

Delivery of antisense peptide nucleic acids (PNAs) to the cytosol by disulphide conjugation to a lipophilic cation

Aleksandra Filipovska^{a,b}, Michael R. Eccles^b, Robin A.J. Smith^c, Michael P. Murphy^{a,*}

^aMedical Research Council Dunn Human Nutrition Unit, Wellcome Trust/MRC Building, Hills Road, Cambridge CB2 2XY, UK

^bDepartment of Pathology, Medical School, University of Otago, P.O. Box 913, Dunedin, New Zealand

^cDepartment of Chemistry, University of Otago, P.O. Box 956, Dunedin, New Zealand

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Abstract Peptide nucleic acids (PNAs) are effective antisense reagents that bind specific mRNAs preventing their translation. However, PNAs cannot cross cell membranes, hampering delivery to cells. To overcome this problem we made PNAs membrane-permeant by conjugation to the lipophilic triphenylphosphonium (TPP) cation through a disulphide bond. The TPP cation led to efficient PNA uptake into the cytoplasm where the disulphide bond was reduced, releasing the antisense PNA to block expression of its target gene. This method of directing PNAs into cells is a significant improvement on current procedures and will facilitate in vitro and pharmacological applications of PNAs.

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1. Introduction

Peptide nucleic acids (PNAs) are DNA analogues where *N*-(2-aminoethyl)glycine units replace the deoxyribose phosphate backbone [1–3]. As the base spacing of PNAs is the same as DNA they retain the sequence-specific binding of nucleic acid oligomers [2,4,5]. The neutral amide backbone increases both the stability of PNAs and their binding affinity to DNA and RNA. Consequently PNAs are effective antisense reagents with therapeutic potential that bind to specific mRNAs, preventing their translation [4,6,7]. However, PNAs do not cross phospholipid bilayers spontaneously [8,9]. Instead, delivery to cells requires modification to the PNA to allow co-administration with cationic lipids, or conjugation to cell-permeant peptides [7,10–14].

To overcome these difficulties and develop a better procedure to deliver PNAs to the cytoplasm, we chose to conjugate the PNA to a triphenylphosphonium (TPP) cation (Fig. 1). These lipophilic cations easily permeate phospholipid bilayers

and are accumulated into the cytosol driven by the plasma membrane potential [15–17]. Most importantly, conjugation to TPP drives PNA uptake into cells through the plasma membrane [18]. However, TPP-conjugated PNAs are only transiently present in the cytosol and rapidly redistribute to mitochondria, driven by the mitochondrial membrane potential [18]. To use TPP to deliver a PNA to the cytosol instead of mitochondria we attached the thiol-containing TPP derivative 4-thiobutyltriphenylphosphonium (TBTP) [19] to a PNA by a disulphide bond (Fig. 1A). Disulphides are stable in the oxidising extracellular environment but are rapidly reduced by the intracellular glutathione pool. This should release the active PNA within the cytosol while the displaced TBTP is expected to initially accumulate in mitochondria before gradually diffusing out of the cell [19] (Fig. 1B).

Here we show that disulphide conjugation of a PNA to TBTP leads to efficient PNA uptake into cells. In the cytoplasm the disulphide bond is rapidly reduced, releasing the antisense PNA which then decreases the expression of its target gene. This procedure is a significant improvement on current procedures for PNA delivery to cells, and will facilitate both in vitro and pharmacological applications of PNAs.

2. Materials and methods

2.1. Synthesis of bis-[(4-thiobutyl)triphenylphosphonium] (bisTBTP)

Acyated TBTP was synthesised as described [19]. To generate the free thiol, TBTP, acylated TBTP (500 mg; 1 mmol) dissolved in 0.5 ml 95% ethanol was incubated with 0.5 ml 1 M NaOH for 20 min at room temperature under argon. The TBTP solution was then diluted with 40 ml 150 mM HEPES, pH 7.3 and the thiol oxidant diamide (200 mg; 1.16 mmol) was added. Diamide rapidly oxidised TBTP to bisTBTP, as assessed by the disappearance of TBTP thiols assayed by reaction with 5,5'-dithiobis (2-nitrobenzoic) acid to form 5-thio-2-nitrobenzoic acid ($\epsilon_{412} = 13.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) [20]. After incubation for 1 h at room temperature the reaction was quenched with 20 ml 1 M HCl, 0.5 g NaBr was added and the aqueous solution extracted three times with 40 ml dichloromethane. The pooled dichloromethane fractions were concentrated by evaporation under reduced pressure to ~50 ml and bisTBTP was precipitated by addition of diethyl ether (50 ml). The white powder was filtered, washed with diethyl ether and air dried (244 mg, 48% yield). The identity of bisTBTP was confirmed by ¹H nuclear magnetic resonance spectroscopy in CDCl₃, acquired using a Varian Gemini 200 MHz spectrometer. The analysis gave the following peaks: δ 7.6–8.0 (30 H, m, (Ph₃P-CH₂-CH₂-CH₂-CH₂)₂S₂), δ 3.92 (4H, b, (Ph₃P-CH₂-CH₂-CH₂-CH₂)₂S₂), δ 2.80 (4H, t, *J* = 7.2 Hz (Ph₃P-CH₂-CH₂-CH₂-CH₂-CH₂)₂S₂), δ 2.06–2.11 (4H, m, (Ph₃P-CH₂-CH₂-CH₂-CH₂)₂S₂), δ 2.09 (4H, m, (Ph₃P-CH₂-CH₂-CH₂-CH₂)₂S₂), δ 1.74 (4H, m, (Ph₃P-CH₂-CH₂-CH₂-CH₂)₂S₂). The identity of bisTBTP was further confirmed by base hydrolysis with 1 M NaOH, which exposed 1.90–2.0 mol sulphhydryl groups per mol bisTBTP. Free thiols were not detected in bisTBTP.

*Corresponding author. Fax: +44 (1223) 252 905.

E-mail address: mpm@mrc-dunn.cam.ac.uk (M.P. Murphy).

Abbreviations: β m, β 2-microglobulin; bio, biotin; bisTBTP, bis-[(4-thiobutyl)triphenylphosphonium]; DMEM, Dulbecco's modified Eagle's medium; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazide; flu, fluorescein; PNA, peptide nucleic acid; PNash, PNA incorporating a C-terminal cysteine; PNAsTBTP, PNAs disulphide conjugated to TBTP; TBTP, 4-thiobutyltriphenylphosphonium; TPP, triphenylphosphonium cation

2.2. Synthesis and purification of disulphide-conjugated TPP-PNAs

PNAs were synthesised by Applied Biosystems (Bedford, MA, USA). Two oligomers were made with N-terminal biotin or fluorescein and a C-terminal cysteine incorporated by standard procedures. These were BioPNash (Biotin-oo-GTTGGCTCTCT-o-Cys-CO₂H) and FluPNash (fluorescein-oo-TTCACACCCCGTGCC-o-Cys-CO₂H), where the linker o is an 8-amino-3,6-dioxanoic acid residue. To conjugate these PNAs to TBTP, PNA oligomers (50 nmol) in 50 µl 10 mM HEPES, 1 mM EDTA, pH 7.5 were incubated with 100 nmol of tris[2-carboxyethyl]phosphine hydrochloride at 37°C for 1 h to ensure complete reduction of the PNA thiol. Then bisTBTP (500 nmol) in 20 µl 10 mM HEPES, 1 mM EDTA, pH 7.5 was added and incubated at 37°C for a further 4 h. The reaction products were separated by reverse-phase high performance liquid chromatography (HPLC) on a C₄ analytical column (Vydac, 300 Å, 4.6 mm × 250 mm), using a Waters 450 HPLC system and a linear gradient from 0.1% trifluoroacetic acid (TFA) in water to 90% acetonitrile and 0.1% TFA, run over 30 min. Peaks were detected by absorbance at 260 nm, collected, lyophilised and dissolved in water for further analysis. The concentrations of the PNAsTBTP conjugates were determined at 55°C using extinction coefficients at 260 nm of 100 400 M⁻¹ cm⁻¹ (BioPNAsTBTP) and 161 500 M⁻¹ cm⁻¹ (FluPNAsTBTP). These were derived by adding the extinction coefficient of BioPNash (97 900 M⁻¹ cm⁻¹) or FluPNash (159 000 M⁻¹ cm⁻¹) to that of TBTP (2500 M⁻¹ cm⁻¹ at 260 nm [19]). Samples were analysed by matrix assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS) using a Finnigan MAT Lasermat 2000 instrument. For this PNA conjugates (~0.5 pmol) in water were mixed with 3,5-dimethoxy-4-hydroxycinnamic acid (~0.5 µl of a 10 mg/ml solution) and after crystallisation spectra were acquired in positive ion mode using melittin (*M_r* 2846) as an external mass calibrant.

2.3. Cell culture and incubations

The cells used were the human osteosarcoma 143B cell line, the murine leukaemia P388 cell line and early passage (~3–7) human skin fibroblasts. All cells were grown at 37°C under a humidified atmosphere of 95% air/5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% inactivated foetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin. For incubations in suspension, 143B cells were harvested using trypsin and 10⁶ cells were suspended in 1 ml DMEM, supplemented with 10 mM HEPES, pH 7.0 and 10% FCS. For cell subfractionation, 143B cells were grown to confluence in 24 well tissue culture plates overnight and then incubated with 1 µM PNA conjugates ± 10 µM carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) for 1 h at 37°C. After washing off the medium the cells were harvested by scraping in 250 mM sucrose, 20 mM MOPS, 3 mM EDTA, pH 6.7 containing 1 mg/ml digitonin and homogenised. A mitochondria-enriched fraction was prepared from 200 µl of the crude homogenate by centrifugation (10 000 × *g*, 1 min) through 300 µl oil (58% silicone oil (Dow Corning)/42% dioctyl phthalate) into 100 µl 0.5 M sucrose/0.1% Triton X-100, leaving a cytosol-enriched upper layer [21]. About 92–96% of the mitochondrial marker enzyme citrate synthase and 0.3–1% of the cytosolic marker enzyme lactate dehydrogenase were found in the mitochondria-enriched fraction. Protein was quantitated by the bicinchoninic acid assay using bovine serum albumin (BSA) as a standard [22]. Cytotoxicity was determined by two methods: measuring the release of the cytosolic enzyme lactate dehydrogenase into the culture medium [21], and assessing cell proliferation by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay using the Roche Cell Proliferation kit I (MTT) according to the manufacturer's instructions.

2.4. Gel electrophoresis and immunoblotting

For detection of PNA conjugates in 143B cells, cell lysates were suspended in 20 µl loading buffer (50 mM Tris, 4% sodium dodecyl sulphate (SDS), 12% glycerol, 2% 2-mercaptoethanol, 0.01% Coomassie brilliant blue) and were separated on 18.5% Tris-Tricine gels using a Bio-Rad Mini Protean system [23]. For Pax2 protein detection, P388 cell lysates were suspended in loading buffer and were resolved on 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) gels. Gels were then electrotransferred onto nitrocellulose using a Bio-Rad Mini Trans-Blot system in transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% methanol) and then blocked with 2%

(w/v) fat-free milk powder in TBS (5 mM Tris-HCl, pH 7.4, 20 mM NaCl), 0.1% Tween 20. Rabbit anti-Pax2 polyclonal antiserum (Zymed) was diluted 1:1000 in TBS, 0.1% fat-free milk powder, 0.1% Tween 20. Rabbit anti-histone H3 polyclonal antiserum (Abcam) was diluted 1:500. Horseradish peroxidase conjugated to goat anti-rabbit IgG (1:10 000, Bio-Rad) was used as a secondary antibody. To detect biotin, horseradish peroxidase-conjugated extravidin (1:3000, Sigma) was used. In both cases secondary antibody binding was visualised by chemiluminescence using a Pierce Super Signal R chemiluminescence substrate with Kodak X-OMAT[®] AR imaging film.

2.5. Fluorescence microscopy

For real-time fluorescence microscopy of live cells, human fibroblasts were grown overnight in 35 mm Petri dishes to 80% confluence. Following incubation for 1 h at 37°C with 1 µM FluPNAsTBTP the cells were incubated with 25 nM MitoTracker Red (Molecular Probes) for 30 min. The cells were then washed in TBS (3 × 10 min) and fresh DMEM added. Images were acquired using a Zeiss inverted confocal microscope with a Plan-Neofluar 40 × 1.3 oil DIC objective, line 488 nm and Zeiss Imaging Software with equal exposure times for all images shown. For immunocytochemistry human fibroblasts (~5000 cells per well) were plated overnight onto 13 mm glass coverslips in 24 well plates. Following incubation for 1 h at 37°C with 1 µM BioPNAsTBTP, cells were fixed with 4% paraformaldehyde in TBS for 30 min, washed with TBS and incubated with 10% FCS/0.1% Triton X-100/TBS (TBST) for 10 min. Cells were then incubated overnight at 4°C with the IgG fraction of anti-TBTP serum, diluted 1:500 in TBS. The IgG fraction of preimmune serum gave no immunoreactivity (data not shown). After washing with TBS (3 × 5 min) the cells were incubated for 15 min in the dark with anti-rabbit IgG conjugated to the Oregon green fluorophore (Molecular Probes), diluted 1:100 in TBS. To detect biotin, the cells were washed in TBS and incubated for another 15 min with streptavidin-conjugated CY3 (1:200, Molecular Probes) in the dark. Cells were then washed in TBS, mounted in DABCO/PVA medium (15 g polyvinyl alcohol, 15 g 1,4-diazabicyclo (2,2,2) octane in 30% glycerol in 0.1 M Tris, pH 8.5) and mounted on coverslips [18]. Images were acquired using a Bio-Rad MRC 600 laser-scanning confocal microscope using a Nikon Diaphot TMD inverted microscope and Nikon ×60 NA 1.4 oil immersion Plan-Apochromat objective. The 568 nm and 488 nm lines of a krypton-argon laser and K1/K2 filter blocks were used at identical gain, black settings and time frame.

2.6. Real-time polymerase chain reaction (PCR)

Total RNA was isolated from cells using the RNeasy kit (Qiagen). First-strand cDNA was produced from 2 µg of random hexamer-primed total RNA using reverse transcription reactions (20 µl) containing 15 mM (NH₄)₂SO₄, 20 mM Tris-HCl (pH 8.8), 2 mM MgCl₂, 0.05% Tween 20, 500 µM dNTPs (Roche), 0.2 mM dithiothreitol, 10 µM random hexamer primer (Roche), 1 U DNase I (Roche), 1 U RNasin (Promega), and 200 U Superscript II reverse transcriptase (Life Technologies). Reactions were incubated at 42°C for 50 min. Relative quantitation by real-time PCR was carried out using SYBR green detection of PCR products in real time using either the ABI Prism 7700 or ABI Prism 7000 Sequence Detection System (Applied Biosystems). In each experiment, the β2-microglobulin (βm) housekeeping gene was amplified as a reference standard. Primers for βm were βmF, 5'-TGCAGAGTTAAGCATGACAGTATGG-3' and βmR, 5'-TGATGCTTGATCACATGTCTCG-3'. In all real-time PCR experiments cDNA and genomic DNA samples were included as negative controls. Template RNAs were treated with DNase I prior to amplification, according to the manufacturer's instructions (Applied Biosystems). Each real-time PCR reaction (25 µl) contained 2.5 µl of cDNA, 12.5 µl of 2 × SYBR Green Master Mix (Applied Biosystems), including Amplitaq polymerase (Perkin-Elmer), and primers at a final concentration of 20 µM. Primers for the Pax2 gene were designed using Primer Express (Applied Biosystems). Primers were as follows: Pax2F, 5'-GTTCCAGTGTCTCATCCAT-3'; Pax2R, 5'-GCTGGTTTCACACCCCG-3'. Reactions were prepared in duplicate and heated to 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 58°C for 60 s, with a final incubation at 95°C for 15 min. To detect the log phase of amplification, the fluorescence level (quantification of product) was determined at each cycle. The cycle at which the fluorescence reached threshold (CT) was recorded and averaged between duplicates.

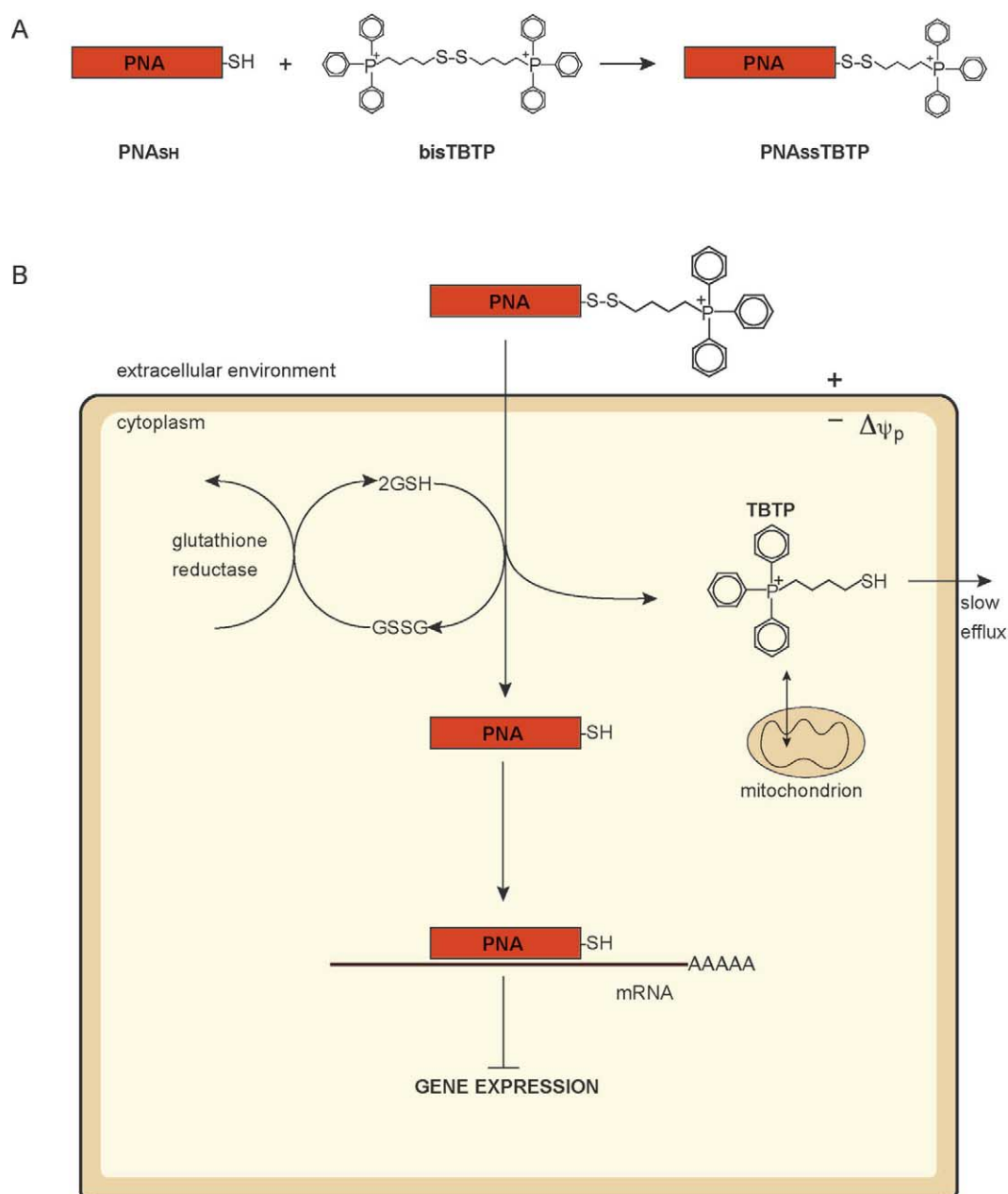


Fig. 1. Synthesis and uptake of PNAs conjugated to TBTP by a disulphide bond. A: The synthesis of PNAssTBTP by thiol–disulphide exchange on incubating excess PNA with bisTBTP is shown. B: The lipophilic TBTP cation enables rapid transport of the PNAssTBTP conjugate across the plasma membrane. In the cytoplasm the disulphide bond is reduced by the endogenous glutathione pool, leaving the PNA free in the cytoplasm. The TBTP cation will initially distribute to the mitochondria and from there slowly wash out of the cell.

3. Results and discussion

3.1. Synthesis, purification and characterisation of PNAssTBTP conjugates

To conjugate TBTP to a PNA we synthesised a novel disulphide reagent, bisTBTP, by the oxidative dimerisation of TBTP (Fig. 1A). A PNA incorporating a C-terminal cysteine (PNAsH) was obtained by conventional automated PNA synthesis. Incubation of excess bisTBTP with PNAsH led to the formation of a disulphide bond between TBTP and the PNA by spontaneous thiol–disulphide exchange (Fig. 1A). The resulting PNAssTBTP was easily purified by reverse-phase HPLC, as is shown for the biotin-tagged BioPNAssTBTP (Fig. 2A), and its identity confirmed by MALDI-TOF MS

(Fig. 2B). To demonstrate that BioPNAssTBTP had a TBTP at one end and a biotin at the other, the construct was probed using antiserum against TBTP, and with streptavidin (Fig. 2C). A PNAssTBTP containing a fluorescein tag (FluPNAssTBTP) was synthesised and purified by the same procedures: all the PNA derivatives synthesised are shown in Fig. 2D. This synthetic scheme can be applied easily to any PNA containing a thiol by incubation with bisTBTP prior to the final HPLC purification.

3.2. PNAssTBTP conjugates are taken up rapidly by cells

The toxicity of the derivatised PNAs was expected to be low. This is because within the cell the PNAssTBTP will be rapidly converted to a free PNA and to TBTP. Consequently

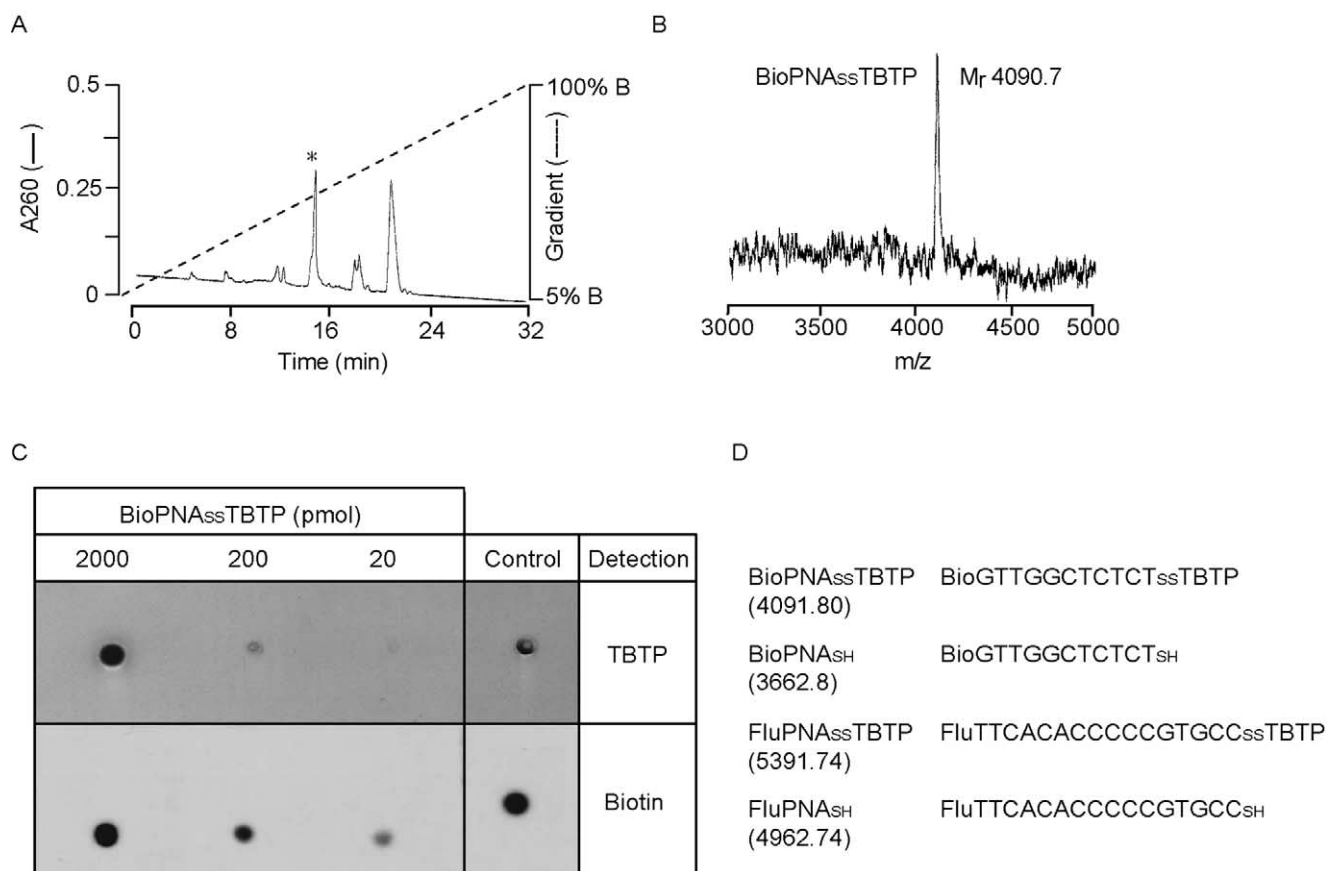


Fig. 2. Purification and characterisation of BioPNAssTBTP. A: Purification of BioPNAssTBTP. After reaction of BioPNAsh with bisTBTP the reaction product was purified by reverse-phase HPLC. The major peak at ~16 min (asterisk; BioPNAssTBTP) was collected and lyophilised. B: Characterisation of BioPNAssTBTP by MALDI-TOF. The observed M_r for BioPNAssTBTP (4090.7) was within 0.01% of the calculated M_r (4091.8). C: Immunoblotting of BioPNAssTBTP. Serial dilutions of BioPNAssTBTP were adsorbed onto nitrocellulose and the TBTP moiety detected using anti-triphenylphosphonium serum [25]. BSA conjugated to IBTP was used as a positive control [25]. Horseradish peroxidase conjugated to extravidin was used to detect biotin and the BioPNAsh oligomer was used as a positive control. Control PNAs, lacking biotin and TBTP, were not detected by either procedure (data not shown). D: List of PNA oligomers synthesised along with their sequences and M_r .

the toxicity of a PNAssTBTP conjugate will be a composite of the toxicity of its two components. PNAs at concentrations up to several hundred micromolar are non-toxic to cells in culture [2]. The toxicity of a wide range of small molecule TPP derivatives such as TBTP has been investigated for many different types of cell in culture and shown to be low, below about 5–10 μ M [24–28]. Concentrations of up to 500 μ M of TPP derivatives can be administered to mice in their drinking water without toxicity [16]. In addition, a TPP–PNA conjugate concentration of 10 μ M was non-toxic when incubated with human myoblasts for up to 3 weeks [18]. We confirmed that BioPNAssTBTP concentrations up to 5 μ M were not cytotoxic by incubations with 143B cells for up to 4 days. This treatment did not increase lactate dehydrogenase release into the extracellular medium, or affect the amount of MTT accumulation, relative to control incubations. Therefore in subsequent experiments we used a PNAssTBTP concentration of 1 μ M, confident that there was no cytotoxicity.

To see if BioPNAssTBTP was accumulated into the cytosol, we incubated it with human osteosarcoma 143B cells for 1 h and then separated the cells into cytosol- and mitochondria-enriched fractions. These were resolved by electrophoresis on a Tris-Tricine gel, transferred to nitrocellulose and probed for the biotin tag using streptavidin (Fig. 3A). This showed that BioPNAsh was present only in the cytosol and not in the

mitochondria. Abolition of the mitochondrial membrane potential with the uncoupler FCCP did not affect the intracellular distribution of BioPNAsh, further indicating that membrane potential-dependent uptake into mitochondria was not occurring (Fig. 3A). When FluPNAssTBTP was incubated with cells it too was taken up into the cytosol and not into mitochondria (Fig. 3B). There was no uptake of non-TBTP-conjugated PNAs under these conditions (data not shown), in agreement with the literature [8,9]. To confirm that the disulphide bond linking TBTP and the PNA was cleaved within the cell we investigated the labelling of mitochondrial and cytosolic thiol proteins by the TBTP released on reduction of FluPNAssTBTP by intracellular glutathione. Cells were incubated with FluPNAssTBTP for 1 h, separated into cytosol- and mitochondria-enriched fractions and the proteins separated by non-reducing SDS–PAGE to preserve disulphide bonds between TBTP and thiol proteins [19,24]. The proteins were then transferred to nitrocellulose and probed with anti-serum against TBTP (Fig. 3C). As a positive control we incubated cells with the mitochondria-targeted thiol reagent 4-iodobutyltriphenylphosphonium (IBTP) which accumulates within mitochondria and there labels thiol proteins [25]. TBTP reacted with mitochondrial, but not cytosolic, thiol proteins and the pattern of labelling was similar to that by IBTP (Fig. 3C). These data indicate that the disulphide bond

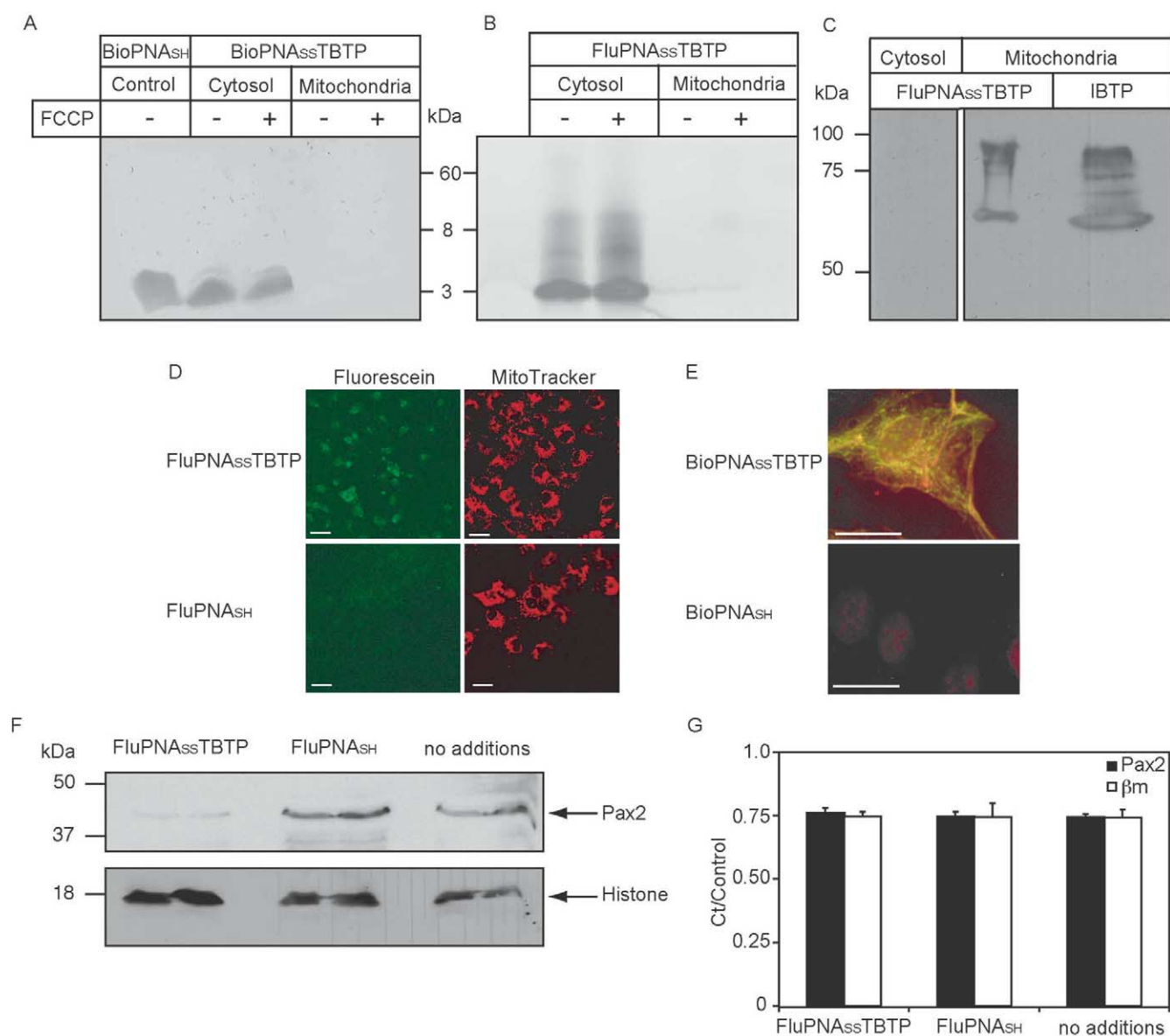


Fig. 3. Uptake, intracellular distribution and antisense efficacy of PNAssTBTPs. **A:** Uptake of BioPNAssTBTP by cells. A suspension of 143B cells (10^6 cells) was incubated with 1 μ M BioPNAssTBTP in the presence or absence of 10 μ M FCCP for 1 h. The cells were then isolated and separated by homogenisation with digitonin into mitochondria- and cytosol-enriched fractions. The fractions (20 μ g protein) were separated on Tris-Tricine gels, transferred to nitrocellulose and probed with streptavidin-linked horseradish peroxidase. BioPNAssH (5 nmol) was used as a positive control. The experiment was repeated four times with identical results. **B:** Uptake of FluPNAssTBTP by cells. A suspension of P388 cells (10^6 cells) was treated with 1 μ M FluPNAssTBTP in the presence or absence of 10 μ M FCCP for 1 h and the cells were then separated into mitochondria- and cytosol-enriched fractions as described in **A**. The fractions (20 μ g protein) were separated on Tris-Tricine gels and the fluorescence of the FluPNAssTBTP and FluPNAssH conjugates detected using a GelDoc fluorescence imager. The experiment was repeated three times with identical results. **C:** Detection of TBTP within cells incubated with FluPNAssTBTP. P388 cells were treated with 1 μ M FluPNAssTBTP for 1 h and were then separated into mitochondria- and cytosol-enriched fractions as in **B**. The fractions were then resolved by non-reducing SDS-PAGE, transferred to nitrocellulose and the TBTP moiety detected using anti-triphenylphosphonium serum. **D:** Uptake of FluPNAssTBTP by cells. P388 cells were incubated for 1 h with 1 μ M FluPNAssTBTP or FluPNAssH and then for 30 min with 25 nM MitoTracker Red (Molecular Probes). Images of the live cells were then acquired (green = fluorescein; red = MitoTracker). **E:** BioPNAssTBTP uptake by human fibroblasts visualised by immunofluorescence microscopy. Cells were incubated with 1 μ M BioPNAssTBTP or BioPNAssH for 1 h. Cells were fixed, incubated with antiserum against TBTP (green) and a streptavidin-linked fluorophore to detect the biotin-tagged PNA (red) and the images acquired as described in Section 2. Magnification, 1400 \times . Scale bars, 20 μ m. **F:** Pax2 protein expression in cells treated with FluPNAssTBTP. P388 cells grown in 24 well culture plates were incubated with 1 μ M FluPNAssTBTP, FluPNAssH or no additions for 4 days. At the end of the treatment cells from two wells were pooled and lysed in 50 μ l loading buffer, and 25 μ l aliquots (20 μ g protein) were resolved by SDS-PAGE gels, transferred to nitrocellulose and probed with a Pax2-specific antibody (upper panel), or anti-histone H3 antibody to check for equal protein yields and for non-specific effects of FluPNAssTBTP on protein expression. Data are from day 4 of an incubation and were repeated four times with identical results. Results after two or three days' incubation were similar (data not shown). **G:** Levels of Pax2 mRNA in FluPNAssTBTP-treated cells. Cells were incubated with 1 μ M FluPNAssTBTP, FluPNAssH or no additions as in **F**. Then total RNA was extracted from cells and the level of Pax2 mRNA and that of a housekeeping gene, β m, were determined by real-time PCR. Data are the cycle at which fluorescence reached threshold (CT), divided by the CT for a control cell incubation after one day and are means \pm S.D. for three measurements. This experiment was repeated twice with identical results.

linking TBTP and the PNA is rapidly broken in the reducing environment within the cell. The TBTP released is then taken up by mitochondria where a proportion forms disulphide bonds with thiol proteins [19].

We next assessed the uptake of FluPNA^{ss}TBTP into cells using laser scanning confocal fluorescence microscopy (Fig. 3D). The large field containing a number of live cells indicates that FluPNA^{ss}TBTP (green fluorescence) was taken up into the cytoplasm of most (>90%) of the cells present over 1 h, while the underivatized FluPNA^{sh} was not (Fig. 3D). Labelling of mitochondria within these cells with MitoTracker Red (red fluorescence) indicated that similar cell numbers were present in both fields and that the cells were viable (Fig. 3D). In live cells it was not possible to assess by immunohistochemistry the location of the TBTP released on cleavage of the FluPNA^{ss}TBTP. Therefore we next incubated cells with BioPNA^{ss}TBTP or BioPNA^{sh} for 1 h, then fixed the cells and probed for both biotin and TBTP by confocal immunofluorescence microscopy (Fig. 3E). There was PNA uptake into cells incubated with BioPNA^{ss}TBTP (red fluorescence; Fig. 3E, upper panel), but not into cells incubated with BioPNA^{sh} (Fig. 3E, lower panel). The distribution of thiol proteins labelled by TBTP (green fluorescence; Fig. 3E, upper panel) showed punctate staining consistent with a mitochondrial localisation. These data corroborate those in Figs. 3A–C, confirm that the disulphide bond linking the PNA and TBTP is rapidly broken within the cell and indicate that TBTP and BioPNA^{sh} are present in separate compartments.

Together, these experiments indicate that conjugation of a PNA to TBTP by a disulphide bond leads to PNA accumulation into the cytoplasm far more rapidly than incubating cells with an unmodified PNA. The disulphide bond is reduced rapidly in the cytoplasm and the PNA is retained in the cytoplasm or nucleus; the TBTP goes to the mitochondria and is then expected to diffuse gradually out of the cell [16,19]. Hence the PNA^{ss}TBTP constructs behave as predicted in Fig. 1B.

3.3. PNA^{ss}TBTP conjugates are effective antisense agents

The final step was to determine whether the PNA delivered to the cytosol by disulphide conjugation to TBTP is effective as an antisense agent against a target gene. The FluPNA^{ss}TBTP sequence is complementary to a unique region of the mRNA of the mouse *Pax2* gene (accession number X55781.1), required for kidney and eye development in mice [29]. Therefore we determined whether prolonged incubation of mouse P388 cells, which express high levels of the Pax2 protein [30], with FluPNA^{ss}TBTP decreased expression levels of the mouse Pax2 protein. To do this, we incubated mouse cells with FluPNA^{ss}TBTP, FluPNA^{sh} or no additions for 4 days and measured the levels of Pax2 protein by immunoblotting (Fig. 3F). There was a dramatic loss of 85–90% of the Pax2 protein relative to controls on incubation with FluPNA^{ss}TBTP, while FluPNA^{sh} had no effect (Fig. 3F). FluPNA^{ss}TBTP did not decrease the level of *Pax2* mRNA, as measured by real-time PCR (Fig. 3G). This is expected as antisense PNAs are thought to act by preventing translation and not by affecting message stability [4]. The specificity of the FluPNA^{ss}TBTP for the *Pax2* mRNA is indicated by the lower panel in Fig. 3F, which shows that FluPNA^{ss}TBTP did not affect the level of expression of a control protein, histone H3. Therefore PNAs targeted to the cytosol by disulphide

conjugation to TBTP are effective and specific antisense reagents.

3.4. Conclusions

Disulphide conjugation of TBTP to a PNA led to efficient PNA uptake into the cytosol, where the disulphide bond was reduced and the bioactive antisense PNA was retained in the cell. This procedure can be carried out by the conventional automated synthesis of a PNA containing a free thiol. The crude synthetic product is then incubated with excess bisTBTP followed by a single reverse-phase HPLC purification to give the final PNA^{ss}TBTP. This procedure is far more straightforward than covalently linking a cell-permeant peptide to a PNA, or the alternative delivery strategy of first annealing the PNA to a complementary DNA to enable it to bind to cationic detergents [10]. Delivery facilitated by cationic detergents is also strongly cell type- and PNA sequence-dependent. As PNAs have been shown to be effective antisense reagents against many genes in a range of cells [4,6,7], and TPP-conjugated molecules are taken up by a wide variety of cells in vitro and in vivo [16,17], this delivery system should be generally applicable. This approach will greatly simplify the in vitro applications of PNAs as antisense reagents. Furthermore, as the disulphide is relatively stable in the extracellular environment, it should be possible to use this approach to deliver PNAs to cells in vivo, facilitating pharmacological applications of PNAs.

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References

- [1] Nielsen, P.E., Egholm, M., Berg, R.H. and Buchardt, O. (1991) *Science* 254, 1497–1500.
- [2] Larsen, H.J., Bentin, T. and Nielsen, P.E. (1999) *Biochim. Biophys. Acta* 1489, 159–166.
- [3] Egholm, M. et al. (1993) *Nature* 365, 566–568.
- [4] Braasch, D.A. and Corey, D.R. (2002) *Biochemistry* 41, 4503–4510.
- [5] Lohse, J., Dahl, O. and Nielsen, P.E. (1999) *Proc. Natl. Acad. Sci. USA* 96, 11804–11808.
- [6] Villa, R., Folini, M., Lualdi, S., Veronese, S., Daidone, M.G. and Zaffaroni, N. (2000) *FEBS Lett.* 473, 241–248.
- [7] Pooga, M. et al. (1998) *Nat. Biotechnol.* 16, 857–861.
- [8] Wittung, P., Kajan, J., Edwards, K., Haaima, G., Nielsen, P.E., Norden, B. and Malmstrom, B.G. (1995) *FEBS Lett.* 375, 27–29.
- [9] Sazani, P., Kang, S.H., Maier, M.A., Wei, C., Dillman, J., Summerton, J., Manoharan, M. and Kole, R. (2001) *Nucleic Acids Res.* 29, 3965–3974.
- [10] Nastruzzi, C., Cortesi, R., Esposito, E., Gambari, R., Borgatti, M., Bianchi, N., Feriotto, G. and Mischiati, C. (2000) *J. Control. Release* 68, 237–249.
- [11] Ljungstrom, T., Knudsen, H. and Nielsen, P.E. (1999) *Bioconjug. Chem.* 10, 965–972.
- [12] Scarfi, S., Giovine, M., Gasparini, A., Damonte, G., Millo, E., Pozzolini, M. and Benatti, U. (1999) *FEBS Lett.* 451, 264–268.
- [13] Wang, G., Xu, X., Pace, B., Dean, D.A., Glazer, P.M., Chan, P., Goodman, S.R. and Shokolenko, I. (1999) *Nucleic Acids Res.* 27, 2806–2813.
- [14] Simmons, C.G., Pitts, A.E., Mayfield, L.D., Shay, J.W. and Corey, D.R. (1997) *Bioorg. Med. Chem. Lett.* 7, 3001–3006.
- [15] Liberman, E.A., Topali, V.P., Tsifina, L.M., Jasaitis, A.A. and Skulachev, V.P. (1969) *Nature* 222, 1076–1078.

- [16] Smith, R.A.J., Porteous, C.M., Gane, A.M. and Murphy, M.P. (2003) *Proc. Natl. Acad. Sci. USA* 100, 5407–5412.
- [17] Murphy, M.P. (1997) *Trends Biotechnol.* 15, 326–330.
- [18] Muratovska, A., Lightowlers, R.N., Taylor, R.W., Turnbull, D.M., Smith, R.A.J., Wilce, J.A., Martin, S.W. and Murphy, M.P. (2001) *Nucleic Acids Res.* 29, 1852–1863.
- [19] Burns, R.J., Smith, R.A.J. and Murphy, M.P. (1995) *Arch. Biochem. Biophys.* 322, 60–68.
- [20] Ellman, G. and Lysko, H. (1979) *Anal. Biochem.* 93, 98–102.
- [21] Porteous, W.K. et al. (1998) *Eur. J. Biochem.* 257, 192–201.
- [22] Smith, P.K. et al. (1985) *Anal. Biochem.* 150, 76–85.
- [23] Schagger, H. and Von Jagow, G. (1987) *Anal. Biochem.* 166, 368–379.
- [24] Burns, R.J. and Murphy, M.P. (1997) *Arch. Biochem. Biophys.* 339, 33–39.
- [25] Lin, T.K., Hughes, G., Muratovska, A., Blaikie, F.H., Brookes, P.S., Darley-Usmar, V., Smith, R.A.J. and Murphy, M.P. (2002) *J. Biol. Chem.* 277, 17048–17056.
- [26] Kelso, G.F., Porteous, C.M., Coulter, C.V., Hughes, G., Porteous, W.K., Ledgerwood, E.C., Smith, R.A.J. and Murphy, M.P. (2001) *J. Biol. Chem.* 276, 4588–4596.
- [27] Murphy, M.P. (2001) *Exp. Opin. Biol. Ther.* 1, 753–764.
- [28] Smith, R.A.J., Porteous, C.M., Coulter, C.V. and Murphy, M.P. (1997) *Eur. J. Biochem.* 263, 709–716.
- [29] Dressler, G.R. and Woolf, A.S. (1999) *Int. J. Dev. Biol.* 43, 463–468.
- [30] Muratovska, A., Zhou, C., He, S., Goodyer, P. and Eccles, M.R. (2003) *Oncogene* 22, 7989–7997.