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Co-delivery of an antisense oligonucleotide and 5-fluorouracil using sustained release poly (lactide-co-glycolide) microsphere formulations for potential combination therapy in cancer

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Abstract

Antisense oligonucleotides (AODNs) can selectively inhibit oncogene expression by Watson–Crick hybridisation to target mRNA and are being increasingly considered for use in combination with conventional drugs for potential anticancer therapy. Combination therapy of AODNs and cytotoxic agents using biodegradable polymeric delivery systems potentially offers several advantages including site-specific or organ-directed targeting, protection from digesting enzymes, and improved pharmacokinetics/pharmacodynamics resulting from sustained delivery of the entrapped drugs. Using a model AODN targeting the epidermal growth factor receptor (that is over-expressed in several cancers including breast and brain cancer) and the commonly used cytotoxic agent, 5-fluorouracil (5-FU), we have examined the use of poly (lactide-co-glycolide) (P(LA-GA)) microsphere formulations for co-delivery of these agents. Both agents were either co-entrapped in a single microsphere formulation or individually entrapped in two separate microsphere formulations and release profiles determined in vitro. Using a double emulsion method for preparing the P(LA-GA) microspheres suitable entrapment and sustained release over 35 days was observed in both types of formulation. Release of AODN and 5-FU from all formulations appeared to be biphasic. However, the release rates of the two agents were significantly slower when co-entrapped as a single microsphere formulation compared to those obtained with the separate formulations. Electrophoretic mobility shift assays suggested that this might be, in part, due to an interaction of 5-FU with the oligodeoxynucleotide (ODN). Further, our data suggest that by mixing individual formulations of 5-FU and ODNs at different mass ratios allowed greater flexibility in achieving the desired release profile as well as avoiding potential drug–drug interactions. Thus, co-administration of individual P(LA-GA) microsphere formulations of AODNs and 5-FU, at appropriate mass ratios, appears worthy of further investigation for the potential co-delivery of these anti-cancer agents in vivo.

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1. Introduction

Antisense oligodeoxynucleotides (AODNs) are short synthetic nucleotide sequences that down-regulate the expression of disease-causing proteins by inhibiting gene expression at the level of mRNA. Conventional therapies utilise compounds which act upon the protein itself often require the non-rational approach of screening thousands of compounds to find an active molecule and even then, the resultant compound may lack specificity of action. In contrast the antisense strategy, at least in principal, allows for the rational design of highly sequence-specific nucleic acid drugs that can target and even destroy a given mRNA. The fidelity of antisense molecules arises from their ability to Watson–Crick hydrogen bond to their target mRNA within hybridisation-accessible sites (for reviews, Akhtar et al., 2000; Hughes et al., 2001). The first antisense drug molecule, Fomiversin (ISIS Pharmaceuticals/Ciba Vision) is already on the market and several others are in clinical trials (Akhtar et al., 2000 for a recent review). However, due to their relatively short half-lives in vivo, AODNs need to be delivered to the target site for extended time periods in order to achieve optimal biological effects, this is especially true for targets with a slow turnover (Akhtar and Agrawal, 1997). This can be achieved using biodegradable sustained release polymeric formulations (Chavany et al., 1992; Lewis et al., 1995, 1998; Fattal et al., 1998; Khan et al., 2000). Of the many options available we have been evaluating the use of biodegradable microspheres of poly (lactide-co-glycolide) (P(LA-GA)) copolymers for the potential delivery of AODNs (e.g. Akhtar and Lewis, 1997; Lewis et al., 1998; Khan et al., 2000). These particulate delivery systems offer several advantages including the potential for localised, site-specific or organ-specific delivery, protection from nucleases that digest oligodeoxynucleotides (ODNs), and tailored release profiles that further allow control of the entrapped drug's pharmacokinetics and pharmacodynamic parameters (Khan et al., 2000; Lewis et al., 1995, 1998).

AODNs have been extensively considered for the down regulation of oncogenes in cancer therapy and several sequences e.g. those targeting c-raf kinase are being tested in ongoing clinical trials (Akhtar et al., 2000; Akhtar and Agrawal, 1997). A more recent approach is the use of AODNs in combination with conventional chemotherapy (Geiger et al., 1998; Bufalo et al., 1996; Tortora et al., 1998). For example, a greater inhibition of tumour cell growth was observed in vitro and in vivo when a c-myb AODN was co-administered with cisplatin than with either of the agents alone (Bufalo et al., 1996). In another study, 5-fluorouracil (5-FU), an inhibitor of thymidylate synthase, was shown to be more cytotoxic (by 50–60%) when combined with AODN specific for thymidylate synthase mRNA (Ferguson et al., 1999). An AODN specific for alpha-fetoprotein mRNA in combination with 5-FU has also shown a significantly enhanced effect on hepatoma cell growth as compared to either AODN or 5-FU alone (Wang et al., 1999). Thus, therapeutic strategies involving combinations of AODNs with conventional cytotoxics appear promising for potential cancer therapy. Cytotoxic drugs like 5-FU are potent antineoplastic agents but they tend to exhibit side effects in the body (Parker and Cheng, 1990). In the case of 5-FU, it is rapidly absorbed through the blood capillaries into systemic circulation (Ardalan and Glazer, 1981). This results in relatively low levels of drug near the site of action with the subsequent loss of efficacy and increased risk of systemic toxicity. By using sustained release formulations of 5-FU the incidence of side effects may be reduced and therapeutic effects increased (Brem and Lawson, 1999; Hagiwara et al., 1996). Based on the fact that local sustained delivery may enhance therapeutic effects a pilot study involving eight patients with newly diagnosed glioblastoma, a malignant brain tumour, was conducted in which 5-FU in P(LA-GA) microspheres was delivered locally af-

ter surgical resection (Menei et al., 1999). Promising results were reported by this study with 2/8 patients achieving disease remission (Menei et al., 1999).

Our laboratory has been examining the use AODNs for the treatment of brain cancer by targeting the epidermal growth factor receptor, EGFR, (encoded by the *c-erb*B1 proto-oncogene), that is commonly over-expressed in glioblastomas (Coulson et al., 1996). In an attempt to use EGFR AODNs with conventional cytotoxics, we report here on the potential co-delivery of a 21-mer phosphorothioate ODN sequence (complementary to the 5′-coding sequence of *c-erb*B1) and 5-FU, a model cytotoxic drug, using P(LA-GA) microspheres. Individual formulations of the two agents, as well as co-entrapment of the AODN and 5-FU within a single microsphere formulation, were characterised and release profiles determined. Release of the two agents appeared biphasic and was sustained for over the entire 35-day study-period. Compared to the separate formulations, release of both agents was significantly retarded when co-entrapped and released from a single microsphere formulation. Electrophoretic mobility shift assays (EMSA) suggested that this effect, in part, may be explained by a potential interaction of 5-FU with adenine residues in the co-entrapped ODN sequence. We conclude that separate formulations of the two agents allow greater flexibility in achieving the desired release profiles, simply by mixing different mass ratios of the two microsphere preparations, and furthermore can avoid the problems of 5-FU and AODN drug–drug interactions.

2. Methods and materials

2.1. Oligodeoxynucleotide synthesis

Antisense phosphorothioate oligodeoxynucleotides were synthesised on an automated DNA synthesiser (Model 392, Applied Biosystems, Warrington, UK) using standard phosphoramidite chemistry (0.2 μM scale). The sequences used were a 21-mer antisense (5′ TTT CTT TTC CTG CAG AGC CCG 3′) molecular weight 6293, complementary to 5′-coding region of the *c-erb*B1 mRNA and a phosphorothioate poly$[A]_{21}$ ODN. Step-wise coupling efficiencies for ODN synthesis were typically $>99.6\%$ as determined by trityl cation assay (see also Applied Biosystems User Manual, Foster City, CA).

2.2. Radiolabelling

The ODNs were labelled at the 5′-end with γ-^{32}P-labelled ATP (Amersham, UK) in a 20 μl reaction containing 4 μl 5 × reaction buffer (100 mM Tris pH 7.5, 20 mM $MgCl_2$, 10 mM DTT, 0.2 mM spermidine and 0.2 mM EDTA), 5 μl of [^{32}P]γ-ATP (4500 Ci/mmol) (Amersham, UK), 20 units of T4 polynucleotide kinase (GibcoBRL, Paisley, Scotland), an appropriate concentration of the relevant ODN and made to 20 μl with double distilled water. The reaction was allowed to proceed at 37 °C for 45 min. The products were separated on 20% native polyacrylamide gels for 2 h at 10 W constant power and visualised by autoradiography. The appropriate region of the gel was excised and the gel crushed in 2 ml of double distilled water. Following overnight shaking the eluted ODN was recovered and concentrated by vacuum centrifugation. The initial activity of the ODN was determined by scintillation counting. Unlabelled and Tritiated 5-FU, 5-fluorouracil-6-^{3}H, was obtained from Sigma, Paisley, UK.

2.3. Microsphere preparation

Internal phase of 100 μl was prepared containing the appropriate amount of 5-FU and/or ODN and 10 μl of 4% PVA. This internal phase was added to 500 mg of P(LA-GA)polymer dissolved in 5 ml of dichloromethane (DCM) and vortexed for 5 min to form a primary emulsion. The primary emulsion was added to 160 ml of external phase (4% PVA, 0.9% NaCl). The double emulsion was then stirred at 1000 rpm for 3 h at room temperature using heidolph stirrer (Lab Plant, Huddersfield, UK) and for a further 3 h on a magnetic stirrer plate to allow for complete evaporation of the DCM.

The resulting microspheres were harvested at 4000 rpm for 10 min (43124-708 rotar, 3000 g, Mistral 3000 centrifuge, MSE Leicester Ltd), washed three times with distilled water to remove any non-encapsulated ODN and/or 5-FU and surfactant. The microspheres were then freeze-dried for 48 h using an Edwards Modulo freeze dryer (Boc Ltd, Sussex, UK).

2.4. Release profiles

The appropriate microspheres of 100 mg were dispersed in 1.5 ml of phosphate buffered saline (PBS). The microsphere suspensions were shaken in a water bath (Grant OLS 200) at 100 strokes/min at 37 °C. At various time points the samples were removed and centrifuged at 13000 rpm for 5 min (32 g, Sigma 112 centrifuge) to remove any suspended particles. The supernatant was removed and replaced with an equivalent volume of PBS. The amount of ODN and/or 5-FU released from the microspheres was assessed by scintillation counting.

2.5. Electrophoretic mobility shift assay

Samples of the 5′-end labelled ODN (*c-erb*B1 AODN and poly (A)) were mixed with increasing concentrations of 5-FU. Samples were heated to 95 °C and cooled slowly to room temperature. An equal volume of gel loading buffer (5% glycerol, 1 × TBE and 0.25% bromophenol blue) was added to each of the samples. The samples were then separated on a 20% native polyacrylamide gel. The bands were visualised by autoradiography.

2.6. Scanning electron microscopy

Samples of microspheres were mounted on carbon adhesive stubs and were gold sputter-coated using an Emscope SC 500 Sputter coater in order to obtain a conducting specimen surface. The surface morphology was viewed using a Cambridge Instruments Steroscan 90 Scanning electron Microscope UK connected to a 35 mm camera (Cambridge Instruments UK).

2.7. Particle-size determination

Approximately 10 mg of microspheres were re-suspended in 10 ml of filtered sterilised ddH_2O (using a 0.2 μM Millipore filter). This was injected into a Malvern Mastersizer E particle-sizer (Malvern Instruments, Malvern, UK) the instrument was fitted with a 45 mm angle lens and a flow cell and the presentation was for polystyrene in water (2PAD).

3. Results and discussion

3.1. In vitro release profiles of AODNs and 5-FU

In an attempt to use P(LA-GA) microspheres for the co-delivery of AODNs and 5-FU for potential combination therapy in cancer we have examined the in vitro release profiles of each drug individually encapsulated in separate microsphere formulations and from a single formulation co-entrapping both agents. P(LA-GA) microspheres were prepared by a double emulsion method (Section 2) and exhibited a smooth surface morphology for both formulation types (Fig. 1A). Microspheres with similar size ranges of between 10–20 μm mean diameter were produced in all cases (Fig. 1B) suggesting that microsphere characteristics (at the doses of drug used) were not adversely affected when both drugs were co-entrapped in a single formulation compared to having them in separate formulations. This was further supported by the fact that entrapment efficiencies of the two agents were similar ($\sim$ 70%) in co-entrapped and individual microsphere formulations (Table 1). Although P(LA-GA) microsphere formulations allow entrapment of therapeutic levels of drugs (Hagiwara et al., 1996; Ciftci et al., 1996) in this preliminary evaluation we aimed to establish proof-of-concept, and additionally to minimise costs we entrapped only very low doses of the two radiolabelled drugs (in the pmolar range; see Figure legends for details).

The release profiles of AODN and 5-FU from separate P(LA-GA) microsphere formulations are shown in Fig. 2. Release appears biphasic for both drugs which is characterised by an initial

'burst effect' (phase 1) followed by sustained release (phase 2). Phase 1 is usually attributed to the drug being present at or near the surface of the microspheres whereas the slower second phase of release represents the movement of drug which is entrapped deeper in the polymer matrix. Similar release profiles have been reported for AODNs previously (Lewis et al., 1998). The comparative release profiles for the two agents from separate formulations suggest that the burst effect is similar for both drugs e.g. after the first hour time point, 10% of the 5-FU entrapped was released compared to 7% for the 21-mer anti-EGFR AODN (Table 1). These data suggest that despite the major differences in molecular weight between

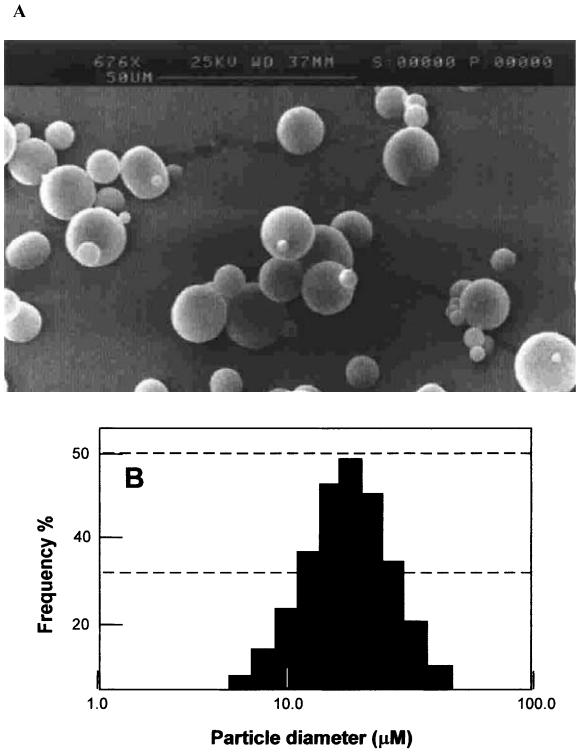

Fig. 1. (A) Scanning electron micrograph showing the morphology of 5-FU loaded P(LA-GA) copolymer microspheres (30 pmoles 5-FU/mg polymer) prepared by the double emulsion method (Section 2). (B) A typical particle size distribution of 5-FU loaded microspheres prepared by the double emulsion method. Similar morphology and size distribution were seen with ODN loaded microspheres as well as microspheres with both drugs combined. The % frequency versus particle diameter (log scale) distribution profile is shown.

5-FU (130 MW) and the AODN ($\sim$ 6300 MW) entrapment and distribution of drugs within the microsphere matrices appear similar. However, consistent with molecular weight differences the subsequent release profile highlights the more rapid ($\sim$ 2-fold) release of 5-FU compared to AODN. For example, the release rate between days 2 and 7 for 5-FU (3.2% day^{-1}) was significantly greater than for AODN (1.6% day^{-1}) reflecting the easier movement of the lower molecular weight of 5-FU molecule through the polymer matrix as compared to the larger AODN (Table 1). After 7 days about 35% of the entrapped 5-FU and 18% of the AODN was released but the release rates tend to tail off in the subsequent 'slower' phases for both agents. Indeed, after 35 days about 80% of the entrapped 5-FU was released compared to only about 40% for the AODN. This showed that it takes approximately a further 28 days to double the average cumulative % release of each agent. This slowing of the release rate was observed with both agents and probably represents the difficulty of progressively fewer drug particles having progressively less access to matrix pore networks that allow efflux from the microsphere.

Fig. 3 shows the release profiles of both AODNs and 5-FU from a single microsphere formulation. Indeed for comparative purposes, the drug loadings and microsphere particle size used in the co-entrapped single formulation (Fig. 3) were selected to be similar to those used in the separate formulations given in Fig. 2. The release profiles for the co-entrapped formulation appear to have different shapes than those observed for the separate formulations (compare Figs. 2 and 3). Firstly the burst effect for AODNs from the co-entrapped microspheres was significantly reduced ($\sim$ 3-fold) from about 7 to 2.5% whereas for 5-FU the burst effect increased slightly ($\sim$ 1.5-fold) from 10 to 15%. In addition the amount and thus the overall average rate of drug released over 35 days for both agents was significantly reduced. Only about 35% of the entrapped 5-FU was released after 35 days compared to about 80% for the separate formulation; a change of over 55%. Similarly only about 30% of the AODN was released from the co-entrapped formulation com-

Table 1
A summary of percent average cumulative release and release rates of drugs from separate and co-entrapped microsphere formulations ($n = 4 \pm SD$)

Microsphere formulation	Drug	Average cumulative release (%)			Release rate from day 2–7 expressed as % release/day
		1 h (burst)	7 days	35 days	
Separate	ODN	7 ± 0.4	18 ± 2.3	41 ± 1.7	1.6 ± 0.2
	5-FU	10 ± 0.8	36 ± 2.3	77 ± 4.3	3.2 ± 0.3
Co-entrapped	ODN	2.5 ± 0.1	8 ± 1.4	30 ± 2.4	0.8 ± 0.1
	5-FU	15 ± 1.1	26 ± 1.3	34 ± 1.3	1.2 ± 0.2

pared to over 40% from the separate formulation; a change of about 30% from the separate formulation. These data suggest that efflux of 5-FU was affected to a greater extent when co-entrapped with ODN than was the case for the 21-mer AODN. It is possible that these markedly reduced released rates for both agents arise due to differences in drug distribution within the co-entrapped versus separate microsphere formulations. To some extent this is suggested by the observed differences in the burst effects of the two drugs in each of the formulations. Alternatively (and/or additionally), these differences, especially noting the marked change in the 5-FU release profile, may be due to an interaction with the AODN. It is possible that since 5-FU is a thymine analogue, it may hydrogen bond with purine residues (e.g. adenine) within the 21-mer anti-EGFR ODN. To investigate this hypothesis further EMSA were performed.

3.2. Electrophoretic mobility shift assay for detecting AODN–drug interaction

We have previously used EMSA to detect single nucleotide changes in ODN sequence as well as modest conformation changes in ODNs of the same length as such differences resulted in altered migration of the ODN in polyacrylamide gels (Coulson et al., 1996). Thus, we postulated that if enough of the 5-FU molecules were hydrogen bonding to the AODN we should be able to detect a change in the migration of the AODN. Fig. 4A shows the 5′-end [^{32}P]-radiolabelled 21-mer anti-EGFR ODN incubated with increasing concentrations of unlabelled 5-FU and the samples run on a non-denaturing (native) polyacrylamide gel (Section 2). Lane 4 shows a modest, but reproducible, retardation in the migration of the radiolabelled ODN upon incubation with highest 5-FU concentration used in the study when compared to the control ODN in Lane 1. This modest effect is to be expected as there are only two adenine residues in the 21-mer anti-EGFR ODN (Section 2) to which the 5-FU can potentially hydrogen bond. So the predicted complex would only have slightly higher MW (increase of only 260 daltons corresponding to 2 molecules of 5-FU) compared to the free AODN.

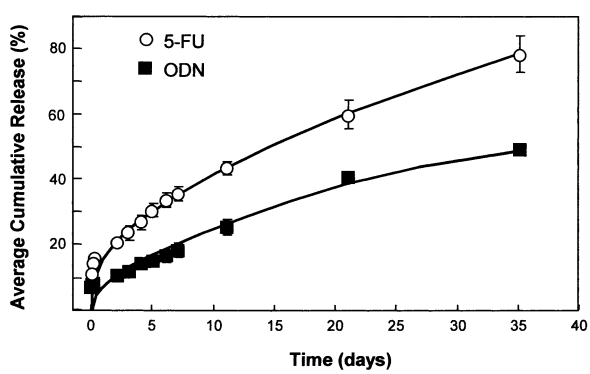

Fig. 2. In vitro release profile of AODN (loading ~ 40 pmoles/mg polymer) and 5-FU (loading ~ 30 pmoles/mg polymer) from individual microsphere formulations (10–20 μm size range). Data points represent percentage average cumulative release. Bars indicate standard deviation; $n = 4$.

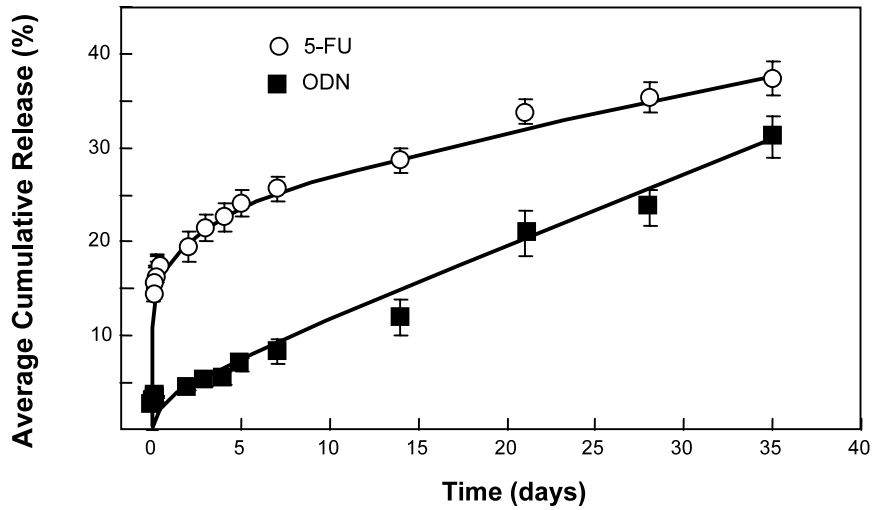

Fig. 3. In vitro release profiles of 5-FU and AODNs from a microsphere formulation containing both agents. Mean particle diameter and drug loadings were selected to be similar for both agents and identical to those used in Fig. 2; (AODNs: 40 pmoles/mg polymer and 5-FU: 30 pmoles/mg polymer in 10–20 μm size particles). Data points represent percentage average cumulative release. Bars indicate standard deviation; $n = 4$.

As a further test of the hypothesis we prepared a 21-mer poly (A) ODN to see if this resulted in a more pronounced gel shift upon incubation with 5-FU. (Fig. 4B) shows a more noticeable gel shift when poly (A) ODN is incubated with increasing concentrations of 5-FU. For example compare Lane 4, where the poly (A) ODN is incubated with 1.6 pmoles of 5-FU, with the migration of the control ODN shown both in Lanes 1 and 5. Taken together these results suggest that 5-FU can hydrogen bond with adenine residues in ODNs and that this drug–drug interaction may contribute to the slower release profiles of the two agents from the co-entrapped P(LA-GA) microsphere formulation.

3.3. In vitro release profiles of AODNs and 5-FU from different mixtures of single formulations

To evaluate the merits of utilising a single P(LA-GA) microsphere containing both agents over separate microsphere formulations of each agent, in vitro release profiles of AODNs and 5-FU from different mixtures of single formulations was carried out. Different microsphere masses containing either 5-FU (at a loading of 0.5 pmoles/mg polymer) or ODN (at a loading of 1.0 pmoles/mg) were mixed in varying ratios and their release profiles investigated (Fig. 5). Since the drug loadings used in this study were significantly lower than used in Fig. 2, the overall amount of drug released was also lower for both agents. This is consistent with our previous reports where drug release from polymer matrices was proportional to amount of ODN entrapped (Lewis et al., 1995, 1998; Khan et al., 2000). The purpose of this study, however, was to show that by changing the mass ratio of microspheres containing each drug at any fixed loading, one could easily modify the release rate of AODNs or 5-FU. Using 30 mg of each microsphere formulation as a standard, (Fig. 5A) shows that AODN release was faster than 5-FU. This is in contrast to that seen in Figs. 2 and 3 but is clearly due to the two-fold higher AODN loading in the microspheres relative to 5-FU. Thus, by using an equivalent mass ratio of microspheres containing twice the drug loading for AODN compared to 5-FU, one can essentially reverse the influence of molecular weight on release rates. Considering the wide difference in molecular weight of AODN ($\sim$6300 Da) and 5-FU ($\sim$130 Da), it further suggests that drug

loading may be a greater influence than molar mass of drugs on release rates from P(LA-GA) microsphere formulations. (Fig. 5B) shows that on increasing the mass of microspheres containing AODNs from 30 to 70 mg and fixing the 5-FU microsphere mass at 30 mg a proportional increase in the amount of ODN released at a given time point was observed. For example, at day 21 average cumulative release of AODN increased from 19 to 45 pmoles, an approximate 2-fold increase. Conversely, increasing the mass of microsphere containing 5-FU to 70 mg and keeping AODN microsphere mass fixed at 30 mg in the release experiment yielded an increase in the amount of 5-FU released at day 21 from 11 to 20 pmoles, an approximate 2-fold increase (Fig. 5C). Furthermore, this particular mass ratio of the two formulations as presented produced a final formulation in which the release profiles of 5-FU and AODN were similar. Thus, if a matched (similar) release profile of the two agents was required for a particular in vivo condition this mass combination of the separate microsphere formulations would be appropriate. These data suggest that drug release can be tailored by simply altering drug loading and mass ratio of the separate microsphere formulations of the two (or more) drugs. Of course, the exact release profiles necessary in vivo will be dependent on many factors including the nature and condition of the disease state, the biological half-life of the drugs being delivered and the half-life of the antisense-targeted gene-product. Such variables need consideration in the selection of the 'correct' formulation mix.

In conclusion, our data suggest that 5-FU and ODNs can be successfully entrapped both separately and in combination within the same P(LA-GA) microsphere formulation. The release of both agents when co-encapsulated within the same microsphere formulation was significantly slower than from separate formulations which may be, in part, explained by a 5-FU–AODN (drug–drug) interaction. Thus, the co-entrapped single formulation was deemed unsuitable for the co-delivery of these two anti-cancer agents. Separate microsphere formulations of the two drugs were considered superior as they avoided potential drug–drug interactions (due to the physical separation of the two drugs) and, by simply mixing different mass ratios, provided a greater flexibility in achieving the required release kinetics. Thus, separate P(LA-GA) sustained release formulations of AODNs and cytotoxic drugs, such as 5-FU, appear worthy of further investigation in vivo for potential combination therapy in cancer.

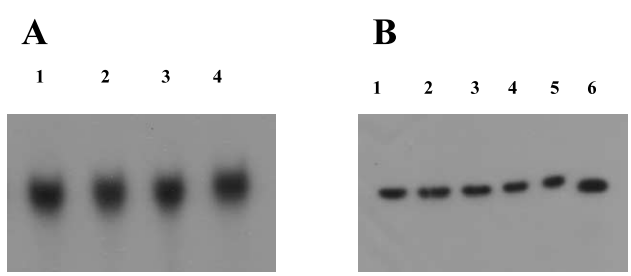

Fig. 4. EMSAs for detecting a potential interaction between 5-FU and 5-end [γ-^{32}P] labelled AODN or Poly (A) ODN. Panel A: Mobility shift of trace amounts of radiolabelled 21-mer anti-EGFR AODN when combined with increasing concentrations of 5-FU (Section 2). Lane 1 = control (AODN alone); Lanes 2–4 represent incubation of AODN with increasing amounts of unlabelled 5-FU at 0.4, 0.8 and 1.6 pmoles, respectively. Panel B: Mobility shift of trace amounts of radiolabelled 21-mer Poly (A) when combined with increasing concentrations of 5-FU. Lanes 1 and 6 represent migration of the control Poly (A) ODN whereas Lanes 2–5 represents its incubation with increasing amounts of unlabelled 5-FU at 0.4, 0.8 and 1.6 pmoles, respectively and Lane 6 representing the same concentration as Lane 4 to confirm gel shift is not due to running of the gel (control).

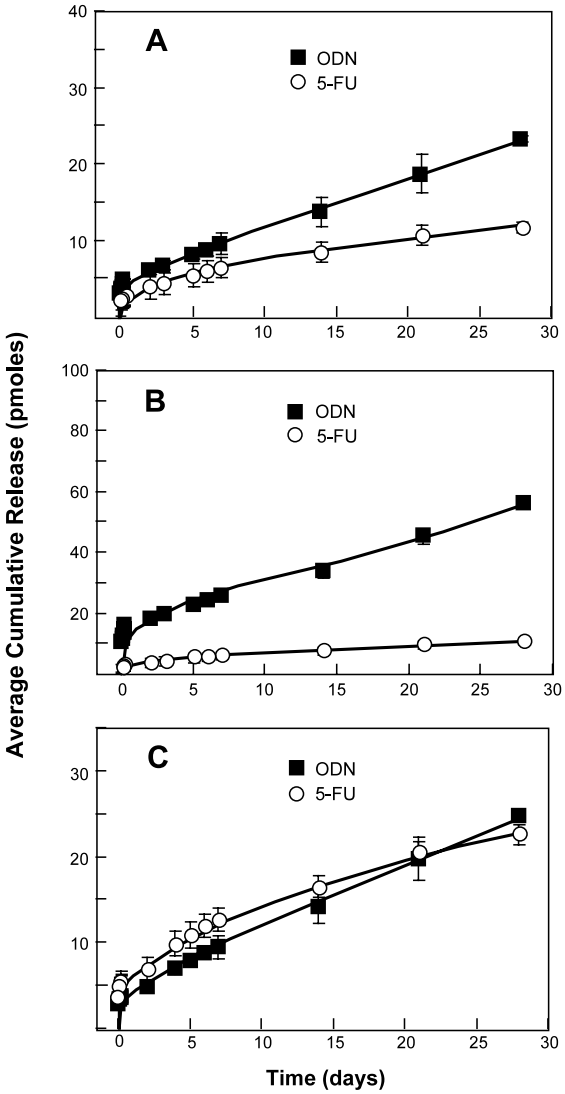

Fig. 5. Release profiles on co-delivery of separate AODN and 5-FU microsphere formulations. Release profiles of radiolabelled AODN and 5-FU were assessed upon combining different mass ratios of the separate microsphere formulations of each drug. Panel A: 30 mg of each formulation was mixed in the in vitro release experiments (Section 2); Panel B: 70 mg of AODN microspheres combined with 30 mg of 5-FU formulation; and Panel C: 30 mg of AODN microspheres combined with 70 mg of 5-FU. See text for details of drug loadings.

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References

Akhtar, S., Hughes, M.D., Khan, A., Bibby, M., Hussain, M., Nawaz, Q., Double, J., Sayyed, P., 2000. The delivery of antisense therapeutics. Adv. Drug Deliv. Rev. 44, 3–21.

Akhtar, S., Lewis, K.J., 1997. Antisense oligodeoxynucleotide delivery to cultured macrophages is improved by incorporation into sustained-release biodegradable polymer microspheres. Int. J. Pharm. 151, 57–67.

Akhtar, S., Agrawal, S., 1997. In vivo studies with antisense oligonucleotides. Trends Pharmacol. 18, 12–18.

Ardalan, B., Glazer, R., 1981. An update on the biochemistry of 5-fluorouarcil. Cancer Treat. 8, 157–167.

Brem, H., and Lawson, C., 1999. The development of new brain tumour therapy utilising the local and sustained delivery of chemotherapeutic agents from biodegradable polymers: editorial. American Cancer Soc. pp. 197–199.

Bufalo, D.D., Cucco, C., Leonetti, C., Citro, G., Agnano, I.D., Benassi, M., Geiser, T., Zon, G., Calabretta, B., Zupi, G., 1996. Effect of cisplatin and c-myb antisense phosphorothioate oligodeoxynucleotides combination on a human colon carcinoma cell line in vitro and in vivo. Brit. J. Cancer 74, 387–393.

Chavany, C., Doan, T.L., Couvreur, P., Puisueux, F., Helene, C., 1992. Polyalkylcyanoacrylate nanoparticles as polymeric carriers for antisense oligonucleotides. Pharm. Res. 9, 441–491.

Ciftci, K., Kas, H.S., Hincal, A.A., Ercan, T.M., Guven, O., Ruacan, S., 1996. In vitro and in vivo evaluation of PLAGA (50/50) microspheres containing 5-fluorouracil prepared by a solvent evaporation method. Int. J. Pharm. 131, 73–82.

Coulson, J.M., Poyner, D.R., Chantry, A., Irwin, W.J., Akhtar, S., 1996. A non-antisense sequence-selective effect of a phosphorothioate oligonucleotide targeted to EGFR in A431 cells. Mol. Pharm. 50 (2), 314–325.

Fattal, E., Vauthier, C., Aynie, I., Nakada, Y., Lambert, G., Malvy, C., Couvreur, P., 1998. Biodegradable polyalkylcyanoacrylate nanoparticles for the delivery of antisense oligonucleotides. Polmer. Del. 53, 137–143.

Ferguson, P.J., Collins, O., Dean, N.M., DeMoor, J., Sha-Li, C., Vincent, M.D., Koropatnick, J., 1999. Antisense downregulation of thymidylate synthase to suppress growth and enhance cytotoxicity of 5-Fudr, 5-FU and tomudex in HeLa cells. Brit. J. Pharmaco. 127, 1777–1786.

Geiger, T., Muller, M., Dean, N.M., Fabbro, D., 1998. Antitumour activity of a PKC-α antisense oligonucleotides in combination with standard chemotherapeutic agents against various human tumours transplanted into nude mice. Anti-Cancer Drug Des. 13, 35–45.

Hughes, M.D., Hussain, M., Nawaz, Q., Sayyed, P., Akhtar, S., 2001. The cellular delivery of antisense oligonucleotides and ribozymes. Drug Disc. Today 6, 303–315.

Hagiwara, A., Takahashi, T., Sawai, K., Sakakura, C., Tsujimoto, H., Imanishi, T., Ohgaki, M., Yamazaki, J., Muranishi, S., Yamamoto, A., Fujita, T., 1996. Pharmacological effects of 5-fluorouracil microspheres on peritoneal carcinomatosis in animals. Brit. J. Cancer 74, 1392–1396.

Khan, A., Sommer, W., Fuxe, K., Akhtar, S., 2000. Site-specific administration of antisense oligonucleotides using biodegradable microspheres provides sustained delivery and improved subcellular biodistribution in the neostriatum of the rat brain. J. Drug Targ. 8, 319–344.

Lewis, K.J., Irwin, W.J., Akhtar, S., 1995. Biodegradable poly(L-lactic acid) matrices for the sustained delivery of antisense oligonucleotides. J. Control Rel. 37, 173–183.

Lewis, K.J., Irwin, W.J., Akhtar, S., 1998. Development of a sustained release biodegradable polymer delivery system for site-specific delivery of oligonucelotides: characterisation of P(LA-GA) copolymer microspheres in vitro. J. Drug Targ. 5, 291–302.

Menei, P., Venier, M.C., Gamelin, E., Saint-Andre, J.P., Hayek, G., Jadaud, E., 1999. Local and sustained delivery of 5-fluorouracil from biodegradable microspheres for the radiosensitization of glioblastoma: a pilot study. Cancer 86, 324–329.

Parker, W.B., Cheng, Y.C., 1990. Metabolism and mechanism of action of 5-fluorouracil. Pharamac. Ther. 48, 381–395.

Tortora, G., Caputa, R., Damiano, V., Agrawal, S., Ciardiello, F., 1998. Co-operative antitumour effect of mixed backbone oligonucleotides targeting protein kinase A in combination with cytotoxic drugs or biologic agents. Antisense Nucleic Acid Drug Dev. 8, 141–145.

Wang, X., Zhang, R., Xie, H., 1999. Combined effect of alpha-fetoprotein antisense oligodeoxy-nucleotides and 5-fluorouracil on human hepatoma cell growth. Chin. Med. J. 8, 743–746.