Summary description of the project objectives

The first global aim of our research was to demonstrate that Peptide Nucleic Acid (PNA) conjugates can be engineered to regulate the expression of specific proto-oncogenes by targeting RNA secondary structures within untranslated regions with high specificity. For this proof-of-concept, we focused on naturally occurring G-quadruplex secondary structures which are believed to play multiple roles in translation regulatory mechanisms and have been found in the promoter regions of a number of proto-oncogenes. G-quadruplexes are formed from sets of four guanines binding in co-planar structures called G-quartets which can then stack on top of each other to from the so-called G-quadruplex structure. The major current limitation to the therapeutic use of G-quadruplex binding small molecules is their lack of specificity. Herein, we aimed to improve small molecule specificity by conjugating short Peptide Nucleic Acid (PNA) sequences complementary to the flanking regions of the G-quadruplex sequence, thus providing the first example of "sequence+structure"-specific G-quadruplex binding ligand.

However, as PNA are relatively large and charge free macromolecules, they do not easily cross the cells' membrane, limiting significantly their bioavailability. To address this, our first goal was to investigate different technologies to increase the cellular uptake of our PNA-conjugates (or any PNA-based drug). In particular we have investigated the potential of sonoporation (ultrasound + engineered microbubbles) to improve of delivery of PNAs into cells.

The main experimental milestones in the project were the design of highly specific (PNA-based) small molecules (drug design), the study of their interaction with the RNA target (biophysics); the study of their effect on gene expression (in vitro translation assay/molecular biology/biochemistry) and the engineering of a suitable technology for improving their cellular uptake/bioavailability (bioengineering/cell biology).

Description of the main achievements

- We have designed and synthesised the first generation of **PNA-naphthalene diimide (PNA_1-NDI-PNA_2) conjugates** directed against the well-characterised and naturally occurring RNA quadruplexes found in the 5'-UTR of the N-Ras proto-oncogene. A small library was synthesised and characterised by varying PNA length and PNA strand orientation. Whilst the NDI was designed to interact with the core of G-quadruplex structure whilst PNA_1 and PNA_2 sequences were complementary to both G-quadruplex flanking regions
- To assess the binding of our ligands, we optimised a fluorescence-based Electrophoretic Mobility Shift Assay (EMSA) and demonstrated that all our ligands could bind to the N-Ras quadruplex with high affinity and high specificity. Our study highlighted **cooperative binding** between the NDI and the PNA to the RNA target since neither the NDI or the PNA alone bind with high affinity. Although PNA orientation has little on binding, PNA length proved essential, with longer PNA strands binding more tightly than shorter PNAs.
- The effect of PNA-NDI conjugates on translation efficiency was assessed using a bioluminescence-based in vitro assay. Two control plasmids were designed to investigate the specificity of translation inhibition: one control containing G-to-A mutations to prevent G-quadruplex formation and a second control containing multiple mutations in the quadruplex flanking regions to prevent PNA-hybridisation. As already shown in our binding assays, **all PNA-NDI conjugates tested did inhibit RNA translation in vitro**. Inhibition efficiency was dependent on PNA length (**the longer the PNA the stronger the inhibition**) but is not really affected by PNA strand orientation. Despite IC50 values in the submicromolar range (comparable to the best inhibitors reported to date), a relative lack of specificity was observed in the translation assays. Although the reasons for lower than expected specificities are not yet fully understood, it is likely to be due to a combination of (i) non-specific

electrostatic interactions between the cationic PNA conjugates and the RNA target and (ii) off-target, non-specific, targeting of the ribosome.

- New RNA quadruplex targets were identified and characterised in genes of clinical interest. These include genes coding for Aurora kinase A, Aurora kinase C and α2(I) collagen (Col1A2). Particular attention was drawn to the **Aurora Kinase A RNA quadruplex that encompasses the AUG start codon**. Although this two-tetrad quadruplex is thermally unstable, we demonstrated that it could be stabilised by small molecules in vitro. We are currently exploring whether such small molecules (e.g. pyridostatin) could efficiently inhibit Aurora Kinase A expression both in vitro and in cellulo.
- We finally explored new ways to efficiently deliver therapeutic PNAs into cells. In particular, we investigated the possible use of microbubbles and ultrasounds to improve the cellular uptake of PNAs. Proof-of-concept study was carried out where human Hela or Huvec cells cultured in 6-well plates were incubated with a mixture of clinically approved microbubbles and fluorescently labelled PNAs and subjected to ultrasound pulses. Subsequently, an optimised set-up was developed where human cells were cultured in a flow cell and subjected to a continuous flow of PNAs and microbubbles. In both cases, transfection efficiency was monitored by fluorescence microscopy. **Under optimised conditions transfection efficiency >45% was obtained while almost no transfection was observed under similar conditions but in the absence of any ultrasound**. Interestingly, this original strategy paves the way to localised and harmless delivery of therapeutic PNAs.

Conclusions, impact and future work

Results obtained during this four-year fellowship clearly demonstrate the potential of PNA-NDI conjugates as inhibitors of translation, likely via a mechanism involving 5'-UTR RNA quadruplex targeting. All ligands tested proved capable to inhibit translation in a concentration-dependent manner and with efficiency strongly dependent on PNA length.

The ability to deliver therapeutic PNAs into cells by sonoporation using techniques already used in Clinique for ultrasound imaging are also likely to have a huge impact on the use of PNA molecules as therapeutic agents. Our preliminary data suggest that our optimized sonoporation conditions (under flow conditions) are harmless to the cells and allow efficient and localised transfection.

Future work includes the engineering of second generation PNA conjugates with improved specificity by either reducing the number of net charges or further increasing PNA length. Building on our promising preliminary transfection experiments, we will aim to improve transfection efficiency by either engineering optimized microbubbles or optimizing ultrasound pulse sequences. This work is already ongoing in our lab and will be reported in due course.