room temperature the electrodes were rinsed with Tris buffer and dried with stream of nitrogen. Mercaptohexanol backfilling was made with 0.5 mM solution for 30 min. The probe modified electrodes were subsequently washed with Tris buffer solution and dried with copious amounts of argon.

DNA hybridisation of one micromolar target DNA was performed by incubation of the prepared electrodes in a buffer solution [10 mM Tris buffer with 0.5 M NaCl solution (pH 7) containing the

respective target DNA corresponding to the four bases] for 1 h at 37^{o}C this results in complementary strand formation up until SNP region. The electrodes were washed with the hybridisation buffer and dried and made ready for primer extension [PEX] reaction.

PEX reaction: This nucleotide incorporation reaction was performed with thermosequanase (GE Healthcare), the prepared electrodes were incubated with a solution containing 1 μM of the redox labelled ddNTP (four separate reactions for each base), 20 mM Tris, pH 8.5, 50% glycerol, 0.1 mM EDTA, 0.5% Tween, 0.5% nonidet-P40, 1 mM DTT, 100 mM KCl, 65 mM $MgCl_2$, 0.5 units of thermosequanase DNA polymerase, 1.5 units thermoplasma acidophilum inorganic pyrophosphatase. The electrodes were sealed with parafilm, incubated at 42^{o}C for additional 30 min, subsequently washed with hybridisation buffer, and kept ready for electrochemical assay.

Electrochemical interrogation: DPV was used to detect the incorporation. The measurements were performed at room temperature in 10 mL electrochemical cell with the normal three-electrode configuration (see details in the text). The electrodes were transferred to 10 mM Tris buffer containing 0.5 M NaCl, pH7. DPV were recorded at various potential windows depending on the redox potential of the labels (vs Ag/AgCl), pulse amplitude of 0.1 V, step potential, 10 mV, pulse width 100 ms and pulse period 5 ms.

Synthesis

*β-(10-phenthiazinyl) propionitrile***:** This compound was synthesised following previously reported method.[4] Briefly, to an ice cooled stirring mixture of phenothiazine (4 g, 21.0 mmol) and acrylonitrile (10 ml) was added tetrabutylammonium hydroxide (1 ml, 40% in water), the exothermic reaction was allowed to warm to room temperature. After the reaction subsided, freshly distilled dioxane (25 ml) was added. The reaction mixture was kept under reflux for 3 h. The mixture was poured to vigorously stirring distilled water, which results in precipitate formation. The precipitate was recrystallised from cold acetone. An off white solid was obtained (4.03 g, 16.0 mmol, yield 75%). ESI-MS m/z calcd for sodium salt $C_{15}H_{12}N_2SNa$ in methanol 275.0613; found 275.0615

β-(10-phenthiazinyl) propionic acid: This compound was synthesised following literature report[4] (3.9 g, 14.4 mmol, yield 90 %). 1H NMR (250 MHz, DMSO) : ^{1}H NMR (250 MHz, DMSO): δ = 2.63 (t, 2H, J=7.2, CH_2CO), 4.12 (t, 2H, NCH_2), 6.92 (t, 2H, J=7.5 Hz, Ar), 7.06 (d, 2H, J=8.1 Hz, Ar), 7.14(dd, 2H, J=1.2 Hz, J=7.5 Hz, Ar), 7.25 (dd, 2H, J=1.2 Hz, J=8.1 Hz, Ar) and 9.92 (1H, COOH). ESI-MS m/z calcd for sodium salt $C_{15}H_{13}NSO_2$ $(M + Na)^+$ 294.0559; found 294.0559

2,5-dioxopyrrolidin-1-yl 3-(10H-phenothiazin-10-yl) propanoate N-hydroxysuccinimide ester (PTZ-ETCOONHS): To a stirring solution of β-(10-phenthiazinyl) propionic acid in freshly distilled dichloromethane (DCM) (0.41 g, 1.5 mmol) was added N-(3-dimethylaminopropyl) N´-ethylcarbodiimide hydrochloride (0.29 mg, 1.5 mmol) and N-hydroxy succinimide (0.180 mg, 1.5 mmol). The mixture was left stirring at room temperature for 18 h. Column chromatography in dichloromethane gave a white solid (0.33 g, 0.9 mmol, yield 60%). ^{1}H, NMR (250 MHz, DMSO) δ =2.81 (s, 4H, $COCH_2CH_2CO$), 3.14 (t, 2H, J=7.2 Hz, CH_2CO), 4.27 (t, 2H, NCH_2), 6.96 (t, 2H, J=7.5 Hz, Ar), 7.09 (d, 2H, J=8.1 Hz, Ar), 7.18 (dd, 2H, J=1.2 Hz, J=7.5 Hz, Ar), 7.27 (dd, 2H,

J=1.2 Hz, J=8.1 Hz, Ar). ^{13}C NMR (75 MHz, DMSO) δ = 25.9 ($COCH_2CH_2CO$), 29.4 (CH_2CO), 42.0 (NCH_2) 116.0, 123.4, 124.4, 127.7, 128.2, 144.5, 167.9 (COO), 170.4 ($COCH_2CH_2CO$) ESI-MS m/z calcd for $C_{19}H_{16}N_2SO_4Na$ $(M + Na)^+$ 391.0723; found 391.0735.

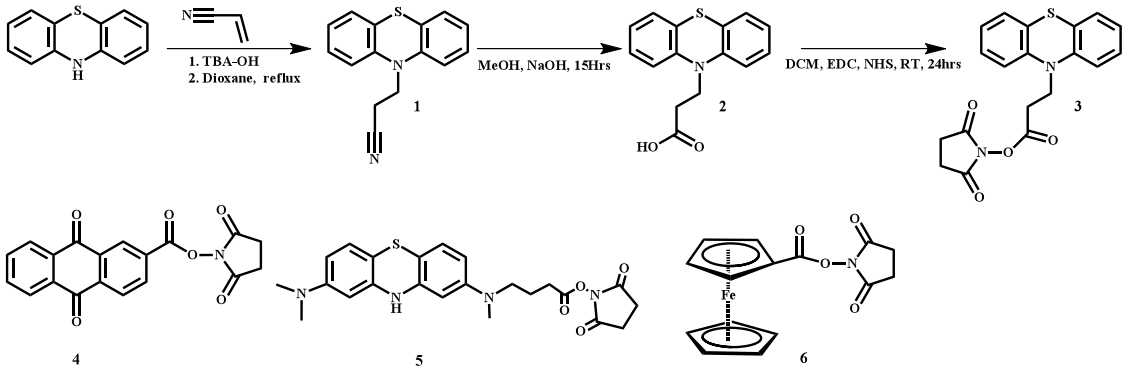

Fig. S1 The functionalisation strategy followed to get the activated ester of phenothiazine (3) and activated esters of anthraquinone (4), methylene blue (5) and ferrocene (6)

2,5-dioxopyrrolidin-1-yl 9,10-dioxo-4a,9,9a,10-tetrahydroanthracene-2-carboxylate (Anthraquinone carboxylic acid NHS) ***4****:* To a stirring solution of anthraquinone-2-carboxylic acid (0.38 g, 1.5 mmol) in a freshly distilled DCM was added N-(3-dimethylaminopropyl)-N´-ethylcarbodiimide hydrochloride (0.29 g, 1.5 mmol) and N-hydroxysuccinimide (0.18 g, 1.5 mmol) the mixture was left stirring at room temperature for 18 h. Column chromatography with dichloromethane gave a white product (0.31 g, 0.9 mmol, yield 60%). ^{1}H, NMR (250 MHz, DMSO-d6) δ 2.95 (s, 4H,$COCH_2CH_2CO$), 7.96-8.03 (m, 2H, Ar), 8.24-8.30 (m, 2H, Ar), 8.42-8.45 (d, 1H, J=3.3Hz, Ar), 8.54-8.58 (1H, d, J=2.8Hz Ar), 8.76 (1H, d, J=1.6 Hz, Ar). ^{13}CNMR (75 MHz, DMSO) δ = 26.1 ($COCH_2CH_2CO$), 128.6, 129.5, 133.6, 134.2, 135.4, 161.3 (COO), 170.6 (CH_2CON), 181.9 (CO). ESI-MS m/z calcd for $C_{19}H_{11}NO_6Na$ $(M + Na)^+$ 372.0479, found 372.0472.

Ferrocene carboxylic N-hydroxysuccinimide ester: To a stirring solution of ferrocene carboxylic acid (0. 35 g, 1.5 mmol) in freshly distilled DCM was added N-(3-dimethylaminopropyl) N´-ethylcarbodiimide hydrochloride (0. 29 g, 1.5 mmol) and N-hydroxy succinimide (0.18 g, 1.5 mmol). The mixture was left stirring at room temperature for 18 h. Column chromatography in DCM gave a white solid (0.29 g, 0.9 mmol, yield 60%,). ^{1}H, NMR (250 MHz, DMSO), δ 2.9 (s, 4H, $COCH_2CH_2CO$), 4.43 (s, 5H, Fc), 4.58 (t, J = 2 Hz, 2H, Fc), 4.93 (t, J=2 Hz, 2H). ^{13}C NMR (75 MHz, DMSO) δ = 25.7 ($COCH_2CH_2CO$), 64.3, 70.8, 70.7, 72.8, 167.4, 169.5 (CH_2COCH_2), HRMS (ESI) m/z calcd for: $C_{15}H_{13}NO_4FeNa$ $(M + Na)^+$350.0086, found 350.0089.

Monocarboxy methylene blue N-hydroxysuccinimide ester: similar methodology was followed to synthesis the NHS ester (13.74 mg, 30.8 µmol, 56% yield) and NMR was taken in DMSO. ^{1}H, NMR (250 MHz, DMSO) δ = 2.5 (m, 2H, $CH_2CH_2CH_2$), 2.83 (s, 4H, $COCH_2CH_2CO$), 2.95 (s, 3H, NCH_3), 3.08 (s, 6H, $N(CH_3)_2$), 3.2 (t, 2H, CH_2CO), 4.1 (t, 2H, NCH_2, J=5.7 Hz), 7.3 (dd, 2H, Ar, J=3 Hz, J=8Hz), 7.57 (d, 2H, J=3Hz), 7.9 (d, 2H, J=8Hz). ^{13}C NMR (75 MHz, DMSO) δ = 19.9 ($COCH_2CH_2CO$), 25.9 (CH_2CO), 53.1 (NCH_2), 63.9, 107.2, 121.0, 138.6, 168.0 (COO), 170.0 ($COCH_2CH_2CO$). ESI-MS m/z calcd for $C_{22}H_{23}N_4SO_3Na$ $(M + Na)^+$ 446.1609, found 446.1612.

Synthesis and characterisation of redox labelled ddNTPs

General Procedure

Labelled ddNTPs were synthesised utilising the NHS esters of the redox labels and subsequent reaction with alkyl amine arm of the ddNTPs. To the NHS ester (2.4 µmol, 3 equiv) in DMSO was added 100 µL of Na_2CO_3-$NaHCO_3$ buffer (0.1 M, pH=8.7) followed by addition of 800 nmol of the propargyl amino ddNTPs. After sonication for 10 min, the reaction was kept in a thermomixer shaking for 6 h. After the reaction time TLC confirmed the complete consumption of the triphosphate. The reaction mixture was kept in the freezer overnight followed by freeze drying. The labelled ddNTPs were purified by using preparative TLC with a mixture of solvents $CHCl_3$/CH_3OH : 85/15. The products were directly used for experiments.

References

1. N. Muelas, P. Hackman, H. Luque, T. Suominen, C. Espinós, M. Garcés-Sánchez, T. Sevilla, I. Azorín, J. M. Millán, B. Udd, J. J. Vílchez, *Clin Genet*. 2012**, 81**,491-4
2. J. C. Hoogvliet, M. Dijksma, B. Kamp, W. P van Bennekom, *Anal. Chem.* 2000**, 72**, 2016-2021
3. M. T. Herne, J. M. Tarlov, *J. Am. Chem. Soc.,* 1997, **119**, 8916-8920
4. E.F. Godefroi, E. L. Whittle, *J. Org. Chem*, 1956**, 21**, 1163-1168