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Messenger RNA expression profiling of genes involved in epidermal growth factor receptor signalling in human cancer cells treated with scanning array-designed antisense oligonucleotides

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Abstract

Scanning oligodeoxynucleotide (ODN) arrays appear promising *in vitro* tools for the prediction of effective antisense reagents but their usefulness has not yet been reported in mammalian systems. In this study, we have evaluated the use of scanning ODN arrays to predict efficacious antisense ODNs targeting the human epidermal growth factor receptor (EGFR) mRNA in a human epidermoid cancer cell line and in primary human glioma cells. Hybridisation accessibility profile of the first 120 nt in the coding region of the human EGFR mRNA was determined by hybridising a radiolabelled EGFR transcript to a scanning array of 2684 antisense sequences ranging from monomers to 27-mers. Two ODNs, AS1 and AS2, complementary to accessible sequences within the EGFR mRNA, were designed and their ability to hybridise to EGFR mRNA was further confirmed by *in vitro* RNase H-mediated cleavage assays. Phosphorothioate-modified 21-mer AS1 and AS2 ODNs inhibited the growth of an established human A431 cancer cell line as well as primary glioma cells from human subjects when delivered as cationic lipoplexes. In contrast, scrambled controls and AS3—an antisense ODN complementary to an inaccessible site in EGFR mRNA—were inactive. Western blots showed that AS1 ODN exhibited a dose-dependent inhibition of EGFR protein expression in A431 cells in the nanomolar range. Microarray-based gene expression profiling studies of A431 cells treated with the 21-mer phosphorothioate AS1 ODN demonstrated successful inhibition of downstream signalling molecules further confirming the effective inhibition of EGFR expression in human cancer cells by antisense ODNs designed by scanning ODN array technology.

Keywords: Expression profiling; EGFR; Phosphorothioate; Oligodeoxynucleotide; Glioma; Primary tumour; A431 cells; Signal transduction

1. Introduction

The sequence-specific knockdown of gene expression by antisense ODNs relies on the fidelity of Watson–Crick base pairing with the complementary mRNA. Inhibition of translation may putatively occur by steric hindrance of the translation machinery and/or by recruitment of RNase H activity. Despite their rapid progression into clinical trials [1], not all complementary ODNs exhibit antisense activity. Compelling evidence suggests that a major cause of this observation is the intramolecular structure of mRNAs, which renders most of the molecule inaccessible to hybridisation with complementary nucleic acids.

In the absence of reliable computational methods for folding mRNAs and also due to the lack of our understanding of their interaction with ODNs, empirical methods are often used to discover effective antisense reagents. Traditionally, this has been achieved by an empirical 'walk-the-gene' strategy whereby a series of ODNs (up to 50 or more) targeted to multiple sites in the target mRNA

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Abbreviations: EGFR, epidermal growth factor receptor; RTK, receptor tyrosine kinase; TK, tyrosine kinase; ODN, oligodeoxynucleotide; RNase H, ribonuclease H.

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are evaluated in cell culture or *in vivo*. Although this strategy has been successful in identifying individually active sequences (e.g. for c-*raf* kinase where only 1/34 was potently active [2]), this strategy is not exhaustive in assessing all possible options, is labour intensive, time consuming and costly. A more recent improvement on gene-walking approach is RNase H-accessibility mapping of accessible sites using random oligomer libraries [3,4]. This technique relies on a sub-set of sequences from a random or a semi-random library to hybridise to accessible sites in RNA, which are then cleaved by RNase H. The resulting fragments are identified by molecular weight sizing or sequencing using defined primers [3,5]. Although, this technique broadly identifies regions of accessibility, precise selection of an antisense ODN sequence is not possible since RNase H cleavage can occur at multiple sites within a given ODN–mRNA heteroduplex. Since even a single nucleotide shift in antisense design can alter efficacy and potency [6,7], RNase H-accessibility mapping is still not ideal for optimal design of antisense reagents.

Recently, antisense ODN scanning arrays were successfully used to provide information on mRNA structure [8] and to identify antisense sequences targeting hybridisation-accessible sites that effectively downregulated target proteins in cell-free assays and in frog oocytes [4]. However, the efficacy of 'array-designed' antisense ODNs in mammalian cells has not been reported. Here, we report on the successful identification of effective antisense ODNs against human EGFR mRNA, designed using an ODN array, which inhibited target gene expression in human cancer cells.

EGFR (HER1 or erbB1), a 170 kD cell-surface-bound glycoprotein, belongs to the erbB family of receptors (others are erbB2, 3 and 4) that are involved in mitogenic signalling *via* their intracellular tyrosine kinase (TK) activity. EGFR is thought to be involved in several putative signal transduction cascades, including the classical cytosolic ras/raf/MAPK pathway, as well as following transactivation of a variety of G-protein-coupled receptors (GPCRs) [9,10]. More recently, it has been suggested that EGFR can translocate into the nucleus and directly acts as a transcription factor [11]. Activation of EGFR signalling can lead to increased proliferation and angiogenesis and decreased apoptosis. Overexpression of EGFR is implicated in the initiation and development of tumours of the breast, lung and brain [12,13]. We have been interested in selective downregulation of EGFR in several human tumour models [14–16]. In a previous study using antisense ODNs designed around the AUG initiation codon of EGFR mRNA, we failed to identify a truly antisense ODN. One ODN sequence that had anti-tumour effects turned out to function as an inhibitor of the EGFR TK activity, probably due to the presence of a TK inhibitory GGA GGG hexanucleotide motif [14,15]. In the present study, we have evaluated the use of scanning array technology to effectively predict active antisense ODNs targeting EGFR mRNA that are efficacious in both an established human A431 epidermoid carcinoma cell line and in primary human glioma cells.

2. Materials and methods

2.1. ODN synthesis and purification

Phosphodiester (PO) oligodeoxyribonucleotides were synthesised on an ABI394 DNA/RNA synthesiser using standard nucleotide CE-phosphoramidites (Cruachem), desalted through NAP-10 columns (Amersham) at least twice and stored at −20°. 2′-*O*-Methyl (2′OMe)-modified PO oligoribonucleotides were synthesised using 2′OMe CE-nucleotide phosphoramidites (Cruachem). Phosphorothioate (PS)-modified ODNs were obtained from Cruachem.

2.2. Preparation of radiolabelled transcripts

A 560 nt segment of EGFR gene starting at the first AUG codon was amplified from a plasmid containing the full-length EGFR cDNA clone using the polymerase chain reaction (94°, 1 min; 60°, 1 min; 72°, 2 min; 30 cycles). The 5′ primer including the T7 promoter sequence that was added upstream (5′ TTC TAA TAC GAC TCA CTA TAG GGA GAA TGC GAC CCT CCG GGA CGG C 3′) and the 3′end primer (5′ TCA GCT GTG GAG CCC TTA AAG 3′) sequences were synthesised as described above. This 560 nt fragment was used as a template in *in vitro* transcription to produce radiolabelled transcripts for hybridisation to the scanning array and RNase H assays. These were carried out in 20 μL volume at 37° for 1–1.5 hr using 2–3 μg of template DNA in the presence of 20 U of T7 RNA polymerase (Promega), 20 μCi [α-^{32}P]UTP (∼3000 Ci/mM; Amersham), 750 μM each ATP, CTP and GTP and 18.75 μM UTP (Amersham). The products were purified by filtration through MicroSpin Sephadex G25 columns (Amersham).

2.3. Fabrication of the ODN array

The scanning ODN array, spanning the first 120 nt downstream of AUG initiation codon of the EGFR mRNA, was made essentially as described earlier [17,18]. Aminated polypropylene (a gift of Beckman Instruments) was used as substrate and standard nucleotide CE-phosphoramidites were used in the synthesis, which was carried out on an adapted ABI394 DNA synthesiser (Applied Biosystems). A diamond-shaped mask with 54 mm diagonal was used to deliver the DNA synthesis reagents to the surface of polypropylene for *in situ* synthesis of ODNs that were attached to the surface by their 3′ ends. The increment at each coupling was 2 mm. Thus, the longest ODNs on the array were 27-mers. The array was de-protected in 30% ammonia solution at 55° for 16 hr in a closed chamber.

2.4. Hybridisation and image analysis

Approximately 50–60 fmol of the *in vitro* transcript was hybridised to the array in 20 mL of 1 M NaCl, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA and 0.01% SDS at 37° for 3 hr in a rotating tube at 3–4 rotations/min. The array was then washed in the hybridisation buffer at 37° for ~1–2 min, dried in layers of Whatman paper and exposed to a storage phosphor screen. The screen was scanned in a PhosphorImager (STORM; Molecular Dynamics). The image was analysed using the computer program, xvseq, as described earlier [18].

2.5. Analysis of hybridisation accessibility using RNase H mapping

A standard reaction consisted of 1 nM 560 bp ^{32}P-labelled 5′ EGFR1 transcript in 10 mM Tris–HCl, pH 7.4, 50 mM KCl, 50 mM $MgCl_2$ and 1 mM DTT, the appropriate ODN at 0.1 μM in a total volume of 9 μL. The reaction was incubated at 37° for 15 min prior to addition of 1 μL RNase H (2 U/μL; Gibco BRL), allowed to proceed for further 1 hr at 37° and stopped by the addition of 10 μL of gel loading buffer (80% formamide v/v, 1 × TBE and 0.25% w/v bromophenol blue). The products were separated by electrophoresis in a 6% denaturing polyacrylamide gel at constant 25 W for 2 hr. The separated products were visualised by autoradiography.

2.6. Cell culture and cell growth/viability assays

A431 cells were maintained in DMEM media supplemented with 10% FBS, 2 mM glutamine and 1% penicillin/streptomycin (all Gibco BRL). The primary cell lines, IN2045 and IN859, were obtained from samples kindly donated to the Department of Neurological Surgery at the Institute of Neurology, London, from patients diagnosed and surgically treated for glioma and were maintained in HAMS F-12 with 10% FBS and 1 mM glutamine. All cells were cultured in 75 cm^2 vented cap tissue culture flasks (Costar) and placed in a 95% humidified incubator with an atmosphere of 5% CO_2 in air and at a temperature of 37°. Confluent cultures were sub-cultured every 3–4 days. Briefly, cells were washed with PBS (A431) or Hanks balanced salt solution (primary cells) and detached from the culture vessel by means of trypsin (Gibco BRL). Cells were resuspended and centrifuged at ~800 *g* for 5 min and again resuspended in fresh media before counting by trypan blue exclusion. Cells were generally diluted 1:10 for stock flasks. Cell growth assays were performed in 24-well plates where cells were seeded at 5×10^4 cells/well and left to adhere overnight. Following this cells were washed with 1 mL PBS or Hanks solution and then fresh media containing the ODN:lipid complex added without FBS at a total volume of 200 μL for 4 hr. ODN concentration was varied but cationic DOTMA lipid (Lipofectin, Gibco BRL) was kept constant at 7.5 μM. After 4 hr fresh medium either without FBS for the A431 cells or with FBS for the primary cells was added and the cells incubated at 37° for a further 24 hr. At this time, a second 4-hr incubation of ODN–lipoplexes was administered as before. After a total culture time period of 48 hr, cells were trypsinised and counted using a haemocytometer and trypan blue to obtain viable cell numbers.

2.7. Western blotting

Cells were seeded into 24-well plates as for growth assays and treated with two doses of the desired ODN over 48 hr at 24-hr intervals. Cells were collected and lysed by the addition of 100 μL lysis buffer (0.5 M Tris–HCl, pH 6.8, 10% glycerol, 10% Triton X-100, 0.1 mM leupeptin, and 0.1 mM PMSF all from Sigma) for every 1×10^6 cells. Cell lysates were sonicated on ice then cell debris removed by centrifugation at ~10,000 *g* for 30 min at 4°. Supernatants were analysed for protein content using the standard BCA assay (Biorad) prior to addition of 5× Laemmlibuffer (367 mM Tris–Base (pH 6.8), 5.7% SDS, 5.7% β-mercaptoethanol (14.6 M), 28.7% glycerol, 0.2% bromophenol blue) and storage at −20°. To determine EGFR protein expression levels SDS–PAGE separation of samples was performed followed by Western blotting. Briefly, protein samples were heated to 95° for 5 min, then 8 μg of protein (as determined by the protein assay) was loaded into preformed wells along with a pre-stained high range molecular weight marker (Biorad). Samples were separated using a 4% stacking gel and a 7.5% resolving gel for 40 min at 40 mA prior to being transferred to nitrocellulose Hybond-ECl membrane (Amersham Life Sciences) at 215 mA for 1.5 hr. Transferred proteins were blocked with 5% non-fat dried milk in PBS–Tween and subsequently probed for EGFR using an optimised primary anti-human EGFR antibody raised in mouse (Sigma) for 1 hr at room temperature. The primary antibody was further probed with anti-mouse IgG hrp-conjugated antibody (Amersham Life Sciences) for 1 hr, which was then detected using ECl reagents and autoradiography (Amersham Life Sciences). To ensure equal loading of proteins actin levels were also detected using primary anti-human actin antibody raised in rabbit and the secondary anti-rabbit IgG hrp-conjugated antibody.

2.8. Gene expression profiling on microarrays

Total RNA was extracted and purified from A431cells using the Promega SV total RNA isolation kit according to manufacturer's protocol. Amino-modified cDNA probes were prepared as described by Clontech in their human 1.0 microarray (housing probes for 1080 human genes) kit and Cy3 or Cy5 fluorescent dyes were coupled to reverse-transcribed probes as per the manufacturer's instructions in their Atlas Glass Fluorescent Labelling Kit User Manual

(Clontech). Hybridisation's were performed overnight at 55° and slides washed sequentially in a series of buffer solutions containing decreasing salt solutions as described in the Atlas Glass Microarray User Manual (Clontech). Scanning and analysis of gene expression on microarrays were performed on a Affymetrix 418 Fluorescent Array Scanner System using Imagene 4.2 and GeneSight 3.0 software (Biodiscovery, Inc.).

2.9. Densitometric analysis of autoradiographs

Autoradiographs were scanned using a Trust SCSI Connect scanner connected to a PC computer and images were saved as uncompressed TIFF files. Scanned images were then analysed using NIH imager v1.58 program (Division of Computing and Research Technology, NIH, Bethesda, USA), which allowed plotting, and quantification of the relevant image intensities of band patterns on the autoradiographs.

3. Results and discussion

3.1. Hybridisation to the scanning array revealed ODNs with high binding affinity for EGFR mRNA

In a previous study, we designed antisense ODNs against the AUG initiation codon of EGFR mRNA using a mini gene-walking strategy and failed to detect an effective antisense inhibitor of EGFR gene expression [14]. In this study, we used a scanning ODN array to identify antisense ODNs with high affinity for human EGFR mRNA.

The scanning ODN array, ranging in size from monomers to 27-mers, represented complements of a 120 nt sequence of the EGFR mRNA starting at the first AUG codon. The longest ODNs were made along the centreline of the array, which represented a 27 nt sliding window of ODNs complementary to EGFR mRNA. The monomers were at the edge of the array and in between were all lengths from monomers to 27-mers. The total number of ODNs on the array can be calculated by using the formula, $N - s + 1$, where N is the total length of the sequence covered on the array and s is the length of ODNs. Therefore, there were 120 monomers, 94 of the full-length 27-mers and a total of 2684 different ODN sequences in the array made by 120 coupling steps. Since these arrays are symmetrical above and below the centreline, each ODN is represented twice allowing for duplicate measurements of the hybridisation at each point.

The autoradiograph of the array hybridised with radiolabelled human EGFR transcript showed three chevron-shaped regions of hybridisation. Analysis of the hybridisation image with the computer software, xvseq, revealed sequence, lengths and the hybridisation intensities of the various ODNs. Approximately 20 nt downstream of the AUG initiation codon lay a region of rather weaker hybridisation and two further regions of stronger hybridisation lay between ~51–74 and 78–95 nt, respectively. Interestingly, the data from this hybridisation explained the previously observed ineffectiveness of our antisense ODNs designed near the AUG start codon of EGFR mRNA [14]; this region of the human EGFR mRNA was found inaccessible with the array technology.

Three 21-mer antisense ODN sequences were selected from the array for further studies. AS1 was selected from the sequence between 51 and 74 nt and AS2 from sequence between 78 and 95 nt. AS3 was selected from an inaccessible region of the EGFR mRNA (Fig. 1). In addition, to investigate the influence of ODN length/hybridisation intensity on biological activity, we also synthesised 7-, 10-, 14- and 17-mer antisense ODNs from the parent 21-mer AS1 (Fig. 1).

3.2. RNase H effectively cleaves EGFR transcripts in the presence of AS1 or AS2 but not AS3

We first examined the ability of AS1 and AS2 to bind with EGFR mRNA *in vitro* using an RNase H assay. A 560 bp EGFR transcript was incubated with the two ODNs as either all PO, all PS or all 2′OMe chemistries and its cleavage in the presence of RNase H was assessed by PAGE. In concordance with the array data, RNase H effectively cleaved the 560 bp EGFR transcript in the presence of AS1 (Fig. 1). AS1/RNase H-mediated cleavage of EGFR transcript occurred with ODNs synthesised by PO or PS chemistries, both of which support RNase H activity, but not when synthesised as 2′OMe, which does not support RNAse H activity. Similar results were obtained with AS2, but AS3, which targets a site predicted by the array to be inaccessible to hybridisation, failed to induce RNase H-mediated cleavage of the EGFR transcript (Fig. 1). This confirmed that array predictions of the accessible sites for hybridisation in EGFR mRNA correlated well with data from RNase H cleavage assays.

We also tested the effect of length of the antisense ODNs on their binding affinity with the target and used 7-, 10-, 14- and 17-mer sequences of AS1 shortened at the 5′ end. Both 7- and 10-mer ODNs showed negligible hybridisation on the array and also did not induce significant RNase H-mediated cleavage of the EGFR transcript, whereas both 14- and 17-mers induced marked cleavage of the EGFR transcript in the expected manner (data not shown).

3.3. Array-designed anti-EGFR ODNs delivered as cationic lipoplexes inhibit growth of cultured human epidermoid A431 and primary human glioma cells

We next evaluated the ability of PS-modified AS1 and AS2 to inhibit the EGFR-dependent growth of human A431 epidermoid carcinoma cells that are known to overexpress this receptor, as well as of the primary human glioma cells taken from two human subjects. The cellular

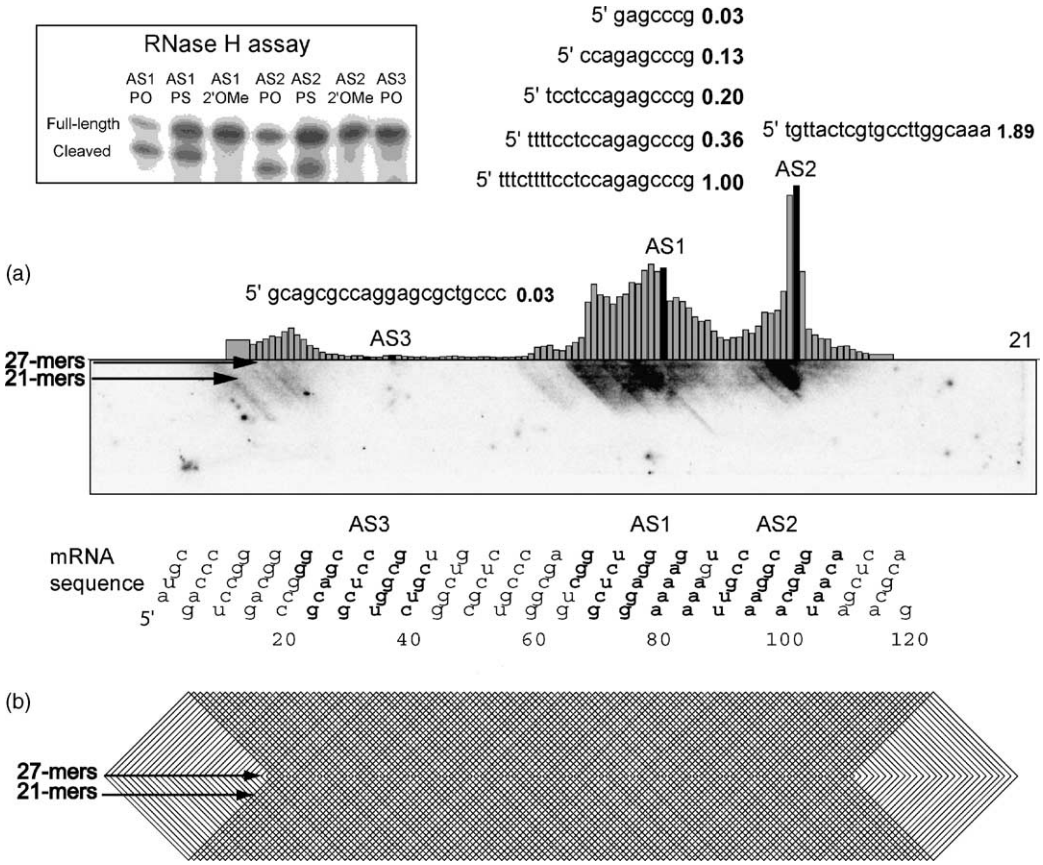

Fig. 1. Autoradiograph of one half of the ODN array hybridised with radiolabelled EGFR transcript. The results of the integrated pixel values obtained using the software xvseq are displayed as histograms. Only values for 21-mers are displayed. Locations of the 21- and 27-mers along the longitude of the array are marked with arrows. The sequences of the antisense ODNs used in various assays are shown on the histograms and their complementary sequences marked in the mRNA sequence. The hybridisation intensities (arbitrary units) of the various antisense ODNs relative to AS1, as obtained from the array image analysis, are also given. Results of the RNase H assay at 0.1 μM concentration of the various ODNs are also shown. A template of overlapping diamonds, produced by the software xvseq, used to analyse the hybridisation image is shown in the lower panel.

delivery of AS1 was optimised for each cell type using Lipofectin, a cationic lipid delivery system. In all cases, a sub-toxic dose of the cationic lipid yielding the highest cell association of ODN and optimal intracellular biodistribution was used as described previously [19]. The transfection conditions for AS2 were the same as for AS1, since both have similar charge and lengths. Figure 2a and b show a dose-dependent inhibition of A431 tumour cell growth with AS1 and AS2 following delivery with Lipofectin but not in the absence of the lipid delivery system. Neither the scrambled control nor AS3 had any effect on A431 cell growth, which is in concordance with the array hybridisation data. The all 2′OMe-modified AS1 and AS2 (Fig. 2a and b) did not inhibit A431 cell growth suggesting an RNase H-mediated mechanism of action. Interestingly, AS2 sequences containing two or four mismatches were ineffective in cell culture, whereas AS1 containing two mismatches retained its activity (∼60% inhibition of A431 cell growth) but became inactive with four mismatches implying that different accessible sites within EGFR mRNA may have differing requirements for hybridisation stringency. Differences in heteroduplex stability may account for this observation but AS1 and AS2 ODNs are predicted to have similar melting temperatures (64 and 62°, respectively) implying that mismatch tolerance may be difficult to predict by T_m calculations and thus has to be determined by experimentation. However, further studies are required for a definitive explanation.

In two primary brain tumour cell types taken from human patients, IN859 and IN2045, AS1 and AS2 were similarly effective at inhibiting the tumour cell growth (between 40 and 60% of control) whereas AS3 or the respective scrambled sequences of AS1 or AS2 or as 2′OMe-modified chemistries were ineffective (Fig. 2c and d). It is interesting to note that although hybridisation intensities from the scanning array (Fig. 1) suggested AS2 should be more active than AS1, this trend was not observed in the three cell populations (A431, IN859 or IN2045) studied here. The reasons for this are unclear but could be due to the obvious differences in the *in vitro* protein-free hybridisation conditions used for the array experiments compared to the natural cellular environment where, for example, RNA-binding proteins may have an important role [4]. Another possibility is that the short

560 bp transcript used for assessing hybridisation accessibility in the scanning array experiment may exhibit differences from that of the full-length transcript found in the cancer cells. Indeed, we have previously shown that subtle differences in hybridisation accessibility were observed with different length transcripts used in array experiments [20]. However, hybridisation of the 120 nt array with a 3578 bp full-length EGFR mRNA transcript did not appear to result in any significant differences in hybridisation data at least within the regions covered by AS1 or AS2 from that observed in Fig. 1 (data not shown).

A 17- and 21-mer PS ODN designed to the AS1 hybridisation site was effective inhibitor of A431 cell growth

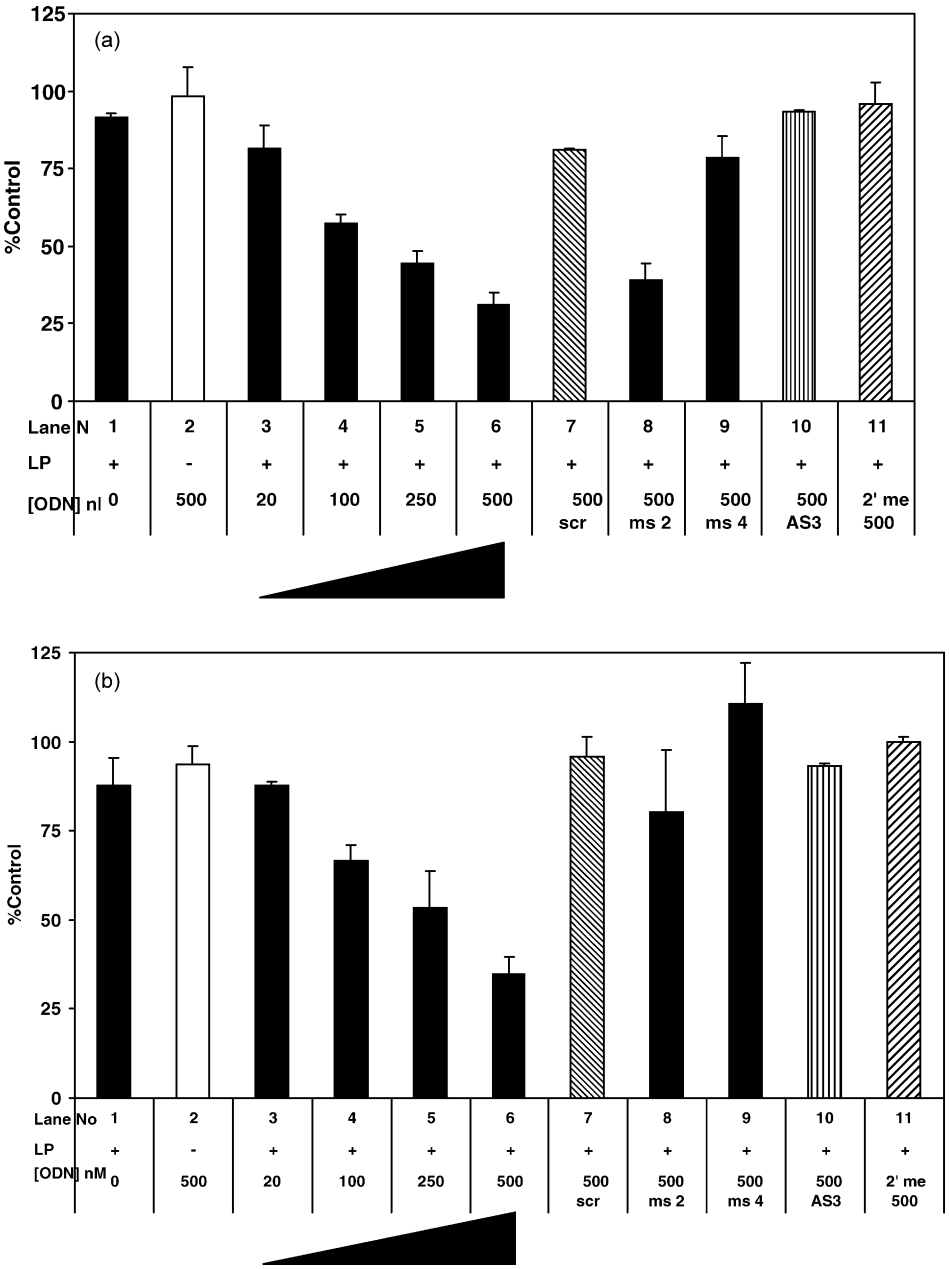

Fig. 2. Inhibition of cell growth with scanning array-designed AS1 and AS2 antisense ODNs in human epidermoid A431cell line (a and b) and primary human glioma cells (c and d). Cells were treated with phosphorothioate AS1 or AS1 delivered as cationic lipoplexes (Lipofectin) and number of viable cells determined by trypan exclusion blue assay after 48 hr as described in Section 2. The presence or absence of the Lipofectin (LP) delivery and the nanomolar dose of ODN are indicated. Scr refers to the corresponding scrambled sequence for AS1 (5′ CTG ATC CTG CTC TGA TCC TCT 3′) or AS2 (5′ ATC GTC TTC AGT GAC TTA GCG 3′). 2′ me refers to the all 2′-*O*-methyl-modified chemistry of the stated ODN whereas AS3 is an antisense ODN targeting a hybridisation-inaccessible region within EGFR mRNA (see Fig. 1). The abbreviations ms 2 (5′ TTT CTT TTC CTA **T**AG AGC CCG 3′ for AS1 and 5′ TGT TAC TCG TGA **A**TT GGC AAA 3′ for AS2) and ms 4 (5′ TTT CTT TTC CGA **T**CG AGC CCG 3′ for AS1 and 5′ TGT TAC TCG T**TA AC**T GGC AAA 3′ for AS2) refer to sequences housing two or four mismatches (shown in bold) in the parent AS1 (a) or AS2 (b). For other sequences used see Fig. 1. The mean ± SD of three independent experiments are shown.

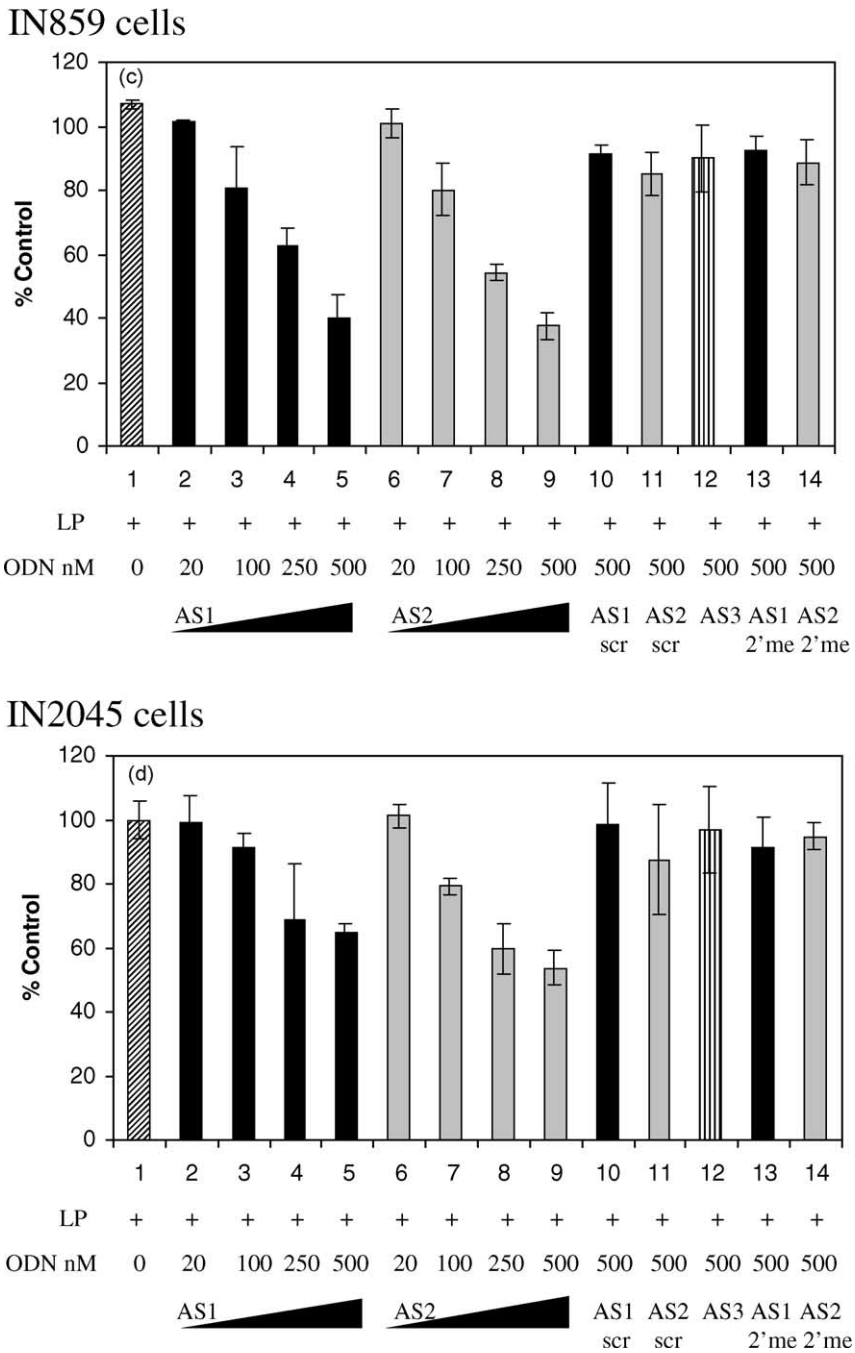

Fig. 2. (*Continued*).

whereas a 7-, 10- and a 14-mer PS ODN was inactive suggesting a size cut-off of around 14- to 17-mers for activity at this site (Fig. 3). These cellular data correlate well with the results obtained in the RNase H cleavage assays and the array experiments as mentioned above.

3.4. Inhibition of A431 cell growth correlates with downregulation of EGFR protein expression

Using the established epidermoid A431 cancer cell line that highly expresses EGFR [14] as our model, we then tested whether the observed inhibition of cell growth was consistent with the downregulation of EGFR protein expression. A431 cells were treated with two doses of AS1 (as complexes with Lipofectin) over a 48-hr period as per the cell growth assays and the levels of EGFR expression were monitored using Western blotting with an anti-EGFR antibody. Figure 4 shows a dose-dependent inhibition of EGFR protein expression; up to 95% inhibition of the protein was observed at the highest (500 nM) dose of AS1 (Fig. 4a and b) but not with the scrambled or AS3 control ODNs (Fig. 4c).

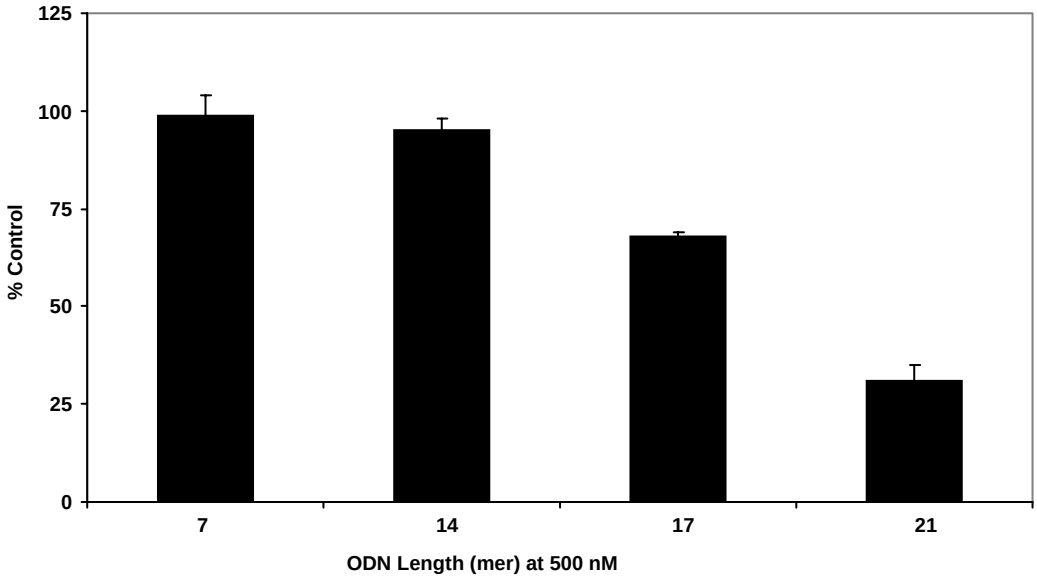

Fig. 3. The effect of AS1 length on A431 cell growth. Cells were treated with a 7-mers (5′GAG CCC G 3′), 14-mers (5′ CCA GAG CCC G 3′), 17-mers (5′ TTT TCC TCC AGA GCC CG 3′) and the parent AS1 (5′ TTT CTT TTC CTC CAG AGC CCG 3′) at a concentration of 500 nM as described in the legend for Fig. 2 (see also Section 2). Inhibitory effects are represented as a percent of viable cells relative to the Lipofectin only-treated control. The mean ± SD of three independent experiments are shown.

3.5. Microarray gene expression profiling showed that AS1 downregulated EGFR mRNA and that of downstream mediators involved in the EGFR signalling pathway

We next examined the effects of AS1 on inhibition of EGFR mRNA expression and several downstream signalling mediators in cultured A431 human carcinoma cells using low density gene expression microarrays (Clontech Glass Arrays containing 1080 genes). Cells were treated with either delivery system (Lipofectin) alone or mixed with AS1 or scrambled ODN. Table 1 shows that antisense activity of AS1 leads to a marked reduction in expression of EGFR mRNA (∼2.5-fold) and of several downstream genes involved in the EGFR-mediated signalling pathway (see below) whereas the scrambled control ODN had relatively little effect on EGFR mRNA or on the expression of the downstream genes studied. The scrambled ODN served as a useful control for the validation of the array expression data. The lack of effect on EGFR protein levels with the scrambled control as determined by Western blotting (see Fig. 4) was confirmed in the gene expression profiling arrays where there was no observed change in EGFR mRNA expression (Table 1).

Activation of EGFR signalling in mammalian systems occurs by a variety of mechanisms that include binding of ligands, such as epidermal growth factor (EGF), transforming growth factor α (TGF-α), heparin-binding EGF (HB-EGF), epiregulin (β-cellulin) and amphiregulin or by transactivation following, for example, GPCR activation. Furthermore, signalling *via* its intracellular TK domain can be through autophosphorylation mediated by homodimerisation or *via* heterodimerisation with other members of the erbB family of receptor tyrosine kinases (RTKs) at specific C-terminal tyrosine residues that subsequently serve as docking sites for proteins housing Src homology 2 (SH2) or phosphotyrsoine-binding (PTB) domains (reviewed in refs. [9,12,13,21]). This diversity of EGFR action leads to a complicated signalling web that involves a multitude of signalling pathways not all of which are well understood. For the purposes of this study, our goal was to assess the knockdown of downstream effectors of EGFR signalling. From our gene expression arrays, we focused on around 45 genes as indicated in Table 1. Key downstream signalling mediators whose gene expression was downregulated by approximately 2-fold or more following treatment of A431 cells with AS1 antisense ODN were members of the Raf family (e.g. B-raf) and several of the MEKs, ERKs and MAPKs (see Table 1) that are thought to be involved in the classical ras/raf/MAPK mitogenic signalling pathway—a major route for intracellular EGFR signalling (for review see ref. [9]). In addition, significant downregulation in gene expression was observed for the intracellular EGFR substrate protein, EPS15 (AF-1P), that appears to be involved in receptor downregulation by regulating clathrin-coated pit-mediated internalisation of the receptor [22–24] as well as the downstream ras-related small G-proteins of the RAP family (i.e. RAP1B) and RAB family (i.e. RAB3B, RAB7 and RAB6) that are also involved in receptor trafficking ([25] and references therein). Phospholipase D gene expression, a well-known downstream effector of EGFR signalling [26,27], was also downregulated by approximately 4-fold. A more than 2-fold inhibition of c-*src* was observed with AS1 treatment. It is known that c-*src* acts as both a downstream substrate and an upstream effector for EGFR signalling cascades [26]. In addition to its role in mediating the mitogenic signalling following EGFR

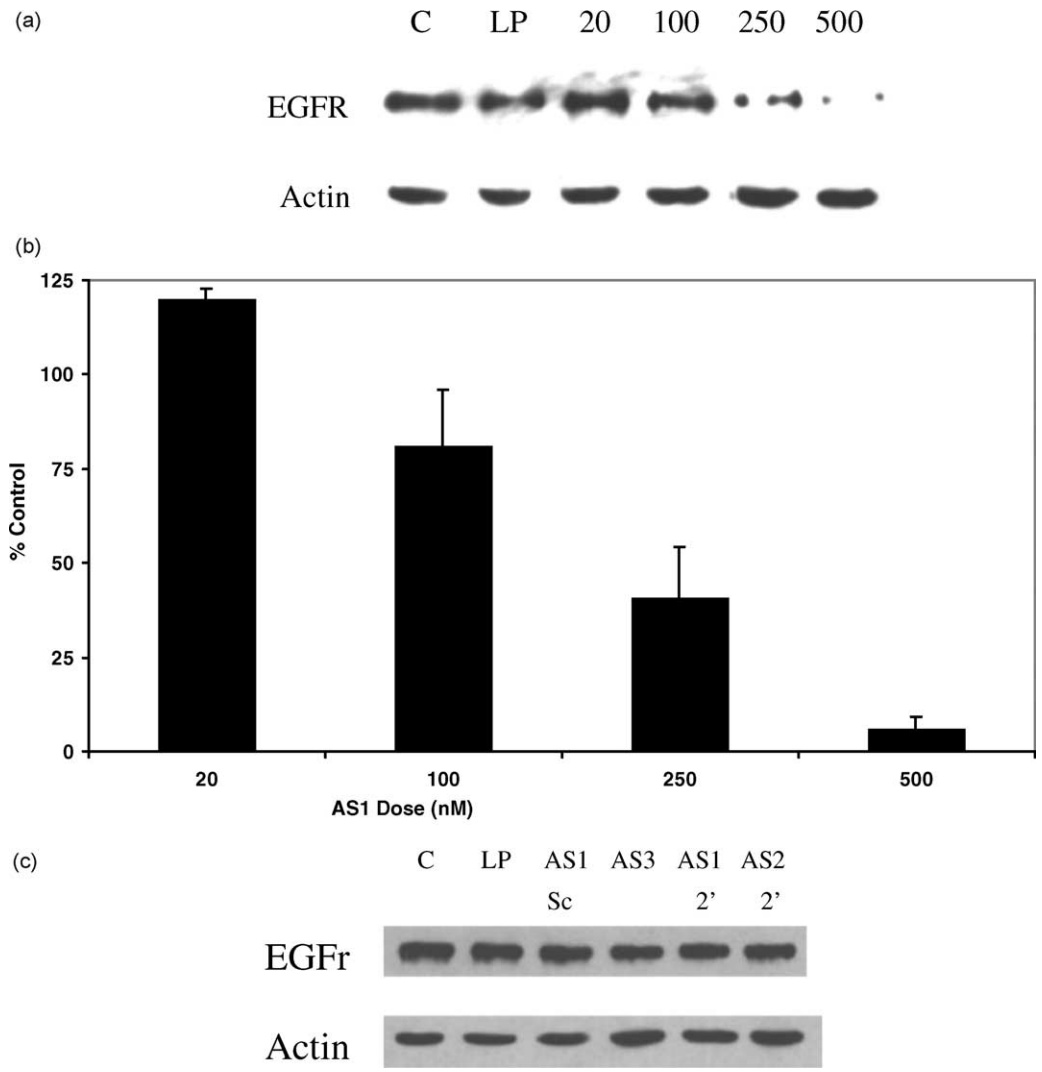

Fig. 4. Inhibition of EGFR protein expression with array-designed AS1 ODN but not with control ODNs. (a) A representative Western blot showing a dose-dependent inhibition of EGFR expression relative to actin expression which is not altered. (b) A histogram showing a quantitative effect of EGFR expression from three independent Western blot experiments following densitometric analyses. Data are represented as a percent of the Lipofectin only-treated controls. The mean ± SD of three independent experiments are shown. (c) A representative Western blot showing the lack of effect on EGFR protein by various control ODNs. The abbreviation Sc refers to the scrambled AS1 sequence, 2′ refers to the all 2′-*O*-methyl-modified chemistry of the AS1 ODN whereas AS3 is an antisense ODN targeting a hybridisation-inaccessible region within EGFR mRNA (for sequences see Fig. 1 and the legend to Fig. 2).

autophosphorylation, c-*src* also plays a key role in the transactivation of EGFR by GPCRs suggesting that AS1-mediated downregulation of EGFR may also impact on GPCR signalling. As GPCR activators include important endogenous chemokines, like angiotensin II, noradrenaline and endothelin-1, inhibition of EGFR transactivation could have important consequences in whole animal studies. However, recent clinical trials data in cancer patients receiving Iressa (ZD1839), an orally administered inhibitor of EGFR signalling, indicates that EGFR inhibitors are well tolerated and the only side-effects appear to be skin rashes and diarrhoea [28]. However, further data from long-term clinical studies are required for a complete toxicological evaluation or EGFR TK inhibitors.

AS1-mediated downregulation of c-Jun N-terminal kinases (JNKs) and JAKs suggested that alternative pathways of EGFR signal transduction (e.g. PAK/JNKK/JNK) were active in the proliferation of A431 cells. Inhibition of Akt upon treatment with antisense ODN, suggested that another important EGFR signalling route *via* phosphoinositide-3-kinase (PI3K)/Akt was also downregulated. This pathway is known to regulate apoptosis and cell proliferation. The serine–threonine kinase, Akt, is thought to send survival (antiapoptotic) signals *via* phosphorylation of multiple targets, including BAD, a member of the BCl-2 family, and may play important roles in regulating caspase-9 activity as well as controlling cell cycle progression [29]. Inhibition of EGFR expression by antisense also appears to impact on signalling pathways involving protein kinase C, where significant inhibition in the β and ε, but not η or γ, isoforms was observed. There was also significant (2-fold) inhibition of the nuclear transcription factors c-*fos* and c-*myc* that are known to be involved in EGFR signalling, following treatment with AS1 but not with the scrambled

Table 1
Gene expression changes in A431 cells treated with AS1 as studied by microarrays.

Gene name	Gene expression ratio[a]	
	AS1 ODN treated	Scrambled ODN treated
EGFR	0.40	1.14
EPS15 substrate protein	0.38	0.91
Src	0.44	0.80
B-raf	0.50	1.09
RAP1A	0.73	0.69
RAP1B	0.50	0.62
RAB3B	0.27	1.40
RAB7	0.23	1.00
RAB5A	0.24	2.18
RAB6	0.36	1.00
MEKK3	0.27	1.33
MEKK5	0.21	0.81
MAPKK5	0.75	0.81
MAPKK6	1.00	3.10
ERK1	0.38	1.07
ERK2	0.68	0.93
ERK3	0.33	1.00
ERK4	0.53	0.80
ERK5	0.36	1.00
p38 MAPK	0.27	1.17
MAPKAPK-2	0.29	1.00
JNK1	0.65	0.91
JNK2	0.67	0.92
JAK1	0.29	0.94
JAK2	0.25	0.77
JAK3	0.32	1.00
Rho7	0.33	0.92
c-*fos*	0.45	0.90
c-*jun*	0.73	0.50
c-*myc*	0.50	0.69
A-myb	0.92	0.73
Protein kinase C-β1	0.39	1.11
Protein kinase C-ε	0.35	0.92
Protein kinase C-η	0.96	0.69
Protein kinase C-γ	0.88	0.75
Phospholipase-β3	0.47	1.21
Phospholipase D	0.28	0.94
Akt	0.50	0.91
GLUT-1, glucose transporter	0.70	1.00
ATP2B2	1.00	0.62
Aquaporin 4	1.03	0.92
CETP1	0.08	0.10
40S ribosomal protein	1.00	0.69

Similar data was obtained in a second independent experiment and the range of data readings obtained in the two experiments was typically less than 10% of the mean.

[a] Gene expression ratio refers to the level of gene expression in AS1 treated relative to that obtained with the lipid alone (Lipofectin) control cells. Values <1 represent reduced expression relative to lipid control whereas >1 represent increased expression relative to the lipid control.

ODN control. Only a modest knockdown in Jun was observed with antisense treatment suggesting its activation by other pathways that would compensate for its inhibition following antisense treatment. However, inhibition of this transcription factor was seen with the scrambled control implying a non-specific activity of this control ODN either directly on the transcription factor or *via* inhibition of other upstream mediators that signal to c-*jun* in the nucleus (see also discussion below). Gene expression profiling thus confirmed that several pathways, including the classical ras/MAPK/ERK, the PI3K/Akt and the JNKK/JNK pathways in the EGFR cascade appear to be important in the growth and proliferation of A431 cells and further affirms the involvement of a multitude of downstream effectors in the complex EGFR signalling web. In a recent study using antisense ODNs targeting the cAMP-dependent protein kinase A R1α isoform [30] have also shown that a multitude of downstream genes are involved in mediating signalling *via* this isoform in prostate carcinoma cells. The authors [30] thus have suggested that antisense ODNs targeting key signalling genes should be considered as 'multisite genomic modulators' presumably as they will alter the expression of many genes both downstream and upstream in the signalling cascade.

Consistent with the Western blotting data that showed that control proteins like actin were unaffected by AS1 treatment (see Fig. 4), microarray expression profiling also showed little or no effect of AS1 (or the scrambled control) on other 'control' genes, such as GLUT-1, ATP2B2, aquaporin 4 or 40S ribosomal protein, that are not thought to be directly involved in EGFR signalling. However, the expression of some apparently non-target genes was affected by ODN treatment. For example, the scrambled control ODN inhibited expression of c-*jun* and the CETP1 (cholesterol ester transfer protein) gene and upregulated the expression of MAPKK6 and RAB5A by a factor of 2-fold or more implying that non-specific effects either due to sequence, delivery system, or the PS chemistry were possible with antisense ODNs. The gene expression changes in apparently non-targeted genes were consistent with a recent report by Juliano and colleagues [31] where expression of approximately 2% of the non-targeted genes tested, as assessed by cDNA microarrays, was altered with an antisense PS ODN targeting the human multidrug resistant (mdr-1) gene. PS ODNs are known to interact with non-targeted proteins, including EGFR [14,15,32,33]. Indeed, we have recently shown that a hexameric GGA GGG PS sequence motif, but not an all PO or 2′OMe-modified motif, inhibited EGFR and insulin RTK activity in cell culture [15]. This hexameric sequence motif, however, was absent from the ODNs studied here implying that other factors were important in the non-target gene changes. A more thorough study examining genome-wide expression as a consequence of antisense treatment is needed for a full assessment of specificity of a given ODN/chemistry combination and, clearly, array technology-based expression profiling represents a powerful tool for such studies.

4. Concluding remarks

The specific inhibition of EGFR signalling has emerged as a promising therapeutic strategy in the treatment of

cancers, such as prostate, breast, brain, gastric, colorectal and ovarian, where receptor expression appears elevated [12,13]. As a result several small molecule drugs that inhibit EGFR TK activity (e.g. Iressa (ZD1839), Tarceva (OSI-774)) and monoclonal antibodies (e.g. IMC-C225) have reached an advanced stage of clinical evaluation [10,12,13]. However, the recent failure of ZD1839 in trials in combination with conventional cytotoxics has highlighted the need for alternative approaches for the inhibition of this important signalling pathway. Several groups, including our own, have been examining the use of antisense ODNs to inhibit EGFR expression as effective inhibitors of cell proliferation for potential anti-cancer therapy [14,34–37]. However, the problems of ineffective delivery of antisense ODNs [1,38] and the inability of all antisense ODN designs to exert the desired biological activity has hampered clinical development [8,39]. Indeed, our previous attempts to design anti-EGFR ODNs by a gene-walking strategy failed to yield effective antisense molecules against EGFR; instead they further highlighted that specific sequences of a defined chemistry can result in non-specific inhibition of RTK activity and, furthermore, that these effects may also mask or be mistaken for a true antisense action [14,15]. The failure of several antisense sequences in our previous study also highlighted the need to target hybridisation-accessible sites within a given, often highly structured, mRNA sequence. The present study thus focused on improving the design of antisense ODNs targeting the EGFR mRNA by using scanning ODN arrays, a technology that had previously been reported to effectively predict hybridisation-accessible sites that were not always identifiable by conventional RNA folding algorithms [4,40–42]. Two antisense constructs (AS1 and AS2), so defined, were effective in inhibiting cell growth in an established A431 cell line overexpressing EGFR as well as in primary human glioma cells from human subjects—a tumour-type reported to overexpress EGFR in about 40–50% of cases [43]. Lipid-mediated delivery produced maximal effects on cell number at 500 nM AS1 as a 1 μM dose resulted in a similar effect on A431 cell growth (data not shown) implying that ODN delivery may be maximal at this ODN:lipid ratio (see also ref. [19]). Mechanistic studies in A431 cells suggested an RNase H-dependent antisense-mediated reduction in EGFR expression. Evidence supporting this hypothesis included the *in vitro* RNase H cleavage assays that confirmed that EGFR mRNA was cleaved at the sites predicted by scanning array technology upon heteroduplexation with AS1 or AS2 (Fig. 1), the lack of effect of 2′OMe-modified AS1 or AS2 on tumour cell growth (Fig. 2)—a chemistry that does not support RNase H activity, and the observed reduction in EGFR mRNA with AS1 treatment as demonstrated by microarray expression profiling (Table 1).

Array-based gene expression profiling was also a useful tool in confirming the activity of array-designed AS1 in downregulating several downstream signalling mediators in the EGFR signal transduction cascade. Thus, this study showed that scanning arrays can be successfully used to identify antisense sequences targeting hybridisation-accessible sites in EGFR mRNA that subsequently function as true antisense molecules in cancer cells of human origin. It further highlighted the usefulness of gene expression arrays for the monitoring of antisense activity and specificity in cell systems as well as providing supportive data for the potential use of array-designed antisense ODNs, as alternatives to small molecule inhibitors, for the potential inhibition of EGFR signalling in the clinic. Such studies may also aid in understanding the mechanism and specificity of action as well as the safety or toxicological profile of EGFR inhibitors, such as ZD1839 and OSI-774, that are already undergoing human clinical trials evaluation.

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