Antisense oligonucleotide activity in tumour cells is influenced by intracellular LBPA distribution and extracellular vesicle recycling

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Next generation modified antisense oligonucleotides (ASOs) are commercially approved new therapeutic modalities, yet poor productive uptake and endosomal entrapment in tumour cells limit their broad application. Here we compare intracellular traffic of anti KRAS antisense oligonucleotide (AZD4785) in tumour cell lines PC9 and LK2, with good and poor productive uptake, respectively. We find that the majority of AZD4785 is rapidly delivered to CD63+ late endosomes (LE) in both cell lines. Importantly, lysobisphosphatidic acid (LBPA) that triggers ASO LE escape is presented in CD63+ LE in PC9 but not in LK2 cells. Moreover, both cell lines recycle AZD4785 in extracellular vesicles (EVs); however, AZD4785 quantification by advanced mass spectrometry and proteomic analysis reveals that LK2 recycles more AZD4785 and RNA-binding proteins. Finally, stimulating LBPA intracellular production or blocking EV recycling enhances AZD4785 activity in LK2 but not in PC9 cells thus offering a possible strategy to enhance ASO potency in tumour cells with poor productive uptake of ASOs.
Anti-sense oligonucleotides (ASOs) are novel, highly specific nucleic acids designed to silence target genes by forming a complementary hybrid duplex with target’s mRNA and mediating mRNA degradation. Modification of oligonucleotide backbone and sugar moieties has resulted in the development of nuclease-resistant forms which are currently in the pre-clinical or clinical validation for treating a broad range of diseases including SARS-CoV-2, inflammatory diseases, neurological disorders and cancer. In particular, phosphorothioate ASO (PS-ASO) rapidly redistributes to tissues upon subcutaneous or intravenous injection; yet cytosolic delivery to target’s mRNA (called productive uptake) remains the most significant challenge. Cellular uptake of PS-ASO begins upon binding to cell membrane proteins including scavenger receptors, stabilins, and EGFR. These complexes are endocytosed via clathrin-dependent and independent pathways and delivered to early and late endosomes. During the final phase, PS-ASO escapes from late endosomes (LE) to the cytosol with the assistance of lysobisphosphatidic acid (LBPA), and annexin A2. This process escape is required for ASO endosomal escape by mediating back-fusion of ASO-loaded LEs with the LE membrane so next, we investigated AZD4785 delivery to LE across both cell lines.

Here we report that the majority of AZD4785 internalisation observed in LK2 cells (PCC = 0.83 and 0.68 ± 0.01 in PC9 and LK2 cells, respectively (Fig. 1)). By plotting the number of AZD4785 and CD63 spots for individual single cells, we found that the number of intracellular AZD4785 spots, as well as intensity, depends on the CD63+ LE level in both cell lines (Fig. 1g, h and Supplementary S1a, b).

Results

Majority of AZD4785 is delivered to CD63+ LE across both cell lines but only limited colocalization with LBPA+ LE is observed. Endosomal ASO escape occurs in the LE so we hypothesised that ASO productive uptake is associated with variable delivery to LE. To test this further, we studied productive uptake of AZD4785 (A cEt-modified ASO) in 2 cell lines, PC9 and LK2 which we previously identified to be good and poor productive uptake cells respectively. Cellular AZD4785 uptake was quantified by immunofluorescence using an antibody detecting phosphorothioate backbone. To visualise LE’s, we used two LE markers, tetraspanin CD63 and LBPA, a lipid which is exclusively generated in LE where it enables ASO endosomal escape by mediating back-fusion of ASO-loaded intraluminal vesicles with the LE membrane.

The baseline number of CD63+ LEs was lower in LK2 cells, but these were stained more intensively indicating higher CD63 loading per single LE (Fig. 1a–c). In agreement with our previous data, there was no difference in bulk AZD4785 uptake by PC9 and LK2 cells (Fig. 1a, d, e). Internalised AZD4785 showed nearly complete overlap with CD63 LEs in both cell lines (Pearson correlation coefficient (PCC) 0.83 and 0.68 ± 0.01 in PC9 and LK2 cells, respectively (Fig. 1)).

In LK2 cells, we observed that the bulk PS-ASO is transported in endosomes and degraded in lysosomes (poor productive uptake) yet the exact intracellular trafficking mechanisms behind the productive uptake remain largely unknown. Endocytic pathways merge in LE where internalised cargo can either be sorted for the plasma membrane recycling, loaded to intraluminal vesicles or progress to lysosomal degradation. Intraluminal vesicles, in turn, can be secreted as extracellular vesicles (EVs) and are implicated in the intercellular communication by delivering biologically active proteins, lipids and nucleic acids. Intraluminal EV budding in LE is driven by LBPA and Alix, and both components are indispensable for ASO endosomal escape most likely acting via the "back-fusion" mechanisms. We and others previously showed that the exogenous siRNA or mRNA delivered in lipid nanoparticle formulation are recycled in EVs so it is still unknown whether formamidase ASO could be recycled via this pathway. To understand the role of LE in productive ASO uptake, we investigated intracellular trafficking of next-generation KRAS targeting PS-ASO (AZD4785) with constrained ethyl modifications in two lung tumour cell lines, PC9 with good productive uptake (IC50 ≤ 0.6 μM) and LK2 with poor productive uptake (IC50 ≥ 10 μM). We found that bulk of the internalised AZD4785 is delivered to CD63+ LE and productive uptake is influenced by LBPA spatial distribution—with LBPA colocalised with CD63+ LE in PC9 cells whilst LBPA presented in LEs distant from CD63+ LE across LK2 cells. Notably, AZD4785 is also recycled from CD63+ LE in EVs by both cell lines however AZD4785 clearance from LK2 cells via this pathway is greater than in PC9 cells. Moreover, stimulating LBPA production or inhibiting EV recycling pathway by using small molecules enhanced AZD4785 productive uptake in LK2 cells but not in PC9 thus indicating specificity towards poor productive uptake cell lines. Hence, targeting these intracellular pathways could be exploited to boost ASO productive uptake and activity in poor productive tumour cells and, possibly, even across other pathologies.

PS-ASO is recycled in EVs in vitro and in vivo. Upon delivery to LE, cargo is either sorted for degradation in lysosomes or secreted from cell in EVs. Although AZD4785 lysosomal delivery has been extensively studied, it is currently unknown whether PS-ASO is loaded into EVs. A recent study confirmed a high rate of EV secretion by tumour cells with 2–3 CD63+ LEs fusing with the plasma membrane each minute so next we tested whether AZD4785 is recycled via EVs pathway. We incubated both cells with AZD4785 and isolated EVs from conditioned cell culture media using differential ultracentrifugation (see ‘Extracellular vesicles isolation and characterisation’ section). Nanoparticle Tracking Analysis (NTA) revealed that control, non-treated PC9 and LK2 cells secret EVs having similar average sizes (166.9 ± 5.1 nm and 163.6 ± 4.7 nm, correspondingly) (Fig. 3a, b). Notably, AZD4785 exposure stimulated EVs secretion both by PC9 and LK2 cells (Fig. 3c) and LK2 secreted larger EVs (271.5 ± 12.4 nm) (Fig. 3b). Interestingly, LK2 treatment with PS-ASO and a different sequence (control ASO) also stimulated the secretion of EVs in vitro and in vivo. Upon delivery to LE, cargo is either sorted for degradation in lysosomes or secreted from cell in EVs. Although AZD4785 lysosomal delivery has been extensively studied, it is currently unknown whether PS-ASO is loaded into EVs. A recent study confirmed a high rate of EV secretion by tumour cells with 2–3 CD63+ LEs fusing with the plasma membrane each minute so next we tested whether AZD4785 is recycled via EVs pathway. We incubated both cells with AZD4785 and isolated EVs from conditioned cell culture media using differential ultracentrifugation (see ‘Extracellular vesicles isolation and characterisation’ section). Nanoparticle Tracking Analysis (NTA) revealed that control, non-treated PC9 and LK2 cells secret EVs having similar average sizes (166.9 ± 5.1 nm and 163.6 ± 4.7 nm, correspondingly) (Fig. 3a, b). Notably, AZD4785 exposure stimulated EVs secretion both by PC9 and LK2 cells (Fig. 3c) and LK2 secreted larger EVs (271.5 ± 12.4 nm) (Fig. 3b). Interestingly, LK2 treatment with PS-ASO and a different sequence (control ASO) also stimulated the secretion of EVs (Fig. 2b). Moreover, AZD4785 treatment of other poor productive uptake cells (Calu6 and A427) also resulted in the secretion of larger EVs (Supplementary Fig. S2b) yet good productive uptake cells H460 but not MiaPaca2 also secreted larger EVs (Supplementary Fig. S2a). Wash-out of AZD4785 from good and poor productive uptake cells (pulse-chase) reversed EV production to the control levels (Fig. 3b, c and Supplementary Fig. S2a, b).

Tumour cells secrete heterogenous EVs originating either from LE (exosomes) or plasma membrane (ectosomes). To test
EVs secreted from the LE compartment, we measured the presence of well-established LE markers, CD63 and Alix\(^1\) by western blotting and dot blot. Both markers were presented in PC9 and LK2-derived EVs indicating the presence of EV with LE origin (Fig. 3d and Supplementary Fig. S3a). Interestingly, we also noted lower levels of Alix protein in LK2 cells as compared to PC9 cells (Supplementary Fig. S3b). To test whether AZD4785 is recycled in EVs, we exploited dot blot analysis by using an anti-phosphorothioate backbone antibody. AZD4785 was detected in EVs isolated from the PS-ASO treated both cell lines and a similar extent of staining was observed in the non-permeabilising and permeabilising conditions indicating that AZD4785 is presented on the outer EV surface (Supplementary Fig. S3a). Albumin has been previously detected in EVs\(^3\) and it is known that over 90% of ASO’s with a phosphorothioate backbone are associated with albumin which is responsible for extending ASO plasma...
Hence, we hypothesised that AZD4785 is delivered to EVs via LE in a complex with albumin. Using isothermal titration calorimetry (ITC), we found that PS-ASO binds to albumin with low affinity (Kd = 8.18 ± 0.52 µM, Supplementary Fig. S3c) and this also corroborates well with previous data. Further, we investigated the colocalization of albumin-Alexa594 and PS-ASO in LE across both cells and observed only a small overlap between albumin and AZD4785 in CD63+ LEs (Supplementary Fig. S4a). Moreover, albumin endocytosis was significantly suppressed by AZD4785 (Supplementary Fig. S4a–c).

Next, we characterised EV composition further by using proteomic analysis. We identified 1310 proteins in PC9-derived EVs and 952 proteins in LK2-derived EVs (Supplementary Data 1). Both exosome-specific (CD9, CD63, CD81 and synthenin-1) and ectosome-specific (alpha-actinin-4, basigin and F42 cell antigen) markers were detected in EV secreted by both cell lines indicating high EV heterogeneity (Supplementary Data 1). Prominently, 818 proteins were common among all datasets (Fig. 3e). AZD4785 treatment resulted in the appearance of 28 unique proteins in PC9-derived EVs and 53 unique proteins in LK2-derived EVs (Supplementary Data 2). To analyse functional enrichment and interaction between unique protein dataset, we used the STRING database. To differentiate between EVs originating from PC9 and LK2 cells, we included only unique proteins from each dataset. PC9 EVs contained 8 proteins associated with endosomal membrane and intracellular vesicular transport (WLS, SNF8, Rab8B, Rab22A, LAMTOR3, ITM2B and ANTRXR1) and this may reflect their endosomal origin (Supplementary Data 2 and Supplementary Fig. S5a). AZD4785 treatment resulted in the secretion of signalling components (NOTCH3, ERBB2) as well as proteins involved in trans-Golgi network transport vesicles (TGOLN2 and AP1G1) (Supplementary Data 2 and Supplementary Fig. S5b). DNA repair protein

**Fig. 2** AZD4785 is delivered to the LBPA-positive LE compartment in PC9 but not in LK2 cells. a PC9 and LK2 cells were incubated with AZD4785 for 6 or 24 h, then fixed and stained. LE and AZD4785 were stained with anti-LBPA and anti-PS-ASO antibodies, correspondingly and visualised using secondary fluorescently labelled antibodies. Nuclei were stained with Hoechst 33342. Cell images were acquired using Opera microscope (×60 objective, NA1.4). b–d Intracellular content of LBPA were quantified by using Columbus software by counting number of stained spots in the cell cytosol (Spot/Area) or integrated intensity (Intensity/Area). Number of spots and intensity values were normalised to the cellular cytosol area. Average of means, error bars, standard deviation. Minimum 300 cells for each condition were counted in each experiment (N = 3–6).
Fig. 3 AZD4785 stimulates EV secretion by PC9 and LK2 cells. a-c PC9 and LK2 were treated with AZD4785 for 24 h. For the pulse-chase (p/c) experiment, cells were treated with 10 µM AZD4785 for 6 h, washed and incubated for 24 h in the absence of AZD4785 (AZD4785 p/c). EVs were isolated from conditioned cell media by differential ultracentrifugation and a 100,000×g pellets were analysed by NTA. Representative NTA images. (N = 6). b Size distribution of EVs secreted by PC9 and LK2 cells. EVs were collected and analysed as in a. Error bars, standard deviation. ANOVA ****p < 0.0001. ***p < 0.001. ns non-significant. N = 3–6. c Quantification of EV secretion by PC9 and LK2 cells. The total number of EVs was measured by NTA as in a and divided by the total number of cells. Error bars, standard deviation. ANOVA. ****p < 0.0001, ns non-significant. N = 3–6. d Detection of exosomal markers, CD63 and Alix, in EVs isolated from PC9 and LK2 cells after 24 h treatment. Equal aliquots of EVs isolated as in a were analysed by western blotting and probed for CD63 and Alix. Representative image from N = 2. e Vein diagram for the EV’s protein mass spectrometry analysis revealed differentially secreted protein in PC9 and LK2 EVs.
network including RPA2 and OLB were identified in EVs secreted by LK2 in the control conditions (Supplementary Data 2 and Supplementary Fig. S6a). In addition, the endosomal sorting complex required for transport III component, CHMP2B was detected in LK2 EVs, indicating the presence of LE-derived EVs. AZD4785 treatment changed LK2 EV profile and 15 proteins involved in RNA binding and processing were identified (Supplementary Data 2 and Supplementary Fig. S6b). To the best of our knowledge, these proteins were not previously implicated in PS-ASO intracellular trafficking.

To quantify AZD4785 content in secreted EVs, we applied our recently developed quantitative UPLC-MS assay\(^{17}\) (Supplementary Figs. S8 and S9). From the standard calibration curve, the sensitivity for intact AZD4785 detection was \(1\) nM (0.005 µg/ml) (Supplementary Fig. S9a). Notably, we found that EVs contained intact AZD4785 and we observed dose-dependent increase in AZD4785 content in EVs secreted by LK2 and PC9 cells (Fig. 4a). Notably, EVs contained 4.2 fold less AZD4785 than LK2-derived EVs (2103 and 9012 molecules per PC9 or LK2-derived EV at 26.1 µM AZD4785 treatment respectively) (Fig. 4a).

Interestingly, comparison of AZD4785 EV load across the panel of tumour cells with good (PC9, MiaPaca2 and H460) and poor productive uptake cells (LK2, Calu6 and A427) showed the higher AZD4785 loading in EVs produced by non-productive cells (Supplementary Fig. S2c) except H460 which also secreted larger EVs in response to AZD4785 treatment (Figs. S2a, c). Removal of AZD4785 from the conditioned media (pulse-chase) reduced EV AZD4785 load in PC9 cells \(\approx 3\) fold times and in LK2 cells \(\approx 6.5\) fold times so both cell lines secreted similar amount of AZD4785 in EVs (Fig. 4a). Next, we compared the amount of AZD4785 recycled in EVs with the intracellular AZD4785 content per cell (Fig. 4b). The comparison between EV and intracellular fraction revealed that PC9-derived EVs contain 1/5th (22.8 ± 6.5%) and LK2-derived EVs contain nearly half (44.8 ± 7.5%) of PS-ASO as compared to their respective intracellular uptake levels. Altogether these data indicate that AZD4785 recycling in EVs can represent novel clearance pathway in LK2 cells.

Finally, to test whether PS-ASO recycling via EVs also occurs in vivo, we isolated EVs from plasma of mice with PC9 xenograft tumour after subcutaneous dosing with two AZD4785 doses, 125 mpk/wk and 250 mpk/wk (see ’Mice PC9 xenograft model and EV isolation from plasma’ section). NT analysis showed that the mode size of isolated EVs was 125–130 nm across all conditions (Supplementary Fig. S10a) similar to the in vitro samples. To test whether EVs are secreted by the grafted tumour human PC9 cells, we exploited anti-CD63 antibody that are highly specific to CD63 originating from human (PC9) but not murine cells. We were unable to observe any CD63 signal in the plasma-derived EVs as opposed to the CD63 in EVs derived from various human tumour cell lines including PC9. Altogether these data suggest that the majority of isolated EVs are unlikely secreted by xenograft cells and can be secreted by other cells (Supplementary Fig. S10b).

Next, we measured AZD4785 concentration in EVs and original plasma samples (Table 1). AZD4785 concentration 72 h post-dosing in the whole plasma was 12.48 and 18.72 nM for the animals dosed with 125 mpk/wk and 250 mpk/wk AZD4785, correspondingly. In samples post 7 days dosing the AZD4785 plasma concentration reduced to 4.286 nM (125 mpk/wk group) and 6.57 nM (250 mpk/wk group). Next, we quantified AZD4785 content in plasma-derived EVs and found that the 72 h post-dosing EVs contained 0.0706 and 0.0711 fMol of AZD4785 per 10^6 EVs for the doses 125 mpk/wk and 250 mpk/wk, correspondingly. In the 7 days post-dosing plasma, the concentration of AZD4785 diminished to 0.0305 fMol/10^6 EVs and 0.0195 fMol/10^6 EVs for doses 125 mpk/wk and 250 mpk/wk, respectively. Knowing the EV concentration in the plasma, we estimated that \(\approx 5\)% and \(\approx 2\)% of plasma PS-ASOs can be transported in EVs, after 72 h and 168 h respectively (Table 1).

**AZD4785 recycling in EVs enables intercellular AZD4785 transfer but reduces productive uptake.** Exosomes efficiently transfer various nucleic acid species between cells\(^{23}\) so we hypothesised that AZD4785 can be transferred between cells in EVs causing KRAS knockdown. We isolated AZD4785-loaded EVs from PC9 and LK2 cells and transferred them to the untreated PC9 and LK2 cells. The exact concentration of AZD4785 delivered via EVs was measured by UPLC-MS as described in the ’Methods’ section (see ’AZD4785 UPLC-MS analysis’ section) so we directly compared efficacy of carrier-free AZD4785 and AZD4785 delivered in EVs in the concentration range from 3–10 nM for PC9-derived EVs and 10–30 nM for LK2-derived EVs (Fig. 4c–e). Staining of recipient cells with anti-ASO antibody revealed an efficient EV-mediated AZD4785 delivery (Fig. 4c). Moreover, treatment with low doses of EV-associated AZD4785 (10 and 20 nM for PC9 and LK2-derived EVs, respectively), resulted in the intracellular levels similar to those after the treatment with 250 nM carrier-free AZD4785 (Fig. 4c and Supplementary Fig. S7a, b) thus showing that AZD4785 delivery in EV is very efficient.

Next, we tested AZD4785 EV KRAS knockdown efficacy. We observed high potency of carrier-free AZD4785 in PC9 cells (Fig. 4d) and low potency in LK2 cells (Fig. 4e) in agreement with the previous data\(^{17}\). AZD4785-loaded EVs induced efficient KRAS dose-dependent knockdown in PC9 cells with nearly 50% of KRAS knockdown even at the low EV-loaded AZD4785 dose (3 nM) (Fig. 4d). Interestingly, there were no significant differences in potency between PC9 and LK2-derived EV (Fig. 4d). Again, the addition of LK2-derived AZD4785 EV to LK2 cells resulted in KRAS knockdown with higher potency as compared to carrier-free AZD4785 (Fig. 4e).

Our data shows that AZD4785 recycling in EV can influence PS-ASO functional activity either by enhancing PS-ASO clearance from cells or by increasing intracellular delivery via EV-loaded AZD4785. To test the overall EV pathway contribution to PS-ASO functional activity, we used a small molecule inhibitor U18666A which blocks EV secretion by inducing cholesterol accumulation in LE\(^{37,38}\). In agreement with previous data, U18666A treatment induced the formation of an enlarged LE compartment with increased CD63 content in both cell lines (Fig. 5a and Supplementary Fig. S11a). Surprisingly, U18666A induced secretion of CD63+/CD81−EVs as detected by CD63 capture bead assay (Supplementary Fig. S12a, b). Next, we tested the effect of U18666A on AZD4785 functional activity (Table 2 and Fig. 5a, b). Again, we observed high AZD4785 potency in PC9 cells (IC\(_{50}\) = 0.15 µM) and low AZD4785 potency (IC\(_{50}\) ≥ 28.4 µM) in LK2 cells in the control conditions (Fig. 5b, c). Notably, U18666A improved AZD4785 efficacy =147-fold in LK2 cells (IC\(_{50}\) ≥ 0.2 µM) (Fig. 5b, c). A recent study indicated that U18666A also induces accumulation of LBPA\(^{39}\) and we found that U18666A treatment increased intracellular LBPA content and number of LBPA+ LEs in PC9 and LK2 cells (Supplementary Fig. S11c–f). To test the contribution of LBPA-dependent pathway, we used thioperamide maleate, an inverse agonist of the histamine H3 receptor HRH3 specifically increasing intracellular LBPA without trafficking pathways interfering\(^{39}\). We found that this novel compound indeed accumulation of LBPA in PC9 and LK2 cells and increased number of LBPA+ LE in LK2 cells only (Supplementary Fig. S11c–f). It also inhibited secretion of CD63+/CD81+ EVs by PC9 cells and stimulated EV secretion by LK2 cells (Supplementary Fig. S12c, d). Interestingly,
Fig. 4 AZD4785 is recycled in EV and transferred between cells. a Detection of AZD4785 in EVs. PC9 and LK cells were treated with AZD4785 for 24 h and EVs were isolated by differential ultracentrifugation. Quantification of AZD4785 in isolated EVs was conducted by UPLC-MS. t-test. *p < 0.05. N = 3–6. b Detection of intracellular AZD4785. AZD4785 was quantified in EV and cell lysates as in a. Intracellular and EV AZD4785 content were normalised per 10⁶ cells. c AZD4785 loaded in EVs is delivered to the cells. EVs were isolated from the conditioned media of the cells treated with 10 µM AZD4785 for 24 h as in a. PC9 and LK2 cells were incubated with AZD4785-loaded EVs for 3 h. Cells were washed, fixed and stained for CD63, AZD4785 and nucleus. d, e AZD4785-loaded EVs induce KRAS knockdown. AZD4785-loaded EVs were isolated from PC9 and LK2 cells after AZD4785 treatment as in a and aliquots with variable volume were added to PC9 (d) and LK2 (e) cells for 72 h. Error bars, standard deviation. ANOVA ****p < 0.0001. ***p < 0.001. **p < 0.01. ns non-significant. Data are duplicates, N = 5.

Table 1 AZD4785 detection in plasma-derived EVs.

| Treatment regime | Plasma collection time after the last dose |
|------------------|------------------------------------------|
|                  | 72 h                                     | 168 h                                    |
|                  | Plasma nM | EVs (fMol/10⁶) | EVs plasma, nM | Plasma nM | EVs (fMol/10⁶) | EVs plasma, nM |
| PBS              | Not detected | NA | NA | Not detected | NA | NA |
| AZD4785 125 mpk/wk | 12.48 | 0.07 | 0.85 | 4.28 | 0.03 | 0.11 |
| AZD4785 250 mpk/wk | 18.72 | 0.07 | 0.80 | 6.57 | 0.02 | 0.10 |
thioperamide maleate treatment improved AZD4785 productive uptake ≈56 fold times in LK2 cells (IC$_{50}$ ≥ 0.5 µM) but had no effect on PC9 cells (Fig.5b, c and Table2). Intraluminal vesicles formed in LE compartment can also be secreted as exosomes and to investigate the role of exosome-specific clearance, we knockdown Rab27a, a well-established regulator of exosome secretion in tumour cells and used a small inhibitor of sphingomyelin phosphodiesterase 3 pathway, 3-O-Methyl-sphingomyelin. Rab27A knockdown by using siRNA resulted in the reduction of Rab27A to ≈15% level in both cell lines (Supplementary Fig. S12e). Importantly, Rab27A knockdown and SMPD3 inhibition reduced the secretion of CD63+/EV by LK2 cells and improved AZD4785 productive uptake ≈2 and ≈5.5 fold, correspondingly (Table 2, Fig. 5e and Supplementary Fig. S12h). Rab27a knockdown in PC9 cells had no effect on CD63+/secretion as well as AZD4785 efficacy (Table 2, Fig. 5d and Supplementary Fig. S12f). Moreover, inhibition of sphingomyelin phosphodiesterase 3 pathway in PC9

Fig. 5 Inhibition of EV secretion and LBPA enhancement improve AZD4785 efficacy. a Intracellular distribution of AZD4785 and CD63 after U18666A inhibitor treatment. PC9 and LK2 were treated with AZD4785 (26.1 µM) for 48 h, fixed and stained. LE and AZD4785 were stained with anti-CD63 and anti-PS-ASO antibodies, respectively, and visualised using secondary fluorescently labelled antibodies. Nuclei were stained with Hoechst 33342. To block EV secretion cells were treated with 7.1 µM U18666A 24 h prior to the addition of AZD4785. Cell images were acquired using Opera microscope (×60 objective, NA1.4). (N = 3). b, c KRAS expression in the inhibitor or siRNA-treated cells. PC9 (b) and LK2 (c) cells were treated with AZD4785 for 48 h. Cells were pre-treated with 7.1 µM U18666A or 10 µM thioperamide maleate (b, c) or control siRNA or Rab27a siRNA for 24 h (d, e) prior to AZD4785 treatment. KRAS expression was quantified by qPCR. Nonlinear regression analysis, each data point are duplicates from N = 3–9.
cells reduced the secretion of CD63+/CD81+EVs but had no effect on AZD4785 efficacy (Supplementary Fig. S12c, g).

Discussion

ASO therapeutics in the clinic are currently delivering high ASO doses presumably to saturate bulk ASO non-productive uptake7,8,42 or by adding cell-targeting ligands43. Both strategies rely on enhancing productive cell uptake as numerous studies have reported that the majority of PS-ASO is taken up into cells via the bulk, non-productive (lysosomal) pathway with only few drug molecules escaping LE compartment and reaching target RNA in the cytosol or nucleus7,9,13-16. Here we further investigated intracellular PS-ASO trafficking in good and poor productive uptake tumour cells and found that the productive uptake depends on level of PS-ASO delivery to the LBPA+LE compartment in both cell lines. Moreover, up to 50% of endocytosed AZD4785 which was delivered to CD63+LE compartment can be recycled via EV especially by LK2 which is a poor productive uptake line. Interestingly, we also show that the ASO recycling mechanism contributes to ASO intracellular clearance as well as intercellular ASO transfer in both cell lines. Importantly, modulation of LBPA-dependent and EV recycling pathways using small molecules enhances PS-ASO functional activity thus offering alternative strategies for improving ASO therapeutic applications.

Late endosomes (LE) are the central cargo sorting hubs merging endocytosis pathways and directing cargo for recycling to the plasma membrane, loading into the small intraluminal vesicles or delivering to lysosomes for degradation18,19. Lysosome’s contribution to the non-productive ASO uptake and degradation were shown in multiple studies9,10,13,17 and here we studied the role of LE in the ASO productive uptake. We selected two LE markers CD63 and LBPA28,29,44 and these markers showed overlap in PC9 cells but were presented in the different LE populations in LK2 cells. Importantly, we found that in both cell lines the bulk AZD4785 uptake correlates with intracellular CD63 levels and endocytosed AZD4785 is detected in CD63+LE. On the contrary, LBPA staining revealed moderate colocalization in PC9 cells and no colocalization between LBPA and AZD4785 in LK2 cells. This striking difference in AZD4785 intracellular trafficking in PC9 and cells LK2 indicates that endocytosed PS-ASO somehow by-passing LBPA+LE sub-population in LK2 cells. LBPA is a key LE component assisting ASO endosomal escape15 most likely by triggering intraluminal vesicles back-fusion with the LE membrane mechanism originally described for pathogens48,45,46. LBPA content in LE is high and reaches up to 15 mol% of all LE phospholipids in BHK cell line28 yet the spatial LBPA distribution across cellular LE population is still unknown18. LBPA LE level is regulated by Alix binding to the external LE surface44 and Alix knockdown reduced LBPA LE content and ASO functional activity in A431 carcinoma cells15. We noted that Alix expression in LK2 cell was lower as compared to PC9 cells so it is tempting to speculate that Alix acts as an upstream regulator for LBPA spatial distribution across LE population in LK2 cells. Importantly, thioperamide maleate, an inverse agonist of the histamine H3 receptor HRH3 elevating intracellular LBPA content19 and U18666A increased LBPA content in LK2 cells that resulted in significantly improved (≈56 and ≈147-fold times, respectively) productive AZD4785 uptake. Altogether these data suggest that ASO delivery to LBPA-positive LE is a novel key factor delineating good and poor productive uptake cells. Although this finding is yet to be confirmed in a diverse panel of cells/tissues, it is tempting to suggest that the future ASO therapeutics can be particularly effective in the cells with high LBPA level, such as macrophages47. Alternatively, ASO can be applied in combination with the HRH3 agonists such as thioperamide maleate to boost endosomal escape and therapeutic effects.

What is the intracellular fate of AZD4785 delivered to CD63+LE? PS-ASO can be sorted for lysosomal degradation9,10,13 but it remained unknown whether it can also be recycled in EVs and whether this also influences its functional activity. Endogenous RNA species can be secreted in EVs, which are generated in CD63+LE and secreted upon the fusion of LE with the plasma membrane9,23,25,26 and here we report for the first time that the exogenous, formulation-free PS-ASO is recycled via EVs. Interestingly, secretion of endocytosed PS-ASO via unknown mechanism has been reported before11. Here we found that non-treated tumour cell lines PC9 and LK2 secret EVs with the similar size and proteomic composition and AZD4785 treatment induces EV secretion. Importantly, only EVs derived from AZD4785-treated LK2 cells were enriched with RNA-binding proteins and this unique profile can possibly be linked to the appearance of a large EVs population in response to ASO treatment. Although we detected the presence of endosomal markers, CD63 and Alix31, the exact origin of these EVs remain to be discovered. To detect intact PS-ASO in EV, we deployed a novel mass spectrometry technique with a 10-fold improved lower detection limits (1 nM/μg/ml) as compared to previously reported assays such as capillary gel electrophoresis or ultrasensitive hybridisation enzyme-linked immunosorbent assay (ELISA) with detection limits of 0.06 μg/ml and 0.07 μg/ml ASO in plasma, correspondingly8,48. Interestingly, an improved sensitivity ultrasensitive hybridisation-based ELISA method has been recently reported for the phosphorodiamidate morpholino oligonucleotide49 however oligonucleotides

| Treatment | IC50, M |
|-----------|---------|
|            |         |
| Vehicle    | 1.47*10^-7 |
| Thioperamide | 1.63*10^-7 |
| U18666A    | 0.93*10^-7 |
| Control siRNA | 1.56*10^-7 |
| Rab27a siRNA | 1.58*10^-7 |

| IC50 |
|------|
| Vehicle | 2.83*10^-5 |
| Thioperamide | 5.00*10^-7 |
| U18666A | 1.93*10^-7 |
| Control siRNA | 4.41*10^-6 |
| Rab27a siRNA | 2.58*10^-6 |

Table 2 IC50 values for KRAS mRNA knockdown by AZD4785.
length over 20–25 nucleotides is critical for sensitivity with this method. Quantification of intact AZD4785 in the cells and in isolated EVs using mass spectrometry revealed that LK2 secrete 3 times more AZD4785 per single vesicle as compared to PC9 (2103 and 9012 AZD4785 molecules per EV, correspondingly). Moreover, our data indicate that up to 50% of ASO can be recycled by LK2 cells as compared to intracellular levels, and this range is close to a recent study by Sahay et al. who established that up to 70% of siRNA delivered in lipid nanoparticles formulation can be recycled from the LE/MVB, presumably via exosomes. We found that AZD4785 is recycled on the external EV surface, suggesting that PS-ASO can be recycled directly from the LE lumen or even bind to the secreted EVs. Yet, we observed dose-dependent AZD4785 loading and it correlated well with the intracellular levels, arguing against the hypothesis that PS-ASO binds to the EVs extracellularly. We identified 15 RNA-binding proteins in LK2 EVs and further tracking of PS-ASO and these proteins can potentially clarify EV loading mechanisms. Pulse-chase experiment and detection of intact AZD4785 in plasma-derived EVs confirmed that the intact, full-size AZD4785 is actively recycled in EVs. AZD4785 wash-off ceased the secretion of the larger EVs population by LK2 cells further strengthening the link between AZD4785 endocytosis and EV secretion. To find out the exact contribution of the EV recycling pathway to ASO functional activity, we attempted to inhibit EV secretion pathway by blocking endosome maturation with U018666A as well as by modulating Rab27A-dependent and sphingomyelin phosphodiesterase 3-dependent exosome biogenesis pathways. Interestingly, in agreement with previous reports U018666A treatment induced CD63 accumulation. Unexpectedly, it stimulated secretion of CD63+/CD81+EVs by both cell lines, most likely by inducing LBPA accumulation in LE and activation of recently-established novel LBPA/Alix EV secretion pathway. Interestingly, in PC9 cells secretion of CD63+/-CD81+EVs was Rab27A independent and inhibition of EV release with sphingomyelin phosphodiesterase 3 inhibitor did not influence AZD4785 potency. However, in LK2 cells Rab27A knockdown and inhibition of sphingomyelin phosphodiesterase 3 reduced CD63+/-CD81+ EV secretion and improved AZD4785 potency but only by ∼2 and ∼5.5 fold, correspondingly. Altogether these data strongly indicate mainly LBPA engagement in LE and to a less extent ASO clearance via the EV recycling pathway influence the productive ASO uptake in LK2 cells (Supplementary Fig. S13). Importantly, rapid AZD4785 delivery to LBPA+ LE in PC9 cells and LBPA engagement dominates over EV recycling clearance pathway enabling good productive uptake hence future studies should be focused on the further understanding of the LBPA spatial distribution as well as the LBPA-mediated ‘back-fusion’ mechanism of endosomal escape (Supplementary Fig. S13). Modulation of intracellular PS-ASO trafficking by using small molecules regulating LBPA15,39 or targeting Rab27A40, SMPD341 or novel Alix-dependent52 EV biogenesis pathways40,41 are novel strategies over- coming non-productive uptake in select tumour cells and further analysis of models of EV role in ASO productive uptake across a varied panel of models and/or diseases are required in the future.

Methods

Cell culture. PC9 (formerly known as PC-14) cells were obtained from ECACC General Cell Collection: (https://www.pheculturecollections.org.uk/products/celllines/generalcell/detail.jsp?refid=90071810&collection=ecacc_gc). LK2 cell was obtained from the Japanese Collection of Research Bioresources Cell Bank (https://cellbank.nibiohn.go.jp/~cellbank/en/search_res_det.cgi?refID=540). Both cell lines were authenticated by short tandem repeat (STR) analysis. Both cell types were grown at 37 °C and 5% CO₂. The cells were grown in RPMI 1640 medium, supplemented with GlutaMAX™ (Gibco®) and 10% fetal bovine serum (FBS). Cell viability was measured using Trypan Blue solution or Vi-CELL® XR Cell Viability Analyzer (Beckman Coulter, software v2.04). For EV isolation, cells were cultured in exosome-free medium prepared with RPMI 1640 medium, supplemented with GlutaMAX®, 10% exosome-depleted FBS (Fetal Bovine Serum, exosome-depleted, One Shot™ format (ThermoFisher Scientific, A2720803)) in the presence or absence of AZD4785. For the pulse-chase experiment cells were treated with AZD4785 as above for 6 h, then washed 3 times with PBS and cultured in RPMI 1640 medium, supplemented with GlutaMAX®, 10% exosome-depleted FBS in the absence of AZD4785 for 24 h.

Antibody, fluorescently labelled proteins, oligonucleotides and inhibitors. Anti-Bovine Serum Albumin antibody [EPR12774] (Abcam, ab192603, 1:1000), Mouse Monoclonal antibody [clone 2C1] (IgG2b) to Human KRAS (LSBio Life Sciences, L-80656, 1:1000), Anti-ASK1 (Abcam, ab37412, 1:2000), anti-human CD63 antibodies (BD Pharmingen 556019, immunofluorescence 1:500, western blotting 1:1000), Alik Antibody (Cell signalling Technology, MA-83977, 1:1000), rabbit anti-ASO (Ionis 13345, 1:25,000), Secondary Donkey Anti-Rabbit IRDye800 CW (Li-COR 926-32213, 1:5000), secondary donkey anti-Goat IRDye680CW (LI-COR, 926-62222, 1:2000), Donkey Anti-Rabbit IgG (H + L) Alexa Fluor 568 (A10042, 1:2000, Donkey Anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (A21202, 1:200) and Goat anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (A21236, 1:2000) (ThermoFisher Scientific), Albumin from Bovine Serum (BSA), Alexa Fluor® 594 conjugate (ThermoFisher Scientific), U-18666A (Sigma, U3633), Thioridazine maleate salt (Sigma, T123), 3-O-Methyl-sphingomyelin (BML-SL225-0001, Enzo Life Sciences). Short interfering RNA (siRNA) oligonucleotides were ON-TARGETplus (HorizonDiscovery) non-targeting control pool (D-001810-10-05) and human Rab27A (L02067-00-0005). Human Generation 2.5 KRAS PS-ASO (AZD4785/IONIS 651987, sequence GTCTTAGAGGTCC, capital letters are cDNA-modified bases) was synthesised as previously described.

Isothermal titration calorimetry (ITC). To evaluate the binding between BSA and the AZD4785 sequence, an Auto ITC-200 (Malvern Panalytical) was used. The affinity was measured in a 0.2 µm filtered buffers—PBS (pH 7.4), 10 mM Pipes (pH 8.0), 100 mM NaCl and 2.5 mM CaCl₂, or 10 mM Pipes (pH 6.15) containing 140 mM NaCl and 2.5 mM CaCl₂, AZD4785 (870 µM final concentration) was titrated into a BSA solution (200 µM). The latter was directly prepared for dissolution in a mixture of the buffer-water 3:1 to match the AZD4785 conditions. The data were collected at 25 °C, using 1 µl sequential injections every 4 min. Background level was derived from the heat of dilution of AZD4785 in the buffer and subtracted from the experimental values. Data were analysed using the ‘One set of sites’ model function in the Origin 7 software, which defines that all binding sites are equal. Each ITC experiment was performed in duplicates and data reported are averaged. Since multiple BSA seemed to bind onto one single ASO strand with, possibly, different types of interactions affinities, the stoichiometry was fixed to a 0.06 AZD4785:BSA ratio for all data analyses. This value was chosen as it corresponds to 1 BSA molecule for nucleotide and it allows for a reasonably good fitting. Since it was not possible to obtain the actual binding stoichiometry the calculated affinity values must be considered as a whole system value.

Extracellular vesicles isolation and characterisation. EVs were isolated from the conditioned medium using differential centrifugation as previously described35,54 with modifications. Briefly, the conditioned medium was collected from cells (V = 60 ml) and centrifuged at 4 °C, 2000 g, 5 min using a Sorvall™ Legend XTR Centrifuge (ThermoFisher Scientific), with TX-100 1000 high capacity rotor, to remove large debris, cell debris, and medium-size EVs by ultracentrifugation at 100,000 g for 2 h. The supernatant containing small EVs was transferred to a Sorvall™ High-Speed PCCO centrifuge tubes and centrifuged at 4 °C, 10,000 × g, 30 min using a ThermoFisher Scientific® RC-6 Plus Superspeed centrifuge with a Sorvall® SS-34 Fixed Angle Rotor to remove large and medium-size EVs. The supernatant was transferred to Beckman Coulter Open Top-ThruWall™ ultracentrifuge tubes and ultracentrifuged at 6 °C, 100,000 × g, 1 h using an Optima™ L-80 XP ultracentrifuge (Beckman Coulter) fitted with a Ti 70 rotor. The pellets were washed with PBS and ultracentrifuged again under the conditions described, before resuspending in a small aliquot (200–400 µl) of sterile filtered PBS for further analysis with NTA and flow cytometry, cell treatment or lysed (see below). An aliquot of EVs was used to determine AZD4785 content by MS and the number of EVs by NTA so we can calculate the dose of AZD4785 loaded in EVs. To study EV uptake, EVs were labelled with 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (Sigma-Aldrich, #218888) as previously described.

Particle tracking analysis (NTA). Purified EV pellets were suspended in PBS and analysed by NTA using NanoSight NT100 NTA Master (Malvern Panalytical Ltd, Almeio, The Netherlands) equipped with a blue (488 nm) laser and sCMOS camera. Five 60 s videos were recorded, and the Brownian motion of particles was analysed using NanoSight NTA software v3.2 (Malvern, Worcestershire, United Kingdom) according to the manufacturer’s protocol, with the screen gain at 10, contrast at 0, EM gain at 14, focus –11, and detection level 7 to particles with minimal background.
for western blotting analysis. Working solution of inhibitor (U18666A) was pre-
pared using complete media as described above, added to cells, and incubated for
48 h before treatment with AZD4785. PC9 and Lk2 cells were transfected with siRNA by reverse transfection using Lipofectamine RNAiMAX Transfection Reagent (ThermoFisher Scientific, 13778100) according to the manufacturer’s protocol. In brief, to transfect cells in 96-well plate siRNA (3 pmol) was diluted in 25 µl Opti-MEM 1 Medium without serum and mixed with 0.25 µl Lipofectamine RNAiMAX and incubated for 15 min at room temperature. Cells were diluted in complete growth media and plated to each well containing siRNA-Lipofectamine complex (125 µl, 25,000 cells per well) and incubated for 24–72 h.

**Cell and EVs lysis** PC9 or Lk2 cells were washed with PBS twice and then incubated with Accutase for 5 min at 37 °C and re-suspended in the complete media (RPMI 1640 medium, supplemented with GlutaMAX® ( Gibco®) and 10% exosome-depleted FBS). Cell number was quanti-

**EV quantification by CD63 capture bead assay** EV quantification in the cell culture media was performed as previously described with modifications.40 In brief, anti-human CD63 antibody (35 µg) was immobilised to 4 µm aldehyde-sulphate beads (1 × 10^6), Aldehyde/Sulfate Latex Beads, 4% w/v, 4 µm (ThermoFisher Scientific, A37304) and CD63 capture beads kept in 0.5 ml of PBS containing 0.1% glycine and 0.1% sodium azide at 4 °C. Supernatants were incubated with CD63 capture beads (1 µl/0.4 ml media) on a shaker overnight at +4 °C. Cells were washed with PBS, detached from the plate by using Accutase and stained with 0.4% Trypan Blue (Gibco, 15250-061). Total number of live cells was counted using Cellaca MX Automated Cell Counter (Nexcelom Bioscience). Beads were washed with PBS supplemented with 2% BSA twice and stained with anti-CD81 PE-antibody (1:500 in PBS supplemented with 2% BSA) for 1 h at room temperature. Stained beads were washed 2% BSA and analysed by flow cytometry (LSR Fortessa, Beckton Dickinson, San Jose, CA). Single bead population was gated, and arbitrary units were calculated as mean fluorescence units × percentage of positive beads and normalised to the number of live cells.

**Mice PC9 xenograft model and EV isolation from plasma** Female SCID mice (Envigo) were housed under specific pathogen-free conditions in individually ventilated cages (Techniplast) at Alderley Park, United Kingdom. All animal experiments were approved by AstraZeneca animal welfare ethical review board and were conducted in 8- to 12-weeks-old female mice in full accordance with the UK Home Office Animal (Scientific Procedures) Act 1986. Mice were inoculated subcutaneously with PC9 cells (3 × 10^6 cells/mouse/mixed with a 1:1 ratio in Matrigel (BD Biosciences)). Tumour growth was monitored twice weekly by caliper measurement and tumour volume was calculated using the equation: 3.14 × length × width^2/6000. Growing tumours were randomised and recruited onto study when tumours reached an average of ~0.1 cm^3 (~11 days after implant). AZD4785 was formulated using PBS and dosed at a dose volume of 0.1 ml/10 g subcutaneously. Animals selected onto study were dosed with either AZD4785 at 250 or 125 mg/kg with dosing schedule 5 days on/2 days off and once a week for the groups 72‘h and 168‘h, correspondingly. Control animals were given PBS once weekly. Animals were sacrificed 72 or 168 h post last dose and terminal blood samples (minimum 100 µl plasma) taken into K2EDTA coated tubes (SARSTEDT CB300 K2E). The tube was placed on wet ice and the plasma prepared with a calibrated pipette and volume measured. Plasma was then transferred into 0.5 ml Nunc cryotubes and immediately frozen until analysis. Plasma was thawed in the water bath (+37 °C) and was centrifuged 1 h 30 min at 47,000 rpm (rotor TLA-55, 4 °C, using Optima MAX-X ultracentrifuge (Beckman Coulter)). The pellets were washed with PBS and ultracentrifuged again and re-suspended in a small aliquot (60 µl) of sterile filtered PBS for further analysis.

**AZD4785 UPLC-MS analysis** The snap-frozen samples were thoroughly defrosted before use and the samples prepared by centrifuging at 10,000 rpm for 5 min and taking 5 µl into a Greiner shallow well plate containing 45 µl of PBS solution. The sample was agitated for 5 min prior to being analysed directly by LC–MS using the conditions detailed below.

Samples were analysed by UPLC-MS utilising a Waters Xevo TQ-MS (WBA0259) and an Acquity UPLC system from Waters consisting of Sample Manager (M161UF953M), Acquity PDA (F171DU457A), Column Oven (E17CMP703G) and Binary Solvent Manager (E17BUR621G). The chromatographic conditions are as follows:

- 1 µl Injection.
- Flow rate 0.5 ml/min.
- Column = Phenomenex bioZen™ 3.6 µm intact XB-C8 100 × 2.1 mm.
- Column temp = 45 °C.
- Solvent A = 3.75 mM TFA: 100 nM HFIP in aqueous.
- Solvent B = 3.75 mM TFA: 100 mM HFIP in MeOH (50:50).
- Gradient = 80% A to 5% A in 4 mins and 2 min hold.
- Total run time = 6 min.

The Waters TQ-MS was operated in negative ion Electrospray (ESI) mode with an AGC target of 20000, gate width of 200ms, and a dynamic exclusion of 5s. Data was acquired in MRM mode using a negative ionisation mass spectrometer and operated in the multiple reaction monitoring (MRM) mode. Data was acquired in the positive ion mode for all compounds of interest. The Waters TQ-MS was operated in negative ion Electrospray (ESI) mode with an AGC target of 20000, gate width of 200ms, and a dynamic exclusion of 5s. Data was acquired in MRM mode using a negative ionisation mass spectrometer and operated in the multiple reaction monitoring (MRM) mode. Data was acquired in the positive ion mode for all compounds of interest.
The authors declare that all relevant data supporting the findings of the study are available within the manuscript and its Supporting Information. Raw proteomic mass spectrometry data (Fig. 3E and Supplementary Figs. S5, S6, Supplementary Data 1, 2) have been deposited in https://www.ebi.ac.uk/pride/archive/ with the accession code PXD027804. Figure’s raw data and unedited western blot images are included in the Supplementary Data 3. All other data for all figures and results presented here are available from the corresponding author (ANR) upon reasonable request.

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Author contributions
A.K., S.R., E.C. and S.P. conceived the study, designed the experiments and wrote the paper, A.K. conducted cell culture experiments and purified EVs, P.D., D.L. and E.C. conducted mass spectrometry, analysed and interpreted the data, C.M. performed the microscopy and edited the paper, E.L. designed the experiments, interpreted data and edited the paper, L.H. performed the mice experiments, N.R., M.S., N.J.B. and P.W.D. conducted proteomics LC–MS/MS analysis, interpreted the data and edited the paper, S.S. performed Isothermal Titration Calorimetry and analysed the data, A.R. and A.R.M. provided ASO, discussed the data and edited the manuscript. All authors have read and approved the final manuscript.

Competing interests
A.K., P.D., D.L., E.L., I.H., N.R., M.S., P.W.A.D., N.J.B., S.S., S.R., E.C. and S.P. are current or past employees and/or shareholders of AstraZeneca. A.R. and A.R.M. are employees and shareholders of Ions Pharmaceutics.

Additional information
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