Investigating microRNA-7 as a potential therapeutic in cancer

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Bachelor of Science (Advanced) with Honours (Class I) in Genetics

This thesis is presented for the degree of Doctor of Philosophy of The University of Western Australia

School of Medicine

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Abstract

Cancer is the second leading cause of deaths worldwide, with liver and colorectal cancer (CRC) the second and third most responsible for cancer-related deaths, respectively (World Health Organisation 2017). Current therapeutic options, namely chemotherapy, lack specificity and are often not effective. Recent technologies have broadened our understanding of the complexity of the disease and this has led to a shift in our approach to cancer therapy. Personalised targeted therapies to individualise treatment and enhance efficacy are being sought. Using microRNAs as therapeutics and their nanoparticle-mediated delivery is one such new and innovative alternative.

MicroRNAs (miRNAs) are short, non-coding RNA molecules which post-transcriptionally regulate gene expression. Their dysregulation has been associated with cancer development and progression. MicroRNA-7 (miR-7) is considered a ‘tumour suppressor’ miRNA which directly and indirectly inhibits the expression of many oncogenic signalling molecules resulting in reduced cell viability and tumour growth. Further, miR-7 has been shown to improve drug sensitivity (chemotherapeutics and targeted therapies) and inhibit metastasis. Endogenous expression of miR-7 is reduced in a variety of human cancers compared to normal tissue. Therefore, upregulating the expression of tumour suppressor miRNAs such as miR-7 may be useful in cancer therapy.

There are significant challenges surrounding the systemic delivery of microRNA-based therapeutics to tumours safely and efficiently. Two methods are explored here; the delivery of miR-7 mimics using polymeric nanoparticles and identification of small molecules that upregulate endogenous miR-7 expression.

In this project, polymeric PGMA-PEI nanoparticles were shown to successfully deliver miR-7 to cancer cells, however, there was a failure in the inhibition of key miR-7 target proteins. This result prompted greater focus on the small molecule-mediated method of upregulating miR-7. miRNAs act via binding to the 3’-UTR of a target mRNA transcript inhibiting translation and/or causing mRNA degradation. Based on this principle, a stable cell-based luciferase reporter system was generated to detect upregulation of miR-7 in response to compound treatment. A library of 800 highly selected naturally occurring
Abstract

Compounds was screened using this reporter system and as a result, compounds were identified consistent with modulation of miR-7 expression.

Cantharidin, a compound isolated from blister beetles and used in traditional Chinese medicine for the treatment of cancer, was the strongest hit and found to upregulate miR-7 in both human HT29 CRC and Huh7 hepatocellular carcinoma (HCC) cells. Cantharidin was further shown to inhibit the expression of miR-7 target proteins and induce apoptosis. These results indicate that cantharidin or its derivatives may prove to be useful therapeutics for the treatment of these cancers.

In summary, this project demonstrated that PGMA-PEI nanoparticles are a viable method of miRNA delivery to cells and further optimisation may assist in ensuring sufficient and/or active miR-7 is delivered. In addition, this work is the first to show miR-7 upregulation by the compound cantharidin in CRC and HCC and demonstrate the downregulation of miR-7 target proteins. Thus, further development of cantharidin-like compounds may establish a novel method of treating these recalcitrant cancers.
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This thesis contains work that has been published.

Details of the work:


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Sections of this publication have been used in Chapter 1, the introduction of this thesis, only.

Student contribution to work:

The manuscript was written in entirety by Jessica L. Horsham and was edited by co-authors.

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3. The role of microRNA-7 in normal development and disease (only including subsections where specified)
3.3. Endocrine pancreas (including 3.3.1. miR-7 expression in the endocrine pancreas and role in development and 3.3.2. miR-7 expression and role in diabetes)
4. Conclusion
Figure 1

Other sections were written by co-authors and the manuscript was edited by all authors.

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Coordinating supervisor signature: [Redacted]
Date: 13/2/2018
First and foremost, to my husband Ben, who has believed in me, supported me, encouraged and motivated me. I owe the completion of this thesis to you, above all others.

To my parents, Jackie and Mark, to whom I owe everything.

To my brother, Daniel, who travelled for the last four years... you really only made this harder! I miss you dearly.

To my lab colleagues, Dianne, Kirsty, Rikki, Larissa, Felicity, Lisa, Clarissa, Mike, Tasnuva and Andrew, thank you for celebrating, commiserating and being the greatest source of my supervision. A special thanks to Kirsty and Di, both of whom read and edited the thesis draft, much of which was done in their own time. A special mention again to Kirsty who went above and beyond and spent some of her leave to look after my little one while I wrote.

To my supervisors, Peter Leedman, Shane Colley and Kevin Pfleger, thank you for your intellectual insight, technical advice, assistance with data analysis and thesis preparation, and the opportunity to attend both local and international conferences.

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The completion of this thesis has been an epic journey and has been completed in conjunction with some major hurdles. I began this PhD by relocating from Sydney. My
first year was hampered by a lengthy and drawn out false cancer diagnosis. My second year saw financial hardship. I spent the third year apart from my now husband as he worked in Kalgoorlie while I remained to complete my PhD studies in Perth. In my fourth year, we celebrated the birth of our first child. Throw in an engagement, wedding, first home purchase and pregnancy that derailed experiments, all I can say is that I am glad to be on the other side of it all.

Finally, to my son Max, you have pushed me over the finish line. A first lesson, “just keep swimming”. It is possible to overcome major challenges with hard work and a lot of determination... and complete a postgrad degree on the side.
Publications


## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>5-FUrdd</td>
<td>5-Fluorouridine</td>
</tr>
<tr>
<td>3mer</td>
<td>Concatemer of 3 target sites</td>
</tr>
<tr>
<td>6mer</td>
<td>Concatemer of 6 target sites</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>%CV</td>
<td>Percent coefficient of variation</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B (PKB)</td>
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<td>APC</td>
<td>Adenomatous polyposis coli</td>
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<tr>
<td>ATCC</td>
<td>American type culture collection</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAG3</td>
<td>Bcl2-associated athanogene 3</td>
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<td>BCL-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>B-cell lymphoma-extra-large</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
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<tr>
<td>BRAF</td>
<td>V-Raf murine sarcoma viral oncogene homolog B</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CAS</td>
<td>Chemical abstracts service</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>c-Myc</td>
<td>V-Myc avian myelocytomatosis viral oncogene homolog</td>
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<tr>
<td>ciRS-7</td>
<td>Circular RNA sponge for miR-7</td>
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<tr>
<td>cm</td>
<td>Centimetre</td>
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<tr>
<td>Cons</td>
<td>Consensus</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
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<tr>
<td>Ct</td>
<td>Cycle threshold</td>
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<td>DAPI</td>
<td>4',6-diamidino-2-phenyldinole</td>
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<td>DMEM</td>
<td>Dulbecco’s modified eagles medium</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
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<td>Ethylenediaminetetraacetic acid</td>
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<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>ERK 1/2</td>
<td>Extracellular signal-regulated kinase 1/2</td>
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<td>FAK</td>
<td>Focal adhesion kinase</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FOXP3</td>
<td>Forkhead box P3</td>
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<tr>
<td>g</td>
<td>Grams; or acceleration due to gravity (g-force)</td>
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<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>h</td>
<td>Hours</td>
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<td>HBx</td>
<td>Hepatitis B virus X</td>
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<td>HCC</td>
<td>Hepatocellular carcinoma</td>
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<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<td>HNF4α</td>
<td>Hepatocyte nuclear factor 4 alpha</td>
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<td>hnRNPK</td>
<td>Heterogeneous nuclear ribonucleoprotein K</td>
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<td>HOTAIR</td>
<td>Hox transcriptase antisense RNA</td>
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<td>Homeobox D10</td>
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<td>Heat shock protein 70</td>
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<td>Human antigen R</td>
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<td>IGFR1</td>
<td>Insulin-like growth factor receptor 1</td>
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<td>IKK</td>
<td>I kappa B kinase</td>
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<td>ILF2</td>
<td>Interleukin enhancer binding factor 2</td>
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<tr>
<td>IRS2</td>
<td>Insulin receptor substrate 2</td>
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<td>JAK</td>
<td>Janus kinase</td>
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<td>Jun nuclear kinase</td>
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<td>KLF4</td>
<td>Kruppel like factor 4</td>
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<td>KRAS</td>
<td>Kirsten rat sarcoma viral oncogene homolog</td>
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<tr>
<td>L</td>
<td>Litre</td>
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<td>LB</td>
<td>Luria Bertani</td>
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<td>LDS</td>
<td>Lithium dodecyl sulfate</td>
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<td>LF</td>
<td>Lipofectamine 2000</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
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<tr>
<td>MDR</td>
<td>Multi-drug resistant</td>
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<tr>
<td>mg</td>
<td>Milligrams</td>
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<tr>
<td>min</td>
<td>Minute</td>
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<td>miR-7</td>
<td>MicroRNA-7</td>
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<td>miRNA</td>
<td>MicroRNA</td>
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<td>MMP2</td>
<td>Matrix metalloproteinase 2</td>
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<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
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<td>Magnetic resonance imaging</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<td>Multidrug resistance protein 1</td>
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<td>MSI2</td>
<td>Musashi homolog 2</td>
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<tr>
<td>MT</td>
<td>Mutant</td>
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<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<td>MW</td>
<td>Molecular weight</td>
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<td>NeoR</td>
<td>Neomycin resistant</td>
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<tr>
<td>Neo</td>
<td>Neomycin</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
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<tr>
<td>ng</td>
<td>Nanogram</td>
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<td>Nanomolar</td>
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<td>Nanoparticle</td>
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<td>NPL</td>
<td>Natural products library</td>
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<td>NS</td>
<td>Not screened</td>
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<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
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<tr>
<td>nt</td>
<td>Nucleotides</td>
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<td>OD</td>
<td>Optical density</td>
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<td>PAK1</td>
<td>P21-activated kinase 1</td>
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<td>pAkt</td>
<td>Phosphorylated Protein kinase B</td>
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<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PEG</td>
<td>Polyethylene glycol</td>
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<td>PEI</td>
<td>Polyethyleneimine</td>
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<td>PET</td>
<td>Positron emission tomography</td>
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<td>PGMA</td>
<td>Polyglycidal methacrylate</td>
</tr>
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<td>PGSF1a</td>
<td>Pituitary gland specific factor 1a</td>
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<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
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<tr>
<td>PP1</td>
<td>Protein phosphatase-1</td>
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<td>Pre-miR</td>
<td>Precursor microRNA</td>
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<tr>
<td>Pri-miR</td>
<td>Primary microRNA</td>
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<tr>
<td>Puro</td>
<td>Puromycin</td>
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<tr>
<td>QKI</td>
<td>Quaking homologs, KH domain RNA binding protein</td>
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<td>Ras</td>
<td>Rat sarcoma</td>
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<td>RELA</td>
<td>V-Rel Avian Reticuloendotheliosis Viral Oncogene Homolog A</td>
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<td>RISC</td>
<td>RNA-induced silencing complex</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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<tr>
<td>RT qPCR</td>
<td>Reverse transcription quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
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<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
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<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>Shp2</td>
<td>S-phase kinase-associated protein 2</td>
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<tr>
<td>SRSF1</td>
<td>Serine/arginine-rich splicing factor 1</td>
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<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>STR</td>
<td>Short tandem repeat</td>
</tr>
<tr>
<td>TARBP2</td>
<td>TAR RNA binding protein 2</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween</td>
</tr>
<tr>
<td>TLR9</td>
<td>Toll-like receptor 9</td>
</tr>
<tr>
<td>TRBP</td>
<td>TAR RNA binding protein</td>
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<tr>
<td>μg</td>
<td>Micrograms</td>
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<tr>
<td>μL</td>
<td>Microlitres</td>
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<td>Micromolar</td>
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<tr>
<td>u</td>
<td>unit</td>
</tr>
<tr>
<td>Usp18</td>
<td>Ubiquitin-specific peptidase 18</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UWA</td>
<td>The University of Western Australia</td>
</tr>
<tr>
<td>v</td>
<td>volume</td>
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<tr>
<td>WT</td>
<td>Wildtype</td>
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<tr>
<td>XRCC2</td>
<td>X-ray repair complementing defective repair in Chinese hamster cells 2</td>
</tr>
<tr>
<td>YY1</td>
<td>Yin yang 1</td>
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1. Thesis introduction

This chapter includes a detailed review of the literature and outlines the aims and objectives of this work as well as the thesis structure. In addition, it broadly describes the project approach, however, this is given in more detail in introductory sections of each results chapters.

1.1. Cancer, the global impacts and challenges

Cancer is undeniably a major world health problem with few effective treatment options. There were 8.8 million cancer-related deaths in the world in 2015 and its incidence is predicted to rise by over 70% in the next 20 years (World Health Organisation 2017). While there have been major steps forward in the treatment of some cancers, such as breast cancer, there is overwhelmingly poor prognosis for many cancers.

Cancer is the result of genetic and epigenetic alterations that cause uncontrolled cell proliferation and acquired potential for invasion and metastasis. In early stages of cancer progression surgery to remove the entire lesion is considered curative. However, if the cancer has metastasised to other parts of the body, therapy commonly involves surgery, radiotherapy, chemotherapy and/or targeted therapies. Chemotherapies are cytotoxic chemicals which act to target rapidly dividing cells. However, chemotherapy agents are non-specific, resulting in toxicity to other rapidly dividing cell types and tissues (such as the stomach lining and intestines, blood and bone marrow, and hair follicles), causing many and sometimes severe side effects (Chen, Chiu et al. 2010). Radiotherapy similarly lacks specificity. In recent years improved knowledge of key oncogenic and tumour suppressor genes and their molecular mechanisms has led to the evolution of targeted therapies and personalised treatment. In this thesis, ‘targeted therapy’ refers to therapies that act on specific molecular targets and ‘personalised treatment’ is when those drugs are administered to patients based on the genetic and epigenetic characteristics of their tumours (Melo, Villanueva et al. 2011). The aim of which is to both improve the efficacy of treatment and reduce side effects.

Gene silencing utilising microRNAs (miRNAs) is a potential strategy in the development of future targeted therapies. miRNAs are short 21-23 nucleotide (nt), endogenous, non-
coding RNA molecules that form a complex network of post-transcriptional gene regulation. Genes regulated by miRNAs are manifold and thus miRNAs mediate the expression of proteins involved in pathways essential for cell function, proliferation, differentiation, apoptosis and development. Aberrant expression of miRNAs has been linked to the development and progression of cancer and other diseases (Farazi, Spitzer et al. 2011, Ha 2011, Bhaskaran and Mohan 2014, Peng and Croce 2016). Based on their intricate and important role in gene regulation it is thought that ‘miRNA replacement therapy’, the administration of tumour suppressor miRNAs, may be useful in the treatment of cancer (Iorio and Croce 2012, Rupaimoole and Slack 2017). The focus of this thesis is to develop methods to increase exposure of human cancer cells to the tumour suppressor miRNA, microRNA-7 (miR-7). To address this, two different approaches are undertaken. The first is to deliver miR-7 using polymeric nanoparticles and the second is to identify small molecules that can upregulate miR-7. Studies in this thesis will be focused for the most part in colorectal cancer (CRC).

1.1.1. Colorectal cancer

In Australia, CRC is the second most common cancer (Cao, Jiang et al. 2015) and was the second most common cause of cancer-related death in 2014 (Australian Government: Cancer Australia 2017). The five-year survival rate of CRC varies from 93% to 8% depending on the stage of the disease; the overall five-year survival rate being 66%. For patients with early-stage disease at diagnosis (stages 1 and 2) the cancer may be cured by surgery alone, however, those patients that progress to late-stage cancer (stages 3 and 4) with advanced metastasis rely on combined surgery and chemotherapy, or chemotherapy alone (Cao, Jiang et al. 2015).

Ninety-five percent of the time CRC develops from adenomas or polyps in the colonic mucosa, the first and innermost layer of the intestine. These polyps are generally benign however some can lead to cancer formation. The risk of the polyp becoming cancerous increases with time and size (Honegger, Schilling et al. 2015). Recognised risk factors for CRC include; age, family history of CRC or polyps, inflammatory bowel disease as well as many preventable risk factors including obesity and smoking (Bonfrate, Altomare et al.)
Development of CRC is associated with frequent mutations. The most frequent mutations involved in CRC occur in the APC, p53 and KRAS genes (Mancikova, Castelblanco et al. 2015). Patients with mutations in KRAS have a poorer prognosis as they generally don’t respond to targeted therapeutics such as cetuximab, an epidermal growth factor receptor (EGFR) inhibiting drug (Lievre, Bachet et al. 2006, Sforza, Martinelli et al. 2016).

A study which examined miR-7 expression and its relationship with EGFR in CRC found low miR-7 expression to correlate with significantly poorer prognosis and cancer progression. This study also found that treatment with miR-7 was able to restore cetuximab sensitivity in cell lines harbouring KRAS mutations (HCT116, SW620) but not BRAF mutations (HT29) (Suto, Yokobori et al. 2015).

CRC is frequently driven by EGFR overexpression and is associated with loss of miR-7 expression. Taken together, these data illustrate the restoration of cetuximab sensitivity with miR-7 provides an ideal foundation for the studies outlined in this thesis.

1.1.2. Liver cancer

Although the focus of this thesis is on CRC, in subsequent chapters the effectiveness of small molecule miR-7 modifiers in hepatocellular carcinoma (HCC) are evaluated as a comparative tumour model. Therefore, some background on HCC is provided here.

There are three types of liver cancer, cholangiocarcinoma, angiosarcoma and HCC. HCC is the most common form of primary liver cancer accounting for 75 – 90% of global cases (Wong, Jiang et al. 2017). Further, secondary liver cancer is common as it is a common site for metastasis of many different types of cancers, especially CRC (Lintoiu-Ursut, Tulin et al. 2015) and pancreatic cancer (Deeb, Haque et al. 2015).

In Australia, approximately 1600 people are diagnosed each year with primary liver cancer (http://www.cancercouncil.com.au/liver-cancer/). Although low in absolute number, its prevalence is more common in those countries where Hepatitis B and C infections are more pervasive. On a global scale liver cancer is responsible for the second
Thesis introduction

largest cancer-related mortality both in men and in developing countries, and is one of the few cancers where its incidence is rapidly increasing (Torre, Bray et al. 2015). The single most important risk factor for HCC is cirrhosis of the liver. There are many causes of cirrhosis, commonly being hepatitis B and C infection, excessive alcohol consumption and fatty liver associated with obesity (Pinter, Trauner et al. 2016).

HCC is currently treated with surgery, radiofrequency ablation and/or chemotherapy and there is only one targeted therapy, Sorafenib, to which resistance is invariant (Raza and Sood 2014).

1.2. MicroRNA biogenesis and action

miRNA biogenesis is a multistep process beginning in the nucleus with the transcription of a primary miRNA (pri-miRNA) transcript. Pri-miRNAs are commonly >1000 nt in length and contain stem-loop structures (Ha and Kim 2014). They are subsequently cleaved by the ribonuclease Drosha to generate hairpin precursor miRNAs (pre-miRNAs). Following Drosha cleavage, the resulting ~110 nt long pre-miRNAs are transported to the cytoplasm where the terminal loop is removed by Dicer, creating a short duplex mature miRNA consisting of a 5p and 3p strand. One strand, termed the “guide strand” or “leading strand”, becomes associated with the RNA-induced silencing complex (RISC). The guide strand may be either the 5p or the 3p strand and is determined in part by the relative stability of the 5’ end and excess of purine versus pyrimidine composition. The passenger strand is considered inactive and is typically degraded (Meijer, Smith et al. 2014, Peng and Croce 2016).

miRNAs guide RISC to target mRNAs via sequence-specific recognition, providing an interface for interaction with the corresponding mRNA. Binding typically occurs at the 3’ untranslated region (3’-UTR) of mRNA transcripts, although examples exist of binding sites within the 5’ untranslated region (5’-UTR) (Lytle, Yario et al. 2007, Zhou and Rigoutsos 2014) or mapped coding regions (Forman and Coller 2010, Lee and Gorospe 2011, Ott, Grunhagen et al. 2011). Complementarity is often imperfect and central bulging of the sequence interaction results in translational repression of the mRNA. However, in the event of complete complementarity, mRNA cleavage ensues leading to
Thesis introduction

accelerated mRNA decay (Ipsaro and Joshua-Tor 2015). The exact mechanism of silencing targets through translational repression is unknown, possibly through interfering with translational initiation (Jonas and Izaurralde 2015). Following translational inhibition, the mRNA is targeted for degradation via deadenylation, decapping and subsequent degradation by exonucleases. It has been demonstrated that translational inhibition precedes target deadenylation and degradation and that this is not a requirement of translational inhibition (Wilczynska and Bushell 2015). All methods of silencing and degradation require target site recognition dependent on perfect base pairing at nucleotides 2–8 of the miRNA known as the ‘seed’ region and the result is a reduction in protein expression. miRNAs generally exert only modest repression on their targets and so their action is more akin to ‘fine-tuning’ gene expression (Baek, Villen et al. 2008, Ebert and Sharp 2012, Wilczynska and Bushell 2015). miRNAs have also been implicated in upregulating gene expression (Vasudevan, Tong et al. 2007, Orom, Nielsen et al. 2008, Vasudevan 2012). To further emphasise the complexity of miRNA action, the UTRs of mRNAs may contain target sites for multiple miRNAs and conversely, a single miRNA may concurrently regulate multiple target genes in common pathways due to the widespread genomic occurrence and sequence flexibility of target sites (Seto 2010, Farazi, Spitzer et al. 2011, Lima, Busacca et al. 2011, Wilczynska and Bushell 2015). miRNA targets can be predicted by statistical modelling, for example through the use of such as programs as TargetScan. TargetScan is a freely available resource that provides information on the type (strength), number and location of sites (Agarwal, Bell et al. 2015, Agarwal, Bell et al. 2016). A simplified overview of miRNA biogenesis and action is given in Figure 1.
Figure 1. miRNA biogenesis and action.

Pri-miRNA transcripts are transcribed by RNA polymerase II from both intronic and intergenic regions of the genome. Pri-miRNAs are long structures which contain a stem-loop and are processed in the nucleus by Drosha to form single hairpin pre-miRNAs. The pre-miRNAs are exported to the cytoplasm via Exportin 5 and are further processed by Dicer which cleaves the terminal loop leaving the mature double-stranded miRNA (guide strand and passenger strand). The guide strand then becomes associated with RISC and the passenger strand is usually degraded. Binding of miRNA/RISC to target sites within the 3’UTR results in translational repression (imperfect complementarity) or mRNA cleavage (perfect complementarity) (Bonfrate, Altomare et al. 2013). The miRNA/RISC complex may also bind to targets in the 5’UTR which may either repress or activate transcription.
1.3. MicroRNA dysregulation in cancer

Aberrant miRNA expression is commonly associated with cancer and has been closely linked to its development and progression. Perturbed miRNA expression has been reported for almost all cancer types and specific examples include CRC (Pellatt, Stevens et al. 2016), breast (Mulrane, McGee et al. 2013), HCC (Morishita and Masaki 2015), brain (Moller, Rasmussen et al. 2013), leukaemia (Babashah, Sadeghizadeh et al. 2012), ovarian (Zhang, Lu et al. 2015), pancreatic (Hernandez and Lucas 2016), lung (Krutakova, Sarlinova et al. 2016), and melanoma (Latchana, Ganju et al. 2016). This perturbed expression has been attributed to several hallmarks of cancer including proliferative signalling, inducing angiogenesis, invasion and metastasis, and evading growth suppressors and cell death as outlined in a recent review (Peng and Croce 2016).

Abnormalities in miRNA expression profiles arise due to various genetic and cellular changes. The location of miRNA genes is often associated with fragile regions of the genome, such as common breakpoints and genomic regions commonly altered in cancer (Calin, Sevignani et al. 2004, Lagana, Russo et al. 2010). In addition, miRNAs may be aberrantly transcribed due to changes in cofactor or transcription factor expression, affected by the expression and action of other miRNAs (Iorio and Croce 2012), or epigenetic inactivation by DNA methylation (Lehmann, Hasemeier et al. 2008, Kwon, Song et al. 2017). Finally, miRNA dysregulation can be attributed to malfunctions in the biogenesis process, such as perturbed Drosha or Dicer activity. Ineffective Drosha processing can lead to a widespread downregulation of miRNAs observed in multiple tumour types (Thomson, Newman et al. 2006, Gurtner, Falcone et al. 2016). In general, when the miRNA processing pathway is suppressed, tumorigenesis is stimulated (Kumar, Lu et al. 2007, Melo, Villanueva et al. 2011).

1.4. MicroRNAs as therapeutics

1.4.1. Challenges in microRNA delivery

One of the major hurdles hindering the progression of miRNA therapeutics is a lack of suitable in vivo delivery systems, particularly to target tissues beyond the liver (Zhang, Wang et al. 2013, Chen, Gao et al. 2014). Localised delivery via intratumoural injection
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requires the tumour to be both easily accessible and nonmetastatic, as such, systemic delivery via intravenous injection is the most attractive route of administration (Seto 2010, Trang, Wiggins et al. 2011). miRNAs delivered systemically are subject to nuclease degradation and clearance by the reticuloendothelial system (cells found in circulation and tissues responsible for the removal of foreign materials from circulation by phagocytosis) which causes them to accumulate in the liver and spleen (Rupaimoole, Han et al. 2011, Muthiah, Park et al. 2013, Chen, Gao et al. 2014). Their negative charge also reduces their potential to cross the cell membrane (Costa and Pedroso de Lima 2013). Once inside the cell, they are subject to degradation in endocytic compartments (Rupaimoole and Slack 2017).

To overcome some of these issues chemically modified oligonucleotides termed ‘miRNA mimics’ and ‘anti-miRs’ have been developed. Chemical modifications such as 2’-O-methylation, phosphorothioates and locked nucleic acids can improve stability, reduce nuclease degradation and improve biodistribution. However, modifications may be limited in that they may also reduce target affinity and cellular uptake (Chen, Gao et al. 2014, Rupaimoole and Slack 2017). Chemical modifications can adversely affect loading into RISC thereby affecting the gene silencing ability of the modified miRNA (Rupaimoole and Slack 2017).

An alternative strategy to modification of the nucleotides and RNA backbone is to package the therapeutic miRNAs into delivery vehicles, for example, nanoparticles, in order to protect them from degradation and overcome the limitations associated with chemical modification. Further, both nanoparticle-mediated delivery and modification of the RNA itself present the opportunity for targeted delivery (Rupaimoole and Slack 2017).

Given the current interest in using miRNAs as therapeutics and the challenge with tissue specific delivery via existing approaches, nanoparticle-based miRNA delivery or the potential for small molecule miRNA regulation are attractive alternatives.
1.4.2. MicroRNAs as replacement therapy

To date, there has been a small number of miRNA-based clinical trials to treat various diseases (Rupaimoole and Slack 2017). The first clinical trial for miRNA replacement therapy was conducted using MRX34, a miR-34 miRNA mimic that was delivered to treat HCC by MIRNA Therapeutics, Inc. (clinicaltrials.gov identifier: NCT01829971). The trial, first of its kind in humans, stemmed from a large body of work indicating that miR-34a was a potent tumour suppressor (Yin, Ogawa et al. 2013, Adams, Parsons et al. 2016, Farooqi, Tabassum et al. 2017). However, the trial was terminated in late 2016 due to five severe immune-related adverse events involving patient deaths (Shah, Ferrajoli et al. 2016, Rupaimoole and Slack 2017). Specifically, the deaths were a result of severe cytokine release syndrome, a consequence of high-level immune activation. Potentially fatal complications of which can include respiratory distress, cardiac dysfunction, renal and/or hepatic failure, disseminated intravascular coagulation and neurologic toxicity (Lee, Gardner et al. 2014). MIRNA Therapeutics, Inc. used first generation chemistry and an older lipid nanoparticle delivery system. However, it is unclear whether the adverse events were the result of the miRNA mimic, sequence or delivery vesicle. The field of miRNA delivery is learning from the small interfering RNA (siRNA) delivery field as they utilise the same intracellular machinery i.e. RISC. Recent papers have shown that by using 2\textsuperscript{nd} generation chemistry and tissue-specific uptake, very effective silencing of a target gene can occur over many months. In particular, this work targeted the protein PCSK9 with siRNAs and demonstrated effective reduction in low-density lipoprotein (LDL) cholesterol (Fitzgerald, White et al. 2017).

A phase I clinical trial was completed for the treatment of malignant pleural mesothelioma with a miR-16 mimic, MesomiR-1 by EnGeneIC (clinicaltrials.gov identifier: NCT02369198). Miragen Therapeutics is currently conducting phase I clinical trials for the miRNA mimic, MRG-106, an agonist of miR-155 in various lymphomas and leukaemias (clinicaltrials.gov identifier: NCT02580552) and completed a phase I clinical trial for MRG-201 a mimic of miR-29b for the treatment of fibrous scar tissue in various diseases (clinicaltrials.gov identifier: NCT02603224). A phase I clinical trial is currently being conducted by Regulus Therapeutics involving the anti-miR-103/107 drug RG-125/AZD4076 in non-alcoholic fatty liver disease (clinicaltrials.gov identifier: NCT02612662) and the same drug is currently involved in a phase I/IIa clinical trial to
treat patients with both type 2 diabetes and non-alcoholic fatty liver diseases (clinicaltrials.gov identifier: NCT02826525). Finally, Santaris Pharma have completed phase I (clinicaltrials.gov identifier: NCT01646489) and phase II trials (clinicaltrials.gov identifiers: NCT01200420, NCT02031133, NCT02508090) for Mirvirasen an anti-miR-122 drug in hepatitis C infection.

In summary, miRNA-based therapeutics have entered clinical trials for the treatment of several diseases, however there are concerns raised by the MRX34 trial surrounding the safety of miRNA delivery. These results further emphasise the importance of the work presented herein, that is, different methods of miRNA delivery may overcome adverse immune activation or alternatively, small molecules that upregulate specific target miRNAs are needed.

1.4.3. Nanoparticle-based delivery

There are several reasons nanoparticle-based delivery is a promising method for miRNA delivery in cancer. They offer a means of protected delivery to overcome miRNA degradation in vivo (Jin, Yu et al. 2012, Liu, Meng et al. 2016). They are easily modified allowing for tumour targeting and enhanced transfection efficiency (Xin, Huang et al. 2017). Finally, they have successfully been demonstrated as effective miRNA carriers (Crew, Tessel et al. 2012, Mohammadi, Salmasi et al. 2015, Ebrahimian, Taghavi et al. 2017, Xie, Murray-Stewart et al. 2017). Nanoparticle delivery of miRNAs is discussed in depth in subsequent chapters.

1.4.4. Small molecule modifiers of microRNA gene expression

Small molecules are advantageous to oligonucleotide-based drugs in a number of ways including: they have good tissue penetration; can diffuse across the cell membrane; have greater stability in the bloodstream; have good stability inside cells; their manufacturing costs are relatively cheap; they may be readily modified; are available in large libraries; and given the majority of clinical drugs are small molecule-based there exists a large base of clinical experience (Kang, Im et al. 2014, Jeker and Marone 2015). High throughput screens have previously identified compounds with demonstrated potential
to both promote and inhibit miRNA transcription and these are discussed in later chapters.

1.5. **MicroRNA-7: A tumour suppressor**

miRNAs may be classified as oncogenic (oncomiRs) or as tumour suppressors depending on the nature of their target genes (Zhang, Pan et al. 2007). miR-7 is a tumour suppressor miRNA which is downregulated in many cancers when compared to normal tissues including CRC (Xu, Chen et al. 2014), glioblastoma (Kefas, Godlewski et al. 2008), non-small cell lung cancers (Xiong, Zheng et al. 2011, Xiong, Zheng et al. 2014), gastric cancer (Xie, Chen et al. 2014), prostate cancer (Chang, Zhou et al. 2015) as well as HCC (Ma, Qi et al. 2013, Wu, Liu et al. 2017). It has been shown to suppress cancer growth, proliferation, and metastasis, induce cell death as well as cell cycle arrest and apoptosis, and increase the sensitivity of resistant cancer cell lines to current therapeutics (Webster, Giles et al. 2009, Kalinowski, Brown et al. 2014, Gu, Huang et al. 2015, Horsham, Kalinowski et al. 2015). miR-7 acts via direct and indirect inhibition of many key oncogenes and members of oncogenic pathways, examples of which are outlined in Figure 2.
Figure 2. miR-7 direct and indirect targets.

miR-7 targets many molecules involved in various oncogenic pathways. This figure is taken from (Kalinowski, Brown et al. 2014) and reproduced here with permission. Molecules that are confirmed direct targets of miR-7 are indicated with a blue dot.
1.5.1. MicroRNA-7 expression and regulation

miR-7 is an evolutionarily conserved miRNA and has three loci (miR-7-1, miR-7-2 and miR-7-3). Loci are located in an intron of the heterogeneous nuclear ribonucleoprotein K (hnRNPK) gene on chromosome 9 (miR-7-1) and an intron of the pituitary gland specific factor 1a (PGSF1a) gene on chromosome 19 (miR-7-3), and in an intergenic region of chromosome 15 (miR-7-2). Each of the miR-7 genes gives rise to different pre- and pri-miRNA transcripts and the same mature miRNA sequence (Kalinowski, Brown et al. 2014). The structure and sequence of pre-miR-7 transcripts are illustrated in Figure 3. In general, miRNAs in intergenic regions are more evolutionarily conserved than their intronic counterparts (Ozsolak 2008). Both intronic and intergenic miRNAs have been shown to have their own promoter regions (Monteys, Spengler et al. 2010) and may be under the control of their host gene and/or regulated independently by transcription factors (Chaudhuri, Kabaria et al. 2015) and epigenetic factors (Suzuki, Takatsuka et al. 2011).

It should be noted that alternative sequences of miRNAs termed isomiRs have been identified in RNA-sequencing studies and may have biological significance. These isomiRs potentially arise from Argonaute 2 cleavage (the catalytic component of RISC) independent of Dicer, producing base substitutions and size variations. They are thought to be functionally relevant, possibly cooperating with canonical miRNAs to target common molecules and pathways (Cloonan, Wani et al. 2011, Tan and Dibb 2015). Although miR-7 is expressed widely at low levels, it is enriched in various regions of the brain, particularly the pituitary (noting the location of miR-7-3 in the intron of pituitary-specific PGSF1a which may be responsible) (Landgraf, Rusu et al. 2007) and the hypothalamus (Farh, Grimson et al. 2005). The complete role of miR-7 in the brain is yet to be fully elucidated, however, studies suggest it has roles in brain and neuronal cell development (Chen, Shalom-Feuerstein et al. 2010, Pollock, Bian et al. 2014). Expression of miR-7 is also enriched in pancreatic islets (Bravo-Egana, Rosero et al. 2008, Correa-Medina, Bravo-Egana et al. 2009) with studies suggesting miR-7 may have a key role in pancreatic beta cell development and maturation and accordingly it is postulated to be a therapeutic target in diabetes (Wang, Liu et al. 2013, Latreille, Hausser et al. 2014).
The low level of miR-7 expression in non-neuronal tissues, despite the widespread expression of the miR-7-1 host gene hnRNPK, is thought to be governed at the level of processing rather than transcription (Choudhury, de Lima Alves et al. 2013). Expression of intronic miR-7-1 stems from its own promoter regions (Reddy, Ohshiro et al. 2008, Chou, Lin et al. 2010).
Figure 3. Sequence and structure of the three human pre-miRNA-7 molecules (pre-miR-7-1, pre-miR-7-2 and pre-miR-7-3).

The pre-miRNAs are derived from corresponding pri-miRNA-7 molecules (pri-miR-7-1, pri-miR-7-2 and pri-miR-7-3) which are transcribed from three different genomic locations and processed into hairpin pre-miRs (shown above). The pre-miRs are further processed into mature miR-7 molecules indicated in red. One strand is then loaded into the RISC complex for sequence-specific translational repression or mRNA decay of target mRNA transcripts and this loaded strand has the same sequence for all three pre-miR-7 molecules and is the upper half of the red sequence in all three diagrams. This figure has been reprinted with permission from (Kalinowski, Brown et al. 2014).
1.5.2. MicroRNA-7 suppresses cancer growth and proliferation

miR-7 overexpression has been shown to inhibit proliferation in lung cancer (Lei, Chen et al. 2017), pancreatic cancer (Gu, Jiang et al. 2017) liver cancer (Wang, Wang et al. 2017), tongue squamous cell carcinoma (Jiang, Liu et al. 2010), breast, brain and prostate cancer (Webster, Giles et al. 2009), glioblastoma (Kefas, Godlewski et al. 2008), head and neck cancer (Kalinowski, Giles et al. 2012), CRC (Suto, Yokobori et al. 2015) and melanoma (Giles, Brown et al. 2016). Of note, miR-7 directly regulates the expression of EGFR and insulin-like growth factor receptor 1 (IGFR1) via target sites in the 3'UTR (Webster, Giles et al. 2009) to suppress cancer cell growth and proliferation.

EGFR is a member of the ErbB family of receptor tyrosine kinases and is also known as ErbB1 (Yarden and Sliwkowski 2001). EGFR plays a key role in the development and progression of several major human cancers and is frequently overexpressed in colon, head and neck, lung, liver, prostate and pancreatic cancers (among others), and correlates with a poorer clinical outcome (Chung, Ely et al. 2006, Buckley, Burgart et al. 2008, Bethune, Bethune et al. 2010, de Muga, Hernandez et al. 2010, Oliveira-Cunha, Newman et al. 2011, Canueto, Cardenoso et al. 2017).

When EGFR is bound by a ligand it initiates downstream signalling cascades including PI3K/Akt (Vivanco and Sawyers 2002) and MAPK pathways (Alroy and Yarden 1997, Mendelsohn and Baselga 2003). These signals regulate cellular processes commonly altered in tumourigenesis such as cell growth, proliferation, migration, differentiation and apoptosis (Yarden and Sliwkowski 2001, Vivanco and Sawyers 2002, Wee and Wang 2017). EGFR has, therefore, become a major therapeutic target in cancer. Many current therapeutics aim to inhibit EGFR action including monoclonal antibodies, for example, cetuximab which competes with EGFR ligands to bind and block the extracellular portion of the receptor (Vincenzi, Zoccoli et al. 2010), and tyrosine kinase inhibitors such as erlotinib which inhibits the intracellular ATP binding site required to initiate signal transduction (Raymond, Faiivre et al. 2000). Patients initially benefit from these EGFR-targeted therapies; however, patients commonly develop resistance to EGFR monoclonal antibodies and tyrosine kinase inhibitors resulting in tumour progression (Pao, Miller et al. 2005, Chong and Janne 2013, Huang and Fu 2015).
Webster et al. showed delivery of miR-7 to lung, breast, brain and prostate cancer cell lines in vitro leads to a decrease in EGFR protein expression and a reduction in cell survival. They showed that miR-7 directly interacts with the EGFR 3′UTR mRNA and is a potent inhibitor of EGFR mRNA and protein expression. In the same study, miR-7 was also shown to regulate the activity of other oncogenic signalling molecules downstream of EGFR, including protein kinase B (Akt) and ERK1/2 (Webster, Giles et al. 2009). These findings are supported elsewhere in glioblastoma (Kefas, Godlewski et al. 2008), head and neck cancer (Kalinowski, Giles et al. 2012), CRC (Suto, Yokobori et al. 2015), and HCC (Fang, Xue et al. 2012). Further, phospho-Akt (pAkt) downregulation was found to be independent of EGFR downregulation (Kefas, Godlewski et al. 2008). IGFR1, which also activates Akt signalling, has been shown to be a direct target of miR-7 and IGFR1 attenuation by miR-7, results in reduced proliferation, cell-cycle arrest and enhanced apoptosis in tongue squamous cancer cells (Jiang, Liu et al. 2010).

1.5.3. MicroRNA-7 increases the sensitivity of resistant cancers to therapeutics

miR-7 has been shown to increase the sensitivity of resistant cancer cell lines to current therapeutic drugs. miR-7 expression restores erlotinib (a targeted tyrosine kinase inhibitor of EGFR signalling) sensitivity to resistant head and neck cancer and in addition, acts synergistically with erlotinib to reduce cancer cell proliferation (Kalinowski, Giles et al. 2012). Another study showed miR-7 overexpression increased sensitivity to cetuximab (an EGFR monoclonal antibody) in HCT116 and SW480 cetuximab-resistant CRC cells (Suto, Yokobori et al. 2015). Ectopic expression of miR-7 further re-established vemurafenib sensitivity of vemurafenib-resistant melanoma cell lines through targeting EGFR and inhibiting downstream signalling pathways (Sun, Li et al. 2016). In addition, overexpression of miR-7 is also associated with increased radio-sensitivity in multiple cancers through inhibition of EGFR signalling (Lee, Choi et al. 2011). Further, in a cisplatin-resistant (cisplatin is a chemotherapeutic drug that causes DNA damage and induces apoptosis) breast cancer subline, ectopic miR-7 expression resulted in increased cisplatin sensitivity via targeting MRP1, a protein responsible for pumping cisplatin out of the cell (Pogribny, Filkowski et al. 2010). Similarly, miR-7 has been found to target MRP1 in small cell lung cancer mediating drug sensitivity (Liu, Wu et al. 2015). miR-7 has
further been attributed to mediating cisplatin sensitivity in gastric cancer through the downregulation of mTOR (Xu, Lian et al. 2017), and in lung adenocarcinoma partly through targeting BCL-2 (Cheng, Shen et al. 2017). A recent study has also linked epigenetic regulation at an upstream CpG island of the miR-7-3 gene with cisplatin resistance in various cancer types (Feng, Wang et al. 2017). Additionally, miR-7 was found to increase the sensitivity of non-small cell lung cancer (NSCLC) to paclitaxel by promoting paclitaxel-induced apoptosis (Liu, Liu et al. 2014), sensitise chronic myeloid leukaemia cells to imatinib (Jiang, Dai et al. 2017), and increase the sensitivity of sorafenib-resistant HCC to sorafenib in a synergistic manner (Kabir, Ganda et al. 2018).

1.5.4. MicroRNA-7 as an anti-metastatic agent

Evidence suggests that miR-7 also plays a role in inhibiting metastasis. Reduced miR-7 expression is often seen in metastatic cancers when compared to their primary line (Fang, Xue et al. 2012, Zhao, Dou et al. 2013). It has been shown that ectopic miR-7 expression inhibits migration and invasion in metastatic melanoma via targeting IRS2, which is known to mediate cell migration in many cancers (Mardilovich, Pankratz et al. 2009, Giles, Brown et al. 2013) and further by targeting the NF-κB subunit, RELA (Giles, Brown et al. 2016). In another study, Fang et al. demonstrated that miR-7 inhibits HCC growth and metastasis and showed miR-7 was able to regulate the PI3K/Atk pathway (Fang, Xue et al. 2012). Further, miR-7 regulation of PAK1 negatively affects invasiveness and anchorage-dependent growth of highly invasive breast cancer (Reddy, Ohshiro et al. 2008) and reduces invasion and migration of sorafenib-resistant HCC (Kabir, Ganda et al. 2018). miR-7 also suppresses brain metastasis of breast cancer via KLF4 inhibition in cancer-like stem cells (Okuda, Xing et al. 2013), metastasis in cervical cancer through FAK inhibition (Hao, Yang et al. 2015), and metastasis in gastric cancer via IGFR1 inhibition (Zhao, Dou et al. 2013). Additionally miR-7 suppresses invasion, migration and epithelial-mesenchymal transition via targeting ILF2 in pancreatic cancer (Bi, Shen et al. 2017).

In summary, miR-7 functions as a tumour suppressor by orchestrating concurrent inhibition of multiple key oncogenic targets in multiple pathways. Due to this synchronous multi-level and multi-target inhibition of key signalling pathways, as a
therapeutic miR-7 may be more effective than many of the current targeted treatments that have been developed to target single molecules in oncogenic cascades.

1.6. **MicroRNA-7: An oncomiR?**

Whilst miR-7 expression has frequently been reported to be downregulated in several malignancies (Webster, Giles et al. 2009, Rai, Takigawa et al. 2011, Fang, Xue et al. 2012, Giles, Brown et al. 2013, Zhang, Li et al. 2013, Zhao, Dou et al. 2013, Kalinowski, Brown et al. 2014, Xiong, Zheng et al. 2014, Zhang, Cai et al. 2014), there are a small number of reports in which miR-7 expression is increased and is associated with tumour aggressiveness and poor prognosis, such as oestrogen receptor positive/lymph node-negative breast cancer (Foekens, Sieuwerts et al. 2008), urothelial carcinoma (Veerla, Lindgren et al. 2009), cervical cancer (Zeng, Wang et al. 2018) and human papillomavirus (HPV) positive cervical cancer patients (Rao, Shen et al. 2012). Additionally, viral oncogene E6/E7 expression in the HPV-positive HeLa cell line was associated with upregulated miR-7 (Honegger, Schilling et al. 2015).

In CRC, miR-7 was found to be upregulated in advanced cancers and in selected cell lines (SW480, DLD-1, and COLO201) compared to normal mucosa. In addition, transfection with anti-miR-7 was shown to suppress cell growth in DLD-1 and COLO201 (Nakagawa, Akao et al. 2015). miR-7 is also reported to be increased in the stool of advanced CRC patients (stage II and onwards), a possible diagnostic screening method for CRC (Ahmed, Ahmed et al. 2013). In contrast to these examples, many reports suggest a tumour suppressive role for miR-7 in CRC. Zhang et al. reported miR-7 to be downregulated in CRC tumours and in six out of seven CRC cell lines when compared to normal colon tissue (including SW480 and DLD-1) (Zhang, Li et al. 2013). In addition, Suto et al. reported low miR-7 expression was associated with poor prognosis in CRC and showed miR-7 could inhibit proliferation in SW480 cells (Suto, Yokobori et al. 2015). Zhang et al. found miR-7 overexpression resulted in reduced proliferation and induced G1 phase arrest and apoptosis via targeting YY1 in CRC (Zhang, Li et al. 2013) and finally, Xu et al. showed miR-7 targets the protein XRCC2 to inhibit proliferation and induce apoptosis (Xu, Chen et al. 2014).
In lung cancer, there is a vast amount of evidence supporting miR-7 as a tumour suppressor however, a few reports have emerged suggesting miR-7 could be an oncogene. Chou et al. reported miR-7 to be induced via EGFR/Ras/ERK/c-Myc signalling and subsequently promote cell proliferation and tumour formation. However, miR-7 overexpression was also shown to attenuate EGFR expression in lung adenocarcinoma CLI-5 cells, suggesting the existence of an EGFR/miR-7 regulatory loop (Chou, Lin et al. 2010). Studies carried out in the epithelial NSCLC cell line A549 have demonstrated varied roles for miR-7. The findings of Chou et al. are supported by an earlier study which found that inhibiting miR-7 inhibited A549 cell growth (Cheng, Byrom et al. 2005). Meza-Sosa et al. showed that miR-7 induced proliferation and migration in A549 cells stably overexpressing miR-7, and suggested miR-7 may act as an oncomiR in an epithelial context. To strengthen this argument, they further showed naturally immortalised skin cells HaCaT also exhibited enhanced proliferation upon stable miR-7 overexpression. This was found to be due to direct downregulation of KLF4 by miR-7, a transcription factor which mediates diverse cellular processes including proliferation (Meza-Sosa, Perez-Garcia et al. 2014). In contrast, Rai et al. overexpressed miR-7 episomally and reported no significant growth inhibition in A549 cells, but showed suppressed growth in EGFR-addicted cell lines such as the NSCLC cell lines PC-9, H3255 and H1975. They further observed much higher miR-7 levels in EGFR-addicted cells compared to non-addicted cells, suggesting EGFR-mediated activation of miR-7 consistent with the findings of Chou et al. (Rai, Takigawa et al. 2011). In work by Xiong et al., transient miR-7 overexpression inhibited migration, proliferation and induced apoptosis in A549 cells by targeting the anti-apoptotic molecule BCL-2 (Xiong, Zheng et al. 2011). We have found miR-7 to inhibit EGFR expression and signalling in A549 cells, consistent with having a tumour suppressive effect (Webster, Giles et al. 2009). In summary, clearly the role of miR-7 in lung cancer is more complex than initially envisaged, and may be particularly cell type-specific and possibly dependent on the method of influencing miR-7 expression experimentally.
1.6.1. Genetic influence on the role of microRNA-7

The regulatory capacity of miR-7 is complex, given the numerous targets reported across many cell types. KLF4, a known target of miR-7 (Okuda, Xing et al. 2013, Meza-Sosa, Perez-Garcia et al. 2014, Wu, Liu et al. 2017), elicits context-dependent oncogenic and tumour suppressive responses (Rowland and Peeper 2006) and indeed, oncogenesis has been reported as a result of KLF4 suppression by miR-7 (Meza-Sosa, Perez-Garcia et al. 2014), as well as the opposite (Okuda, Xing et al. 2013, Wu, Liu et al. 2017). Similarly, with respect to the mutational profile of the cell, STAT3, an indirect target of miR-7, can either promote or suppress tumourigenesis depending on biochemical and genetic factors (De la Iglesia, Konopka et al. 2008, Zhang and Lai 2014). Hence, the role/s of miR-7 may be adversely affected by the cell’s mutational background. Rai et al. suggest that the level of EGFR-addiction will play an important role in the effect of miR-7 (Rai, Takigawa et al. 2011). Also, as observed in the studies conducted in A549 cells (Cheng, Byrom et al. 2005, Webster, Giles et al. 2009, Rai, Takigawa et al. 2011, Xiong, Zheng et al. 2011, Meza-Sosa, Perez-Garcia et al. 2014) the experimental approach could be responsible for conflicting observations whereby miR-7 is over or under-expressed, the degree of miR-7 overexpression within the cell or whether miR-7 overexpression is sustained (Kalinowski, Brown et al. 2014).

Given miR-7 participates in feedback and feedforward loops, as well as regulates several transcription factors, changes in miR-7 expression may result in a ripple effect, that is, the indirect regulation of the expression of other genes and even miRNAs. To emphasise this point, a study investigating miR-7 transient overexpression in ovarian cancer cells reported a change in the expression of hundreds of genes in diverse pathways, however, only ~20% of the regulated genes were predicted to be direct targets, concluding that the majority of the observed changes to gene expression are an indirect consequence of miR-7 expression and effect (Shahab, Matyunina et al. 2012).

1.7. MicroRNA-7 as a biomarker

Given the widespread dysregulation or miRNAs, it has been suggested that expression profiles of a single miRNA or set of miRNAs may be used as potential prognostic, predictive and diagnostic biomarkers (Lan, Lu et al. 2015). miR-7 is a potential biomarker...
for a number of cancer types. For example, Wang et al. identified miR-7 as one of three miRNAs (along with miR-93 and miR-409-3p) from an array of 723 human miRNAs, which were found to be powerful predictors of CRC. This panel of miRNAs could be used to distinguish early-stage CRC (nonmetastatic) and late-stage CRC (metastatic) patients from healthy patients with great accuracy (Wang, Xiang et al. 2015). Kitano et al. found miR-7 to be a useful biomarker for distinguishing between benign and malignant thyroid tumours, specifically in those cases where a diagnosis is difficult to ascertain from fine-needle aspiration biopsies. The model was highly sensitive with a negative prediction value of 100% (Kitano, Rahbari et al. 2012). Santos et al. identified miR-7 levels in peripheral whole blood as a useful prognostic biomarker for castrate-resistant prostate cancer development. Higher miR-7 levels in peripheral whole-blood, in combination with high-Gleason score tumours were correlated with significantly earlier progression to castrate-resistance and further trended toward lower overall survival of patients (Santos, Teixeira et al. 2014). Low serum miR-7 levels have also been suggested as a potential diagnostic biomarker in HCC (Tarek, Louka et al. 2017) and in oesophageal squamous cell carcinoma (Hara, Miyazaki et al. 2017).

Further, miR-7 has been described as a potential biomarker for predicting treatment response to neoadjuvant chemotherapy in breast cancer (Raychaudhuri, Bronger et al. 2017) and in oesophageal adenocarcinoma (Matsui, Zaidi et al. 2016) which could assist in therapeutic management of these cancers.

1.8. Transcriptional and post-transcriptional regulation

The regulation of mature miR-7 expression occurs at the transcriptional level as well as at various stages throughout the miRNA maturation process, and there are many examples.

At the transcriptional level, miR-7 expression has been shown to be promoted by EGFR signalling in lung cancer via Ras/ERK/c-Myc and PI3K/Akt pathways (Chou, Lin et al. 2010). Whilst the exact mechanism of miR-7 stimulation via the PI3K/Akt pathway is yet to be identified, the transcription factor c-Myc was found to directly bind and stimulate expression from the miR-7-1 promoter (Chou, Lin et al. 2010). This finding is supported
by an earlier study which also found miR-7 was upregulated as a result of c-Myc expression in lymphoma (Chang, Yu et al. 2008). Other transcription factors have similarly been involved in promoting miR-7 expression via directly interacting with the promoter regions of miR-7 genes including homeobox D10 (HOXD10) via the miR-7-1 promoter region in breast cancer (Reddy, Ohshiro et al. 2008) and HNF4α via the miR-7-2 promoter in HCC. HNF4α was identified as part of a feedback loop also involving miR-124, miR-21 and NF-κB (Ning, Ding et al. 2014). The transcription factor FOXP3 which also positively regulates miR-7 expression in breast cancer (McInnes, Sadlon et al. 2012) has been found to have potential binding regions in the locality of miR-7-1 and miR-7-2 genes (Sadlon, Wilkinson et al. 2010). miR-7 expression is further promoted by HBx in hepatitis B virus-associated HCC. The transduction of signals between HBx and miR-7 activation is postulated to involve nuclear IKKα and IKK/NF-κB signalling pathways, however, this relationship is yet to be elucidated (Chen, Chien et al. 2013). Further, HGF has been found to induce miR-7 expression and subsequently downregulate miR-7 targets EGFR, FAK, KLF4 and PAK1 in MCF-10A normal breast cells (Jeong, Ham et al. 2017). A recent study in gastric cancer found miR-7 to be involved in a negative feedback loop with IKKε and RELA. miR-7 targets and inhibits IKKε and RELA expression, and IKKε and RELA were found to suppress pri-miR-7 expression. Direct binding of RELA to both miR-7-1 and miR-7-2 promoter regions was confirmed (Zhao, Lu et al. 2015). Further, Usp18 negatively regulates miR-7 expression. Knockdown of Usp18 was found to increase expression of miR-7 host genes and pri-miR-7-2 from an intergenic region and subsequently increase expression of mature miR-7 (Duex, Comeau et al. 2011). miR-7 expression is further negatively regulated by the oncogenic long non-coding RNA, HOTAIR, which indirectly inhibits miR-7 expression via HOXD10 suppression. Downregulated HOTAIR showed an anti-correlative relationship with both HOXD10 and miR-7 in MDA-MB-231 breast cancer cells, and miR-7 was inversely correlated with HOTAIR expression in breast cancer patients (Zhang, Cai et al. 2014). More recent work has identified miR-7 downregulation in lung cancer patients due to mutations in the miR-7-2 promoter region and the presence of these mutations was associated with poor prognosis (Zhao, Wang et al. 2015). Transcriptional regulators that bind to miR-7 promoter regions are shown in Figure 4.
Post-transcriptional regulation of miR-7 is promoted by SRSF1, also known as SF2/ASF, in a splicing-independent fashion. SRSF1 promotes maturation of many miRNAs including miR-7 via enhancing Drosha cleavage of the primary transcript. miR-7 in turn targets and inhibits translation of SRSF1 via its 3’UTR, completing a negative feedback loop (Wu, Sun et al. 2010). Conversely, the RNA binding protein HuR negatively affects miR-7 maturation and HuR knockdown is negatively correlated to the specific and substantial upregulation of miR-7 (Lebedeva, Jens et al. 2011). Li et al. similarly found miR-7 expression to be impeded by TLR9-induced HuR upregulation in lung cancer cells (Li, Wang et al. 2013). Furthermore, MSI2 was found to bind to the terminal loop of the pri-miR-7 transcript in a HuR-dependent manner in non-neural cells resulting in failure of the pri-miR-7-1 transcript to mature (Choudhury, de Lima Alves et al. 2013). QKI-5 and QKI-6, have also been implicated in the failure of miR-7-1 to be processed into mature miR-7 and exported to the cytoplasm in glioblastoma. QKI binding sites were found in pri-miR-7-1 and pri-miR-7-2 but not pri-miR-7-3. QKI-5 and QKI-6 are speculated to increase association of miR-7-1 with Drosha (Wang, Vogel et al. 2013).

A circular RNA sponge for miR-7 termed ‘ciRS-7’ (also referred to as CDR1NAT, CDR1-AS and CDR1as) has been recently identified (Hansen, Wiklund et al. 2011, Hansen, Jensen et al. 2013). CiRS-7 is derived from the antisense transcript of the coding CDR1 gene (Hansen, Wiklund et al. 2011) and is highly and stably expressed in human and mouse brain (Hansen, Wiklund et al. 2011). CiRS-7 is suggested to act as a competing endogenous RNA or miRNA ‘sponge’ in neuronal tissues and contains >70 seed-matched miR-7 binding sites. The pattern of ciRS-7 expression in the mouse brain closely aligns with that of miR-7, especially in the hippocampus and neocortex (Hansen, Jensen et al. 2013), and in the developing brain of mouse embryos (Memczak, Jens et al. 2013). ciRS-7 is able to considerably attenuate miR-7 activity and thereby reduce repression of miR-7 targets (Hansen, Jensen et al. 2013). It is suggested that ciRS-7 may act as a buffer of miR-7 activity by competing with miR-7 targets, thereby reducing the availability of miR-7 for low-affinity target mRNAs. To add an additional level of regulation, miR-671 has been shown to cause RISC-induced endonucleolytic ciRS-7 degradation via near-perfect complementarity (Hansen, Wiklund et al. 2011). It is speculated that upon ciRS-7 degradation, sequestered miR-7 is released. Therefore, miR-671 could possibly be considered a positive regulator of miR-7 either by release of ciRS-7 bound miR-7 or by
reducing the number of available ciRS-7 molecules for miR-7 sequestration (Hansen, Kjems et al. 2013). There is recent evidence to suggest ciRS-7 acts as an oncogene in HCC through targeting miR-7 and is upregulated in HCC compared to non-tumour tissue (Yu, Gong et al. 2016) and additionally, overexpression of ciRS-7 results in miR-7 downregulation, increased EGFR expression and proliferation in HCC (Yang, Xiong et al. 2017). However, another study found no difference in ciRS-7 expression between cancerous and matched non-tumour tissue in 108 HCC patients but did show that higher ciRS-7 expression was associated with hepatic microvascular invasion in HCC tissue (Xu, Zhang et al. 2017). Similarly, ciRS-7 is upregulated and associated with poor prognosis in CRC (Weng, Wei et al. 2017) and gastric cancer (Pan, Li et al. 2018). A summary of molecules involved in miR-7 regulation can be found in Table 1.
Proteins shown in grey positively regulate miR-7 expression while those shown in orange negatively regulate miR-7 expression. The transcription factors HOXD10 and c-Myc bind to and stimulate expression from the miR-7-1 promoter. HOXD10 may bind to two binding motifs −1019 to −1028 bp and −958 to −968 bp upstream of the miR-7-1 transcription initiation site (Reddy, Ohshiro et al. 2008). C-Myc has been found to bind to an E-box motif at positions −534 to −539 bp upstream of miR-7-1 (Chou, Lin et al. 2010). HNF4α similarly binds (the exact location is not described) and stimulates expression from the miR-7-2 promoter region (Ning, Ding et al. 2014). RELA binds to three predicted NF-κB binding sites at −459 and −1391 bp in the miR-7-1 and −719 bp miR-7-2 promoters (Zhao, Lu et al. 2015). Proteins which may bind and stimulate or inhibit expression from a miR-7-3 promoter are currently unknown. This figure was generated as a part of the publication (Horsham, Kalinowski et al. 2015).
Table 1. Summary of miR-7 regulatory molecules and their effect on miR-7 expression in cancer cell lines.
The table was generated as a part of the publication (Horsham, Kalinowski et al. 2015).

<table>
<thead>
<tr>
<th>Regulatory Molecule or Pathway</th>
<th>miR-7 up- (↑)/down- (↓) Regulation</th>
<th>Action</th>
<th>Direct/Indirect Interaction</th>
<th>Cancer Type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR signalling</td>
<td>↑</td>
<td>Via Ras/ERK/c-Myc and additionally by PI3K/Akt pathways</td>
<td>Indirect</td>
<td>Lung</td>
<td>(Chou, Lin et al. 2010)</td>
</tr>
<tr>
<td>c-Myc</td>
<td>↑</td>
<td>Binds and stimulates expression from the MIR7-1 promoter</td>
<td>Direct</td>
<td>Lung</td>
<td>(Chou, Lin et al. 2010)</td>
</tr>
<tr>
<td>HOXD10</td>
<td>↑</td>
<td>Binds and stimulates expression from the MIR7-1 promoter</td>
<td>Direct</td>
<td>Breast</td>
<td>(Reddy, Ohshiro et al. 2008)</td>
</tr>
<tr>
<td>HNF4a</td>
<td>↑</td>
<td>Interacts with MIR7-2 promoter</td>
<td>Direct</td>
<td>Liver</td>
<td>(Ning, Ding et al. 2014)</td>
</tr>
<tr>
<td>FOXP3</td>
<td>↑</td>
<td>Predicted binding regions in proximity to MIR7-1 and MIR7-2 loci</td>
<td>Not confirmed</td>
<td>Breast</td>
<td>(McInnes, Sadlon et al. 2012)</td>
</tr>
<tr>
<td>HBx</td>
<td>↑</td>
<td>Postulated to involve IKKα and IKK/NF-κB signalling</td>
<td>Indirect</td>
<td>Liver</td>
<td>(Chen, Chien et al. 2013)</td>
</tr>
<tr>
<td>HGF</td>
<td>↑</td>
<td>Mechanism not identified</td>
<td>Not confirmed</td>
<td>Breast (normal)</td>
<td>(Jeong, Ham et al. 2017)</td>
</tr>
<tr>
<td>RELA</td>
<td>↓</td>
<td>Binds to MIR7-1 and MIR7-2 promoter regions</td>
<td>Direct</td>
<td>Gastric</td>
<td>(Zhao, Lu et al. 2015)</td>
</tr>
<tr>
<td>Usp18</td>
<td>↓</td>
<td>Mechanism not identified</td>
<td>Not confirmed</td>
<td>Cervical, Head and neck, Brain</td>
<td>(Duex, Comeau et al. 2011)</td>
</tr>
<tr>
<td>HOTAIR</td>
<td>↓</td>
<td>Via inhibiting HOXD10</td>
<td>Indirect</td>
<td>Breast</td>
<td>(Zhang, Cai et al. 2014)</td>
</tr>
<tr>
<td>SRSF1</td>
<td>↑</td>
<td>Binds to pri-miR-7 and promotes maturation via enhancing Drosha cleavage</td>
<td>Direct</td>
<td>Cervical</td>
<td>(Wu, Sun et al. 2010)</td>
</tr>
<tr>
<td>HuR</td>
<td>↓</td>
<td>Hypothesised to repress miR-7-1 processing which may involve HuR binding in the intron of hnRNPK which hosts the MIR7-1 gene</td>
<td>Not confirmed</td>
<td>Cervical, Lung</td>
<td>(Lebedeva, Jens et al. 2011), (Li, Wang et al. 2013)</td>
</tr>
<tr>
<td><strong>TLR9 signalling</strong></td>
<td>↓</td>
<td>Via HuR upregulation which is suggested to involve the PI3K/Akt pathway</td>
<td>Indirect</td>
<td>Lung</td>
<td>(Li, Wang et al. 2013)</td>
</tr>
<tr>
<td>---------------------</td>
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<td>-------------------------------------------------</td>
<td>---------</td>
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<td>----------------------</td>
</tr>
<tr>
<td><strong>MSI2</strong></td>
<td>↓</td>
<td>Binds to the terminal loop of the pri-miR-7 transcript in an HuR-dependent manner resulting in failure of the pri-miR-7-1 transcript to mature</td>
<td>Direct</td>
<td>Cervical, Brain</td>
<td>(Choudhury, de Lima Alves et al. 2013)</td>
</tr>
<tr>
<td><strong>QKI 5 and QKI 6</strong></td>
<td>↓</td>
<td>Bind to QKI response elements in pri-miR-7-1 resulting in processing failure (binding sites also identified in pri-miR-7-2)</td>
<td>Direct</td>
<td>Brain</td>
<td>(Wang, Vogel et al. 2013)</td>
</tr>
<tr>
<td><strong>ciRS-7</strong></td>
<td>↓</td>
<td>Contains &gt;70 seed-matched miR-7 binding sites that can sequester miR-7</td>
<td>Direct</td>
<td>Proof of concept demonstrated in HeLa and HEK293 cells</td>
<td>(Hansen, Jensen et al. 2013), (Memczak, Jens et al. 2013)</td>
</tr>
</tbody>
</table>
1.9. MicroRNA-7 in development and other diseases

miR-7 is highly and specifically expressed in the brain and endocrine pancreas, where it plays a significant role in the normal neuronal development and osmotic balance of the brain and normal endocrine cell differentiation, insulin synthesis and secretion in the pancreas (Farh, Grimson et al. 2005, Wienholds, Kloosterman et al. 2005, Ason, Darnell et al. 2006, Landgraf, Rusu et al. 2007, Tessmar-Raible, Raible et al. 2007, Bak, Silahtaroglu et al. 2008, Bravo-Egana, Rosero et al. 2008, Correa-Medina, Bravo-Egana et al. 2009, Li, Xi et al. 2011). miR-7 expression and action has also been associated with other disease states in addition to cancer including; schizophrenia and Parkinson’s disease in the brain; dilated cardiomyopathy and cardiac infarction in the heart; diabetes in the pancreas; as well as impaired wound healing, scleroderma and dermatomyositis of the skin. Therefore, there is much scope for the findings presented here in to impact upon the research undertaken in these diseases. A more comprehensive review of miR-7 in development and these diseases was published by myself and colleagues in 2015 (Horsham, Ganda et al. 2015). However, as the focus of this thesis is cancer, they will not be discussed further here.

1.10. Thesis aims, hypotheses and objectives

Based on the literature presented above, the overriding aim of this research is to devise ways to increase the expression of miR-7 in cancer cells with clinical relevance. Two independent approaches will be undertaken. In the first part of this thesis I aim to evaluate novel polymeric nanoparticles as a means of delivering miR-7 in vitro (Part I). These novel nanoparticles were available via a collaboration with other investigators at the University of Western Australia (UWA). The second is to screen a library of ~800 compounds to identify new small molecule modifiers of miR-7 which will enable the upregulation of endogenous miR-7 (Part II). As mentioned previously, miR-7 has been shown to have potential in many different types of cancer. However, herein I will focus primarily on CRC and HCC.
1.10.1. PART I – Nanoparticle-mediated microRNA delivery

Hypotheses

1. Use of novel polymeric nanoparticles will increase delivery of miR-7 into CRC cells and the higher intracellular levels of miR-7 will result in reduced target protein expression.

Aims

1. Evaluate novel nanoparticles developed by the Iyer lab at UWA for their ability to deliver miR-7 to cancer cells in vitro.
2. Assess the effect of these nanoparticles compared to commercially available nanolipids through the expression of key miR-7 targets in CRC cells.

1.10.2. PART II – Identifying small molecule modifiers of microRNA-7 expression and activity

Hypotheses

1. Small molecule modifiers of miR-7 exist that can be identified in a cell-based reporter assay using CRC cells.
2. Identified compounds will be agonists of miR-7, and there will be a decrease in downstream signalling via miR-7 and inhibition of cancer cell growth in more than one tissue.

Aims

1. Develop a stable reporter system for use in screening whereby an increase in intracellular miR-7 activity can be detected.
2. Conduct a screen of 800 compounds (Natural Products Library, TimTec) to identify those that upregulate miR-7 expression or activity.
3. Evaluate some of the major miR-7 regulating compounds using cell-based assays in CRC and HCC to validate the compounds as miR-7 agonists.
1.11. Thesis organisation

This thesis is divided into two parts (nanoparticle delivery and small molecule screen). Each of these was commenced simultaneously. Part I addresses preliminary nanoparticle studies which, based on the data in chapter 3 was not further expanded, the screening approach then became the primary focus and occupies the remainder of the thesis (chapters 4, 5, and 6) (Part II). Chapter 7 concludes the thesis and contains a summary of significant findings from Parts I and II and discusses future directions.
2. Methods

This chapter describes materials and methods necessary for all experiments associated with the evaluation of nanoparticle delivery, generating the screen, conducting the screen and following up hits compounds.

2.1. Cell culture

Three CRC cell lines (HT29, HCT116 and SW620) and one HCC cell line (Huh7) are used throughout this thesis. HT29 cells are an epithelial adherent colorectal adenocarcinoma cell line from a 44-year-old Caucasian female. HCT116 cells are an epithelial adherent colorectal carcinoma from an adult male (ethnicity and age are not provided by the supplier), SW620 cells are an epithelial adherent colorectal adenocarcinoma derived from a metastatic site (lymph node) from a 51-year-old Caucasian male and were from the American Type Culture Collection (ATCC, www.atcc.org). Huh7 cells are an adherent epithelial hepatocellular carcinoma from a 57-year-old Japanese male (Nakabayashi, Taketa et al. 1982). HCT116, HT29 and SW620 cells were acquired from in-house liquid nitrogen stocks and were cultured in DMEM supplemented with 4.5 g/L glucose, 2 mM L-glutamine (In-house, Perkins Media Store) and 10% FBS (Gibco). Huh7 were also acquired from in-house liquid nitrogen stocks and were cultured in DMEM supplemented with 1 g/L glucose, 2 mM L-glutamine (In-house, Perkins Media Store) and 10% FBS (Gibco). All cultures were maintained at 37°C and 5% CO₂. Cells were authenticated by STR profiling (approximately 1 x 10⁶ cells were pelleted and sent to Cell Bank Australia). Cells were not routinely cultured in antibiotics.

2.1.1. Cell counting

Live cells were counted using 0.4% Trypan Blue stain (Thermo Fisher Scientific) and a Countess Automated Cell Counter (Invitrogen).
2.1.2. Mycoplasma testing

Cell lines were regularly tested for mycoplasma. Approximately $1 \times 10^6$ cells were pelleted and sent to PathWest (Nedlands, WA). All cell lines were negative for mycoplasma.

2.2. Assessing nanoparticle transfection

2.2.1. Time course of nanoparticle uptake

The uptake of nanoparticles was monitored over a 24 h period to assess the speed, extent of uptake and retention over time. Human CRC HT29 and HCT116 cells were seeded in a 12-well plate at $1 \times 10^5$ cells/mL in a total volume of 1 mL and left overnight at 37°C and 5% CO₂. The following day cells were transfected (section 2.3.2) in duplicate with 6 µL of polyglycidal methacrylate (PGMA)-polyethyleneimine (PEI) nanoparticles (2 mg/mL) (Iyer Lab, UWA) at minus 24 h, 8 h, 6 h, 2 h, and 30 min. Wells containing cells that had not been subjected to nanoparticles were used as a control. At time 0 (24 h after the first nanoparticle transfection) all wells were washed twice with PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄, to 1 L with distilled water, pH adjusted to 7.4) In-house, Perkins Media Store) and subsequently imaged on an Olympus ix71 fluorescent microscope and images taken using an Olympus DP71 camera to observe nanoparticle uptake.

2.2.2. Flow cytometry

Flow cytometry was used to determine the transfection efficiency (the percentage of cells that had nanoparticles present) and the relative number of nanoparticles that had been taken up (the range of fluorescence intensities from the cells). HCT116 cells were reverse transfected (section 2.3.2) with unloaded PGMA-PEI nanoparticles (Iyer Lab, UWA) and nanoparticles loaded with miR-7 (Pre-miR™ miRNA Precursor, Invitrogen, PM10047) in a 6 cm dish. A negative control of HCT116 cells only was included. To prepare nanoparticle-miR-7 complexes, nanoparticles and miRNA were combined in a ratio of 14:1 nanoparticles:miRNA and incubated at room temperature for 1 h, then added to 6 mL of cell suspension at $1 \times 10^5$ cells/mL (final concentration of miR-7 was 30
Methods

This concentration is the same concentration as has been used in Western blot analysis to observe miR-7 target inhibition. Cells were incubated for 24 h at 37°C and 5% CO₂. Following incubation, cells were trypsinised and washed three times with fluorescence-activated cell sorting (FACS) buffer (PBS (In-house, Perkins Media Store), 2% FBS (Gibco)) before being passed through a 40 μM Falcon cell strainer (Thermo Fisher Scientific) to remove clumps of cells. Cells were then presented for flow cytometry. Flow cytometry was conducted using a BD Accuri C6 flow cytometer (Becton Dickinson Biosciences, USA) (488 nm blue excitation laser and 580/40 filter) and with the assistance of Kevin Li. The cell suspension was then stained with DAPI (Invitrogen) to determine cell viability via flow cytometry according to the manufacturer’s instructions.

2.2.3. Fluorescence microscopy

DAPI (Invitrogen) (300 nM in PBS) was used to stain fixed cells for visualisation on a DeltaVision Elite Imaging System and Softworx software (GE Healthcare) microscope. DAPI staining and microscopy was conducted with the kind assistance of Rouhan Li according to the manufacturer’s instructions.

2.3. Nanoparticle/microRNA-7 delivery

2.3.1. Loading nanoparticles with microRNA

In order to conjugate PGMA-PEI nanoparticles (Iyer lab, UWA) with miR-7 (Pre-miR™ miRNA Precursor, Invitrogen, PM10047) or negative control (miR-NC) (Pre-miR™ miRNA Precursor, Invitrogen, AM17110) the nanoparticles were first sterilised under UV light for 20 min and then combined with the miRNA at a ratio of 14:1 nanoparticles:miRNA. Combined nanoparticles/miRNA were mixed well by pipetting and left to incubate at room temperature for 1 h.

2.3.2. Nanoparticle reverse transfection

PGMA-PEI nanoparticles (Iyer lab, UWA) conjugated with miRNA-7/miR-NC (Pre-miR™ miRNA Precursor, Invitrogen, PM10047 and AM17110) were reverse transfected into
Methods

cells in 6-well plates. Unconjugated nanoparticles were used as a negative control. Two millilitres of cell suspension (1 x 10^5 cells/mL for HT29 cells and 2-3 x10^5 cells/mL for HCT116 cells) were plated and nanoparticle/miRNA was added so that miR-7 was at a final concentration of 30 nM. Plates were incubated at 37°C and 5% CO₂.

2.3.3. Western blot of nanoparticle-delivered miR-7

To assess miR-7 delivery by PGMA-PEI nanoparticles (Iyer lab, UWA) a Western blot was performed to compare the extent of which miR-7 targets EGFR, Akt and pAkt were reduced compared with delivery using Lipofectamine 2000 (Invitrogen). Protein was harvested 72 h post-transfection, quantified and visualised by Western blot. Protocols were performed as outlined in 2.10.3 quantification and 2.10.4 for Western blot.

2.3.4. RT qPCR for nanoparticle-delivered miR-7

RT qPCR was performed to compare the relative levels of miR-7 delivery by Lipofectamine 2000 (Invitrogen) and PGMA-PEI nanoparticles (Iyer Lab, UWA). HCT116 cells were reverse transfected with nanoparticles as described in 2.3.2 and with Lipofectamine 2000 (Invitrogen) in a 6-well plate as described in section 2.10.1 for 24 h. The wells were washed thoroughly with media to remove residual untransfected miR-7. RNA was then extracted and RT qPCR carried out as described in 2.6 and 2.7.

2.4. Generating psiCHECK-2 microRNA-7 target vectors

The vector psiCHECK-2 (Promega) was used to generate five different plasmids. psiCHECK-2 (3mer), a plasmid with three perfect miR-7 target sites cloned downstream of the Renilla luciferase gene; psiCHECK-2 (6mer), containing six perfect miR-7 target sites cloned downstream of the Renilla luciferase gene; mutant versions of both plasmids containing three point mutations in each of the miR-7 targets (mutations are shown in Figure 29) and; the fifth vector generated was the psiCHECK-2 (6mer +NeoR), the psiCHECK-2 (6mer) vector with the addition of a neomycin resistance (NeoR) cassette. A plasmid list can be found in Table 4.
2.4.1. Oligonucleotide annealing

Oligonucleotides containing a concatemer of three consensus miR-7 target sites (termed ‘Cons 3mer F’ and ‘Cons 3mer R’) were obtained from GeneWorks and designed to incorporate 5’ XhoI and 3’ NotI overhangs for subsequent cloning into the psiCHECK-2 (Promega) vector as well as a Sall site downstream of the consensus target repeats. As Sall overhangs are compatible with XhoI overhangs, subsequent concatemerisation is possible. Corresponding oligonucleotides containing a mutant concatemer of three miR-7 target sites (termed ‘MT 3mer F’ and ‘MT 3mer R’) with three point mutations in each seed sequence were also obtained (GeneWorks). The three point mutations are in line with what has been previously described to inhibit miR-7 binding (Webster, Giles et al. 2009). The mutant concatemer is important for validating direct binding of miR-7 to the target site. Lyophilised single-stranded oligonucleotides were resuspended in nuclease-free water and mixed in equimolar amounts. The reaction was heated to 95°C in a heat block for 10 min and then slowly cooled in the block to room temperature. All oligonucleotide sequences may be found in Table 2.

2.4.2. Polymerase chain reaction (PCR)

All primer sequences may be found in Table 2.

The neomycin resistance cassette (SV40 promoter, Neomycin (Neo)/Kanamycin (Kan) resistance gene, SV40 poly A) was amplified from the pcDNA3-HOXD10 vector (A gift from Corey Largman (Addgene plasmid #21007)). Primers (pcDNA3 Neo cassette F and pcDNA3 Neo cassette R) were designed to incorporate BamHI sites into each end of the product for subsequent cloning into the psiCHECK-2 (6mer) vector.

The PCR reaction was carried out using Phusion High-Fidelity DNA polymerase (New England Biolabs) and run in an Eppendorf Mastercycler Nexus Thermal Cycler. The reaction was performed in 20 µL volume containing: 2 ng plasmid vector template, 1 µL of each 10 µM forward and reverse primers (GeneWorks), 4 µL of 5 x Phusion HF buffer (NEB), 0.4 µL of 10 mM dNTP mix (NEB), 0.2 µL of 2 units/µL Phusion DNA polymerase (NEB), made up to 20 µL with nuclease-free water. The expected size may be found in
**Methods**

**Table 3.** Cycle conditions for pcDNA3 Neo cassette were as follows: 30 s at 98°C; 5 s at 98°C, 30 s at 55°C, 25 s at 72°C for 10 cycles (this lower annealing temperature was used initially for 10 cycles to allow annealing of primer which introduced the restriction site); then 5 s at 98°C, 30 s at 65°C, 25 s at 72°C for 25 cycles and then 10 min at 72 °C.

PCR products were purified with High PCR Cleanup Micro Kit (Roche).
### Methods

**Table 2. Primers and oligonucleotides.**
All primers and oligonucleotides were ordered from GeneWorks.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence 5’ – 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>psiCHECK-2 seq F</td>
<td>TTCCCTAACACCGAGTTCGT</td>
</tr>
<tr>
<td>psiCHECK-2 seq R</td>
<td>CAAACCCTAACACCGCTTA</td>
</tr>
<tr>
<td>pcDNA3 Neo cassette F</td>
<td>TTATGGATCCTCGGCCTATTGGTTAAAAA</td>
</tr>
<tr>
<td>pcDNA3 Neo cassette R</td>
<td>TATAGGATCCAGCTATGACCATGATTACG</td>
</tr>
<tr>
<td><strong>Oligonucleotides</strong></td>
<td></td>
</tr>
<tr>
<td>Cons 3mer F</td>
<td>TCGAGACAACAAATCACTAGTCTTCCACGATACAACAAAAAT</td>
</tr>
<tr>
<td></td>
<td>CACTAGTCTTCATCACACAAACAAATCACTAGTCTTCCAGTC</td>
</tr>
<tr>
<td></td>
<td>GACCGATTCACG</td>
</tr>
<tr>
<td>Cons 3mer R</td>
<td>GCGTGAATCGGTCGACTGGAAGACTGTATTTTGTGTTGTTG</td>
</tr>
<tr>
<td></td>
<td>ATGGAAGACTAGTATTTTGTATCGTGGAAGACTAGTGA</td>
</tr>
<tr>
<td></td>
<td>TTTTGTGTCTCGA</td>
</tr>
<tr>
<td>MT 3mer F</td>
<td>TCGAGACAACAAATCACTAGCCCTTCACGATACAACAAAAAT</td>
</tr>
<tr>
<td></td>
<td>CACTAGCCCTTCATCACACAAACAAATCACAGGCTTCCAGTC</td>
</tr>
<tr>
<td>MT 3mer R</td>
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</tr>
<tr>
<td></td>
<td>GGCGCCTGAATCCTGCTCAGTAAGGGCTAGTGTATTGTG</td>
</tr>
<tr>
<td></td>
<td>GGTATGAAGGGCTAGTATTTTGTGTTATCGTGAAAGGCTG</td>
</tr>
<tr>
<td></td>
<td>AGTATTATTGTGTC</td>
</tr>
</tbody>
</table>
2.4.3. Restriction digest

Restriction digest reactions were carried out as double digests in a total volume of 30 µL and contained a final concentration of 1-2 µg of DNA, 1 µL of each of the appropriate restriction enzymes (10 u/µL) (outlined below for specific vectors), 3 µL of appropriate Buffer (10 x) (outlined below for specific vectors), 0.3 µL BSA (1 ug/µL) (Promega), to 30 µL with nuclease-free water. The reactions were incubated at 37°C in a heat block for 2-3 h.

The restriction digest reactions were purified using QIAquick Gel Extraction Kit (Qiagen).

2.4.3.1. Digest of psiCHECK-2 to generate psiCHECK-2 (3mer)

XhoI/NotI (Promega) double restriction digests of psiCHECK-2 vector (Promega) and 3mer oligos (Consensus and MT) (GeneWorks) were carried out in Buffer D (Promega) for the purpose of generating the psiCHECK-2 (3mer) and mutant vectors.

2.4.3.2. Digest of psiCHECK-2 (3mer) to generate psiCHECK-2 (6mer)

SalI/NotI (Promega) double restriction digests of psiCHECK-2 (3mer) vector and 3mer oligos (Cons and MT) (GeneWorks) were carried out in Buffer D (Promega) for the purpose of generating the psiCHECK-2 (6mer) and mutant vectors.

2.4.3.3. Digest of psiCHECK-2 (6mer) to generate psiCHECK-2 (6mer + NeoR)

BamHI (Promega) restriction digest of the psiCHECK-2 (6mer WT) vector and the pcDNA3 Neomycin cassette PCR product were carried out in Buffer E (Promega) for the purpose of generating psiCHECK-2 (6mer + NeoR).
Table 3. PCR products and their expected sizes.

<table>
<thead>
<tr>
<th>PCR product name</th>
<th>Expected length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3mer oligo (MT and WT)</td>
<td>98</td>
</tr>
<tr>
<td>6mer oligo (MT and WT)</td>
<td>188</td>
</tr>
<tr>
<td>pcDNA3 Neomycin cassette</td>
<td>1630</td>
</tr>
</tbody>
</table>
2.4.4. Ligation

Ligation reactions were carried out in a total volume of 10 µL. The reaction mixture contained the appropriate digested psiCHECK-2 vector and the appropriate insert in a 1:6 molar ratio or 3:1 molar ratio for psiCHECK-2 (6mer) and the Neomycin cassette PCR product, 1 µL of 10 x T4 ligation buffer (Promega), 1 µL of (3 u/µL) T4 ligase (Promega), made up to 10 µL with nuclease-free water. The reaction was incubated at 4°C overnight. A list of plasmids that were generated can be found in **Table 4**.

2.4.4.1. psiCHECK-2 (3mer)

The Cons and MT 3mer oligos (GeneWorks) were ligated into the XhoI and NotI sites of the psiCHECK-2 vector (Promega) to generate psiCHECK-2 (3mer) and mutant vectors.

2.4.4.2. psiCHECK-2 (6mer)

The Cons and MT 3mer oligos (GeneWorks) were ligated into SalI and NotI sites of the psiCHECK-2 (3mer) vector to generate psiCHECK-2 (6mer) and mutant vectors. This was possible due to the compatibility of SalI and XhoI overhangs.

2.4.4.3. psiCHECK-2 (6mer + NeoR)

The digested pcDNA3 Neomycin cassette PCR product with BamHI overhangs was ligated into the BamHI site of the psiCHECK-2 (6mer) vector to generate psiCHECK-2 (6mer + NeoR). Note that the addition of the Neomycin resistance cassette has incorporated a second SalI site into the psiCHECK-2 (6mer) vector. Therefore, further concatemerisation of this vector can no longer be easily achieved.

2.4.5. Transformation

Plasmids (approximately 40 ng or 5 µL of the ligation reaction) were transformed into OneShot TOP10 chemically competent *E. coli* (Invitrogen) as per the manufacturer’s instructions. Cultures were grown in Luria Bertani (LB) medium (10 g/L Bacto-Tryptone,
5 g/L yeast extract and 10 g/L NaCl, to 1 L with milliQ water) pH 7.5. Solid media was made with the addition of 15 g/L of Bacto Agar (Sigma-Aldrich). Liquid and solid LB media contained 50 µg/mL Ampicillin (Sigma-Aldrich) and were grown up overnight at 37°C, and 200 rpm for liquid cultures.
Table 4. Plasmids acquired and generated in this study.
Plasmid maps of psiCHECK-2 vectors may be found in the appendix.

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3 HOXD10</td>
<td>Addgene</td>
</tr>
<tr>
<td>pcDNA3</td>
<td>In-house</td>
</tr>
<tr>
<td>psiCHECK-2</td>
<td>Promega</td>
</tr>
<tr>
<td>psiCHECK-2 (3mer)</td>
<td>This study</td>
</tr>
<tr>
<td>psiCHECK-2 (3mer MT)</td>
<td>This study</td>
</tr>
<tr>
<td>psiCHECK-2 (6mer)</td>
<td>This study</td>
</tr>
<tr>
<td>psiCHECK-2 (6mer MT)</td>
<td>This study</td>
</tr>
<tr>
<td>psiCHECK-2 (6mer + NeoR) (Consensus vector only)</td>
<td>This study</td>
</tr>
<tr>
<td>pBABE puro</td>
<td>In-house</td>
</tr>
</tbody>
</table>
2.4.6. Colony PCR

Successful colonies were identified via colony PCR and sequencing. psiCHECK-2 seq F and R primers (GeneWorks) were used to confirm 3mer and 6mer (Consensus and mutant) inserts. pcDNA3 Neo cassette F and R primers (GeneWorks) were used to confirm the Neomycin cassette insert. See Table 2 for appropriate primer sequences.

The colony PCR reaction was carried out in 20 µL containing: 1 µL of each 10 µM forward and reverse primers (GeneWorks), 4 µL of 5 x Green GoTaq flexi Buffer (Promega), 0.4 µL of 25 mM MgCl₂ (Promega), 1 µL of 10 mM dNTP mix (Promega), 0.1 µL of 5 u/µL GoTaq flexi DNA polymerase (Promega), to 20 µL with nuclease-free water. To each reaction mix, cells from a single colony were transferred by sterile pipette tip. PCR reactions were carried out in an Eppendorf Mastercycler Nexus Thermal Cycler. PCR cycle conditions psiCHECK-2 (3mer) and psiCHECK-2 (6mer) vectors were as follows: 2 min at 95°C; 30 s at 95°C, 30 s at 55°C for, 1 min at 72°C for 30 cycles; and then 5 min at 72°C. Expected PCR product lengths can be found in Table 3. Cycle conditions for psiCHECK-2 (6mer + NeoR) were as follows: 30 s at 98°C; 5 s at 98°C, 30 s at 65°C, 25 s at 72°C for 30 cycles and then 10 min at 72°C.

PCR products were visualised by electrophoresis using a 1% agarose gel. For electrophoresis see section 2.4.8.
Table 5. Colony PCR products and their expected sizes.
Products and their sizes generated by colony PCR for confirmation of the correct insert.

<table>
<thead>
<tr>
<th>Colony PCR product name</th>
<th>Expected length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3mer (MT and WT)</td>
<td>382</td>
</tr>
<tr>
<td>6mer (MT and WT)</td>
<td>472</td>
</tr>
<tr>
<td>Empty target site</td>
<td>284</td>
</tr>
<tr>
<td>Neomycin cassette</td>
<td>1630</td>
</tr>
</tbody>
</table>
2.4.7. Miniprep

Plasmids were extracted from OneShot TOP10 *E. coli* (Thermo Fisher Scientific) using PureLink Quick Plasmid Miniprep Kit (Invitrogen).

2.4.8. Electrophoresis

PCR and colony PCR products, plasmids and restriction digests were verified on 1% w/v agarose gel (1% w/v molecular grade agarose (Bioline) in 1 x TAE (Tris-acetate-EDTA) (For 50 x stock TAE: 242 g Tris-base, 57.1 mL glacial acetic acid, 18.6 g EDTA, to 1 L with MilliQ water)), with 4 µL of 10 mg/mL ethidium bromide (Sigma-Aldrich) per 100 mL. The gels were visualised using a Chemi Doc with the aid of Quantity One software (Bio-Rad).

2.4.9. Sequencing

Sequencing was performed to confirm insert direction and the absence of base errors. Sequencing was carried out by the Australian Genome Research Facility (AGRF; Perth, WA). Plasmid and PCR templates were supplied to AGRF with either the forward or reverse psiCHECK-2 seq primers (GeneWorks) in accordance with the concentrations and quality outlined by AGRF (for PCR products 200-400 bp in length 6 – 12 ng was supplied, for double-stranded plasmids 600 – 1500 ng was supplied). The samples were submitted in a total volume of 12 µL with 1 µL of 10 µM forward or reverse primer and 11 µL of nuclease-free water. Primer sequences may be found in Table 2.

2.4.10. Maxiprep

Maxipreps were carried out using the Qiagen Plasmid Maxi Kit (Qiagen) following the manufacturer’s instructions. Colonies were cultured overnight in 250 mL of LB medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl, to 1 L with MilliQ water) pH 7.5 containing appropriate antibiotic.
2.5. **HOXD10 upregulation of endogenous microRNA-7**

To determine that the miRNA processing pathway was active in all cell lines and transcriptional upregulation of miR-7 was possible, pcDNA3-HOXD10 (Addgene) was reverse transfected into cells which were subsequently assayed for miR-7 expression. pcDNA3-HOXD10 encodes the transcription factor HOXD10 which is proposed to upregulate miR-7 (Reddy, Ohshiro et al. 2008).

Transfections were adapted from Giles *et al.* (Giles, Barker et al. 2011) and were performed in triplicate in a 6-well plate using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. A Lipofectamine 2000 only negative control was included. Transfection mix (per well) contained 8.33 µL of Lipofectamine 2000 and 500 ng of pcDNA-HOXD10 plasmid. To each well, 1.5 mL of cell suspension (1.5 x10^5 cells/mL) was added and overlayed with 500 µL of transfection mix. Cells were incubated overnight at 37\(^\circ\)C and 5% CO\(_2\) and RNA was extracted after 24 h. For RNA extraction method see section 2.6.

2.6. **RNA extraction**

To extract RNA, 1 mL of Trizol (Thermo Fisher Scientific) was added to each well of a 6-well plate containing adherent cells. The solution was pipetted up and down to mix and mechanically lyse cells and then transferred to a 1.5 mL Eppendorf tube. Then, 200 µL of chloroform (Sigma-Aldrich) was added and the samples were shaken for 15 s, left at room temperature for 3 min and then centrifuged at 12 000 x g for 15 min at 4\(^\circ\)C. The supernatant was removed and transferred to a clean 1.5 mL tube and the chloroform step was repeated. Following this, 500 µL of isopropanol (Sigma-Aldrich) was added, the samples were mixed by inversion and left at room temperature for 10 min before being centrifuged at 12 000 x g for 15 min at 4\(^\circ\)C. The supernatant was removed and discarded. Then, 1.5 mL of 80% ethanol (Sigma-Aldrich) was added, samples were vortexed and then centrifuged at 7 500 x g for 5 min at 4\(^\circ\)C. The ethanol was removed and this step was repeated. The pellet was air dried on a heat block at 60\(^\circ\)C (cap open) and resuspended in 20 µL of nuclease-free water. The concentration of RNA was estimated using a NanoDrop (Thermo Scientific) at OD\(_{260}\).
2.6.1. Bioline isolate II RNA extraction

Initially, the Bioline Isolate II RNA mini kit (Bioline, BIO-52073) was used to extract RNA from cells treated with compounds for the purpose of RT qPCR measurement of miR-7 upregulation. This method was soon abandoned after issues were encountered, however during this time RNA was extracted as per the manufacturer’s instruction.

2.7. TaqMan microRNA RT qPCR

RT qPCR was carried out to assess the relative levels of miR-7 expression between HT29, HCT116 and SW620 cells to determine the cell line with the lowest level of endogenous expression. RT qPCR was also used to determine the relative level of miR-7 upregulation by HOXD10 for each cell line.

2.7.1. Reverse transcription (RT) reactions

2.7.1.1. TaqMan

Samples were diluted to 2 ng/µL. RT reactions were set up using TaqMan miRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s instructions. Primers for miR-7 (dme-miR-7, RT000268, Thermo Fisher Scientific) and a housekeeping gene, U44 (RNU44, RT001094, Thermo Fisher Scientific) or U6 (RNU6B, RT001093, Thermo Fisher Scientific) were used and 10 ng of RNA was added to each reaction. RT reactions were carried out in an Eppendorf Mastercycler Nexus Thermal Cycler and cycling conditions were as follows; 30 min at 16°C, 30 min at 42°C, and then 5 min at 85°C.

2.7.1.2. EGFR/GAPDH

Genomic DNA elimination reactions and RT reactions were set up using the QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer’s instructions. To each reaction, 0.5 µg of RNA was added. RT reactions were carried out in an Eppendorf
Mastercycler Nexus Thermal Cycler and cycling conditions were as follows; 15 min at 42°C, and 3 min at 95°C.

2.7.2. Quantitative PCR (qPCR)

2.7.2.1. TaqMan

The cDNA produced in TaqMan RT reactions was made up to 50 µL with nuclease-free water. qPCR master mix was made up containing (per reaction); 10 µL TaqMan 2x Master Mix (Thermo Fisher Scientific), 3 µL of nuclease-free water and 2 µL of either miR-7 (dme-miR-7, TM000268, Thermo Fisher Scientific) or housekeeping gene, U44 (RNU44, RT001094, Thermo Fisher Scientific) or U6 (RNU6B, RT001093, Thermo Fisher Scientific) probe. In a 0.2 mL Eppendorf tube, 15 µL of master mix was added to 5 µL of diluted cDNA. qPCR cycling conditions were as follows: hold 95°C for 10 min, cycling (40 repeats) step 1: 95°C for 15 s, step 2: 60°C for 60 s carried out in a Rotor-Gene 6000 thermocycler (Qiagen).

2.7.2.2. EGFR/GAPDH

The cDNA produced in EGFR/GAPDH RT was used. A standard curve was first run using the following; water only, neat cDNA and dilutions (1:10, 1:100, 1:1000, 1:10000) in duplicate. qPCR master mix was made up containing (per reaction) 10 µL 2 x SYBR mix with ROX (Bioline), 6.8 µL water (Baxter), 0.6 µL of each EGFR forward (5ʹ-GCG TTC GGC ACG GTG TAT AA) and reverse (5ʹ-GGC TTT CGG AGA TGT TGA TTC) primers (GeneWorks) or housekeeping gene, GAPDH forward (5ʹ-ATG GGG AAG GTG AAG GTC G) and reverse (5ʹ-GGG GTC ATT GAT GGC AAC AAT A) primers (GeneWorks) (10 µM). In a 0.2 mL Eppendorf tube, 18 µL of master mix was added to 5 µL of diluted cDNA. qPCR cycling conditions were as follows: hold 95°C for 5 min; cycling (40 repeats) step 1: 95°C for 30 s, step 2: 59°C for 30 s, step 3: 72°C for 30 s; melt (60°C – 99°C) hold 30 s on step 1, hold 5 s on subsequent steps, melt A SYBR green. qPCR reactions were carried out in a Rotor-Gene 6000 thermocycler (Qiagen). Single peak melt curves and reaction efficiencies between 0.9 and 1.1 were achieved. Expression of EGFR mRNA relative to GAPDH mRNA was determined using the 2⁻ΔΔCT method.
2.8. **Luciferase assay**

Luciferase assays were carried out to ensure the specificity of the generated psiCHECK-2 vectors for miR-7 via transient transfection.

2.8.1. **Transfection of psiCHECK-2 microRNA-7 target vectors with miR-7, miR-NC and pcDNA-HOXD10**

Reverse transfection of the psiCHECK-2 miR-7 target vectors was carried out using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. Transfections were performed in triplicate in white 96-well plates (Greiner) (clear bottomed plates were used for initial pilot studies and opaque plates thereafter once cell number and viability were confirmed by observation). The transfection mix (per well) contained 0.3 μL of Lipofectamine 2000 and 20 ng of psiCHECK-2 miR-7 target vector co-transfected with either miR-7 (Pre-miR™ miRNA Precursor, Invitrogen, PM10047) and or miR-NC (Pre-miR™ miRNA Precursor, Invitrogen, AM17110) at a final concentration of 1 nM, or 20 ng of pcDNA-HOXD10 (Addgene). To each well, 100 μL of cell suspension (1 – 2 x 10^5 cells/mL) was added and overlayed with 20 μL of transfection mix. The plate was incubated overnight at 37°C and 5% CO₂ and assayed for luciferase activity 24 h later using the Dual-Glo Luciferase Assay System (Promega) as per the manufacturer’s instructions.

2.8.2. **Dual-Glo luciferase assay**

Luciferase assays were carried out using the Dual-Glo Luciferase Assay System (Promega) as per the manufacturer’s instructions. To reduce well volume, 45 μL of media was removed from each well of the 96-well plate and 75 μL of Dual-Glo luciferase reagent (Promega) was added. The plate was incubated at room temperature for 10 min before Firefly luciferase activity was measured using a CLARIOstar microplate reader (BMG Labtech). Following which, 75 μL of Dual-Glo Stop & Glo reagent (1:1:1 ratio) (Promega) was added. The plate was again incubated for 10 min at room temperature before *Renilla* luciferase activity was measured in the same way. Relative luciferase
values were determined by normalising Renilla luciferase values to Firefly luciferase values.

2.9. Statistical analysis

In many cases, statistical analyses were performed using Prism (GraphPad) or Excel (Microsoft).

2.9.1. Z’factor

A Z’factor predicts assay robustness and suitability and was used for determining plate acceptance in the high throughput screen. The Z’factor was calculated using the following formula (Zhang, Chung et al. 1999):

\[
Z' = 1 - \frac{(3\sigma_{ct} + 3\sigma_{cb})}{|\mu_{ct} - \mu_{cb}|}
\]

Where \( \sigma \) is the standard deviation and \( \mu \) is the mean. The top control is denoted by ‘ct’ and ‘cb’ refers to the bottom control. In this experiment, a positive result is given by a reduction in signal and conversely, a negative result is given by no change in signal. Due to this inverse readout, the top control is the negative control (no change in signal, or brightest Renilla luciferase readings) and the bottom control is the positive control (where Renilla luciferase expression has been reduced). A Z’factor of \( \geq 0.4 \) was considered acceptable.

2.9.2. Percent coefficient of variation

The percent coefficient of variation (%CV) is expressed as a percentage and was used to quantify the variation in measurements of control wells in pilot, initial and repeat screens. It was calculated using the following formula where \( \sigma \) is the standard deviation and \( \mu \) is the mean:

\[
%CV = \frac{\sigma}{\mu} \times 100
\]
2.9.3. \( t \)-test

To determine statistical significance a two-tailed, unpaired, Student’s \( t \)-test was used. A p-value of <0.05 was considered to be statistically significant.

2.9.4. \( 2^{-\Delta\Delta C_t} \) method

To analyse RT qPCR data the \( 2^{-\Delta\Delta C_t} \) was used.

2.9.5. Outliers

Outliers were determined as data points that lay more than 1.5 times the interquartile range below the first quartile or above the third quartile.

2.10. Western blot

Western blots were carried out for HCT116, HT29 and SW620 cells following miR-7 or miR-NC transfection to determine the effects of miR-7 on EGFR, Akt and pAkt levels. The relative levels of these proteins were also compared between cell lines.

2.10.1. Reverse transfection with microRNA-7

Reverse transfections of miR-7 were performed in duplicate in a 6-well plate using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. A Lipofectamine 2000 only and miR-NC negative control was included. Transfection mix (per well) contained 8.33 µL of Lipofectamine 2000 and either miR-7 (Pre-miR™ miRNA Precursor, Invitrogen, PM10047) or miR-NC (Pre-miR™ miRNA Precursor, Invitrogen, AM17110) at a final concentration of 30 nM per well. To each well, 1.5 mL of cell suspension was added (1 – 2 \( \times \) 10\(^5\) cells/mL for HT29 and 2 – 3 \( \times \)10\(^5\) cells/mL for HCT116 and SW620. A higher cell count for HCT116 and SW620 cells was used to ensure
Methods

sufficient protein yield). Cells were incubated at 37°C and 5% CO₂ and protein was harvested 72 h later.

2.10.2. Preparation of cell lysates for Western blot analysis

Cell lysates were prepared according to Giles et al. (Giles, Barker et al. 2011). Cells in 6-well plates were placed on ice, media was removed and cells were subsequently washed gently with 2 mL of cold PBS (In-house, Perkins Media Store). Then 200 μL of CEB lysis buffer (10 mM HEPES pH 7.1, 3 mM MgCl₂, 14 mM KCl, 5% glycerol, 0.2% NP40 (IPEGAL)) with one complete Mini EDTA-free Protease Inhibitor cocktail tablet (Roche) and one Phos STOP Phosphatase Inhibitor cocktail tablet (Roche) per 10 mL of CEB was added to each well. Cells were scraped using a cell scraper and collected. Lysates were then subjected to one freeze-thaw cycle at -80°C to further aid cell lysis. Lysates were cleared by centrifugation at 13 000 x g for 5 min and the supernatant collected.

2.10.3. Bio-Rad protein quantification

A Bio-Rad assay was carried out to quantify proteins samples using Bio-Rad Protein Assay Dye Reagent Concentrate (#5000006, Bio-Rad) in a clear 96-well plate. First, 50 μL of Bio-Rad dye and 200 μL of water (Baxter) was added to each well followed by 1 μL of protein sample in duplicate wells. A standard curve was generated using known concentrations of BSA (Promega) ranging from 0 – 8 μg/μL. The wells were mixed and the plate was left for 5 min at room temperature to allow colour development. Absorbance was then read on a FLUOstar plate reader (BMG labtech) at 595 nm.

2.10.4. Western blot analysis

Following protein quantification, 15 μg of protein sample was made up to a volume of 13 μL with MilliQ water and added to 5 μL of NuPAGE LDS Sample Buffer (4X) (Invitrogen) and 2 μL of 10 x reducing agent (Invitrogen) in a 1.5 mL Eppendorf tube. Proteins were denatured at 70°C for 10 min on a heat block. A vertical gel tank was assembled and 300 mL of running buffer (25 mL NuPAGE MOPS SDS Running Buffer (20X) (Invitrogen) and
475 mL of MilliQ) was poured into the outer chamber. To the remaining 200 mL of running buffer, 500 µL of NuPAGE antioxidant (Invitrogen) was added. The buffer was then poured into the inner chamber. Then, 20 µL of each sample was loaded in NuPAGE 4 – 12% Bis-Tris precast gels (Invitrogen). Gels were run at 150 Volts for 90 min at 4°C.

Proteins were transferred to PVDF Western blotting membranes (03010040001, Sigma-Aldrich) in transfer buffer (14.4 g glycine and 3.03 g Tris-base made up to 900 mL with MilliQ water and then 100 mL of methanol added) overnight at 25 Volts on a stirring block with flea at 4°C.

Membranes were blocked using 5% skim milk/TBST solution (20 g of skim milk powder in 400 mL of TBST (60.5 g Tris-base, 87.6 g NaCl, 10 mL Tween (Sigma-Aldrich), pH adjusted to 7.5 and then to 10 L with MilliQ water) for 1 h on a rocker. Membranes were then trimmed and cut using a scalpel according to the expected protein location with reference to the ladder (Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards #1610375, Bio Rad) to allow separate detection with corresponding antibodies.

All antibodies were made up in 1% skim milk/TBST solution. Primary antibodies; anti-β-actin mouse monoclonal antibody [AC-15] (Abcam, ab6276, 1:15 000), anti-EGFR rabbit monoclonal antibody [EP38Y] (Abcam, ab52894, 1:5000), anti-Akt rabbit antibody (Cell Signalling Technologies, #9272S, 1:1000), anti-phospho-Akt rabbit monoclonal antibody (Ser473) (D9E) (Cell Signalling Technologies, #40605, 1:500), anti-survivin rabbit antibody [EP2880Y] (Sapphire Bioscience, GTX62039, 1:500) and anti-PARP (214/215) cleavage site rabbit polyclonal antibody (EMD Millipore, AB3565, 1:1000). Secondary antibodies; ECL anti-mouse IgG linked to horseradish peroxidase (GE Healthcare, NA931V, 1:10000), ECL anti-rabbit IgG linked to horseradish peroxidase (GE Healthcare, NA934V, 1:10000).

Primary antibodies were poured onto the membrane and rocked for 1 h. The primary antibody was then removed and the membrane pieces were washed for 3 x 10 min with 5% skim milk/TBST before applying the appropriate secondary antibodies. Finally, the membrane was further washed with TBST for 3 x 10 min. Following the washes, 1 mL Luminata Crescendo Western HRP Substrate (EMD Millipore) substrate was applied for
5 min. ECL-Hyperfilm (GE Heathcare) was exposed for various time points up to 15 min and processed on a CP1000 AGFA x-ray processor.

2.10.1. Densitometry

Densitometry of western images was calculated using ImageJ software. Images were imported into the software and the background was subtracted from the image. Bands were selected with tight boundaries and intensities are quantified. Graphs generated using densitometry were generated using data from western blot images displayed in this thesis only.

2.11. EC<sub>50</sub> cell viability assay

To determine the half-maximal effective concentration (EC<sub>50</sub>) of miR-7 on all three CRC cell lines HCT116, HT29 and SW620 an MTT assay was performed.

HCT116, HT29 and SW620 cells were transfected with various concentrations of miR-7 or miR-NC in a 96-well plate. A Lipofectamine 2000 (Invitrogen) only control was included. Transfections were performed in replicates of 6 with Lipofectamine 2000 as per the manufacturer’s instructions. Transfection mix (per well) contained 0.3 µL of Lipofectamine 2000 and either miR-7 (Pre-miR™ miRNA Precursor, Invitrogen, PM10047) or miR-NC (Pre-miR™ miRNA Precursor, Invitrogen, AM17110) at a final concentration of 0, 0.3, 1.5, 3, 6, 9, 12, 30 and 60 nM per well. To each well, 2.5 x 10<sup>3</sup> HT29 cells, 2.5 x 10<sup>3</sup> HCT116 cells and 6 x 10<sup>3</sup> SW620 cells were seeded in a total volume of 150 µL in a 96-well plate. Cells were then overlayed with 50 µL of transfection mix and incubated at 37°C and 5% CO<sub>2</sub> for 72 h.

To measure cell viability media was first removed from each well and replaced with 180 µL of media containing 1% FBS (Gibco) and 20 µL of Cell Titer 96 Aqueous One Solution (Promega). The plates were incubated at 37°C and 5% CO<sub>2</sub> for 1 – 4 h. Colour development was monitored and plates were read when control wells appeared to have developed to completion.

The EC<sub>50</sub> values were calculated using GraphPad Prism software (version 5.04).
2.12. Generating a stable cell line

2.12.1. Neomycin kill curve

A kill curve was carried out in HT29 cells to determine the concentration of neomycin required for stable cell selection of psiCHECK-2 (6mer + NeoR) positive cells. This concentration was determined as the point at which over 80% cell death occurred after 72 h and complete cell death was observed after two weeks. Cells were plated in a 24-well plate at a density of 0.5-1 x10⁵ cells/well in a total volume of 0.5 mL. Cells were left to settle overnight. The following day media was removed and was replaced with fresh media containing 0, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 μg/mL of G418/Geneticin (Life Technologies) in duplicate. Media containing antibiotic was changed every 2 – 3 days. The plates were incubated at 37°C and 5% CO₂.

2.12.2. Linearising psiCHECK-2 (6mer + NeoR)

In order to increase the likelihood of plasmid integration upon transfection, the psiCHECK-2 (6mer + NeoR) vector was linearised by restriction enzyme digest with Ahdl (NEB).

Restriction digest reactions were carried out in a total volume of 30 µL and contained a final concentration of 4 µg of DNA, 20 units of Ahdl (NEB), 1 x CutSmart Buffer (NEB), to 30 µL with nuclease-free water. The reactions were incubated at 37°C in a heat block for 2 – 3 h.

Digested plasmids were purified using QIAquick Gel Extraction Kit (Qiagen).

2.12.3. Transfection of psiCHECK-2 (6mer + NeoR)

Transfection of the psiCHECK-2 (6mer + NeoR) construct into HCT116 and HT29 prior to antibiotic selection was adapted from Giles et al. (Giles, Barker et al. 2011) and was performed in triplicate in a 6-well plate using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. Transfection mix (per well) contained 8.33 µL of Lipofectamine 2000 and 500 ng of linearised psiCHECK-2 plasmid. To each well, 1.5 mL
of cell suspension (1.5 x10^5 cells/mL) was added and overlayed with 500 μL of transfection mix.

2.12.4. **Antibiotic selection for psiCHECK-2 (6mer + NeoR)**

Twenty-four hours post-transfection with psiCHECK-2 (6mer + NeoR) G418/Geneticin (Life Technologies) was added in a final concentration of 500 μg/mL for HT29 cells. Cells were cultured in G418/Geneticin for approximately 3 – 4 weeks before being diluted across 96-well plates in order to isolate single clones.

2.12.5. **Diluting and culturing monoclonal populations**

Monoclonal populations were isolated via single-cell plating in clear 96-well tissue culture plates. Cells were plated and serially diluted across a 96-well plate. Four thousand cells were first plated in a total volume of 200 μL in well A1. Cells were then diluted 1 in 2 down column A so that A12 had 1/128 the original number of cells as in A1. Following which, cells were diluted 1 in 2 across all columns so that well H12 contained 1/262144 the number of cells as well A1. Wells were checked for single cells via observation using a light microscope. Single cells in these wells were established into clonal populations. Clones were then assayed for luciferase activity and responsiveness to miR-7. The clone with the greatest response and least variation was sought for the screen.

2.12.6. **Stable assay platform dose-response**

One hundred microliters of HT29 psiCHECK-2 (6mer + NeoR) stable cells (1.5 x 10^5 cells/mL) were plated in a 96-well plate and six replicate wells were treated with Lipofectamine 2000 only, or 0.5 nM, 1 nM, 5 nM, 10 nM or 30 nM of miR-7 or miR-NC. The plate was incubated overnight at 37°C and 5% CO₂, and assayed for luciferase activity 24 h later using Dual-Glo Luciferase Assay System (Promega) as per the manufacturer’s instructions.
Later the assay platform was assessed for the effect of varying concentrations of DMSO. Stable cells were plated in a 96-well plate as above and treated with 0, 0.01%, 0.025%, 0.05%, 0.1% and 0.2% DMSO. The plate was incubated overnight at 37°C and 5% CO₂, and assayed for luciferase activity 24 h later using Dual-Glo Luciferase Assay System (Promega) as per the manufacturer’s instructions.

2.13. Performing the high throughput screen

2.13.1. Compound library dilution

The NPL-800 compound library (TimTec), a compound library of natural products, was supplied in 10 x v-bottom clear 96-well plates with columns 1 and 12 left empty. To dilute the NPL-800 library to a final concentration of 50 µM two serial dilution steps were performed using the JANUS MDT automated liquid handling robotics system (PerkinElmer). The stock plates (10 mM, 100% DMSO) were initially premixed and diluted 10 x in PBS (In-house, Perkins Media Store) to generate replicate daughter plates (1 mM, 10% DMSO). These daughter plates were then premixed and diluted a further 20 x in PBS (In-house, Perkins Media Store) to generate replicate working plates (50 µM, 0.5% DMSO). Stock plates and daughter plates were sealed with axymats and stored at -80 °C for a maximum of one week. Working plates were sealed with clear film and stored at -80°C. All compounds assured by the vendor as >90% pure and 1H-NMR spectra were available upon request.

2.13.2. Testing compounds using a stable reporter assay system

To perform the screen, stable HT29 psiCHECK-2 (6mer + NeoR) cells were plated in white opaque flat-bottomed 96-well tissue culture plates at 9 x 10³ cells per well in a total volume of 60 µL of phenol red-free high-glucose DMEM (Gibco). Immediately, 15 µL of compounds from ‘working plates’ (50 µM, 0.5% DMSO) were added using the JANUS MDT automated liquid handling robot to give a final concentration of 10 µM and 0.1% DMSO. Cells in columns 1 and 12 were treated with either 15 µL of 0.5% DMSO (final concentration 0.1% DMSO) or transfected in a total volume of 15 µL with miR-7 (Pre-miR™ miRNA Precursor, Invitrogen, PM10047) or miR-NC (Pre-miR™ miRNA Precursor,
Invitrogen, AM17110) (final concentration 10 nM). miR-7 and miR-NC controls were plated in an alternating pattern as per Figure 40. Transfections were carried out using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. Plates were then incubated for 24 h at 37°C and assayed using the Dual-Glo luciferase assay system (Promega).

2.13.3. Running the screen

The library was screened in 10 plates over the course of two days. Repeat plates were screened on a third day. Plates were read on a Clariostar plate reader (BMG labtech). Settings were as follows; gain, 90% 3600; focus height, 11 mm; measurement interval time, 2 s; top read. The direction in which the plates were read was in a vertical S shape with column A1 read first through to H1, followed by H2 through to A2 etc. The final well to be read was A12. The time to read one plate took approximately 3.5 min. All plates were read in an initial screen (termed “screen 1”) and plates with an inadequate Z’factor were run again in a second screen (termed “screen 2”). For screen 1 both luciferase reagents were added with a multichannel as quickly as possible across wells. For screen 2 reagents were added every 17 s with a multichannel pipette as the read time for each column takes ~17 s. This was done to minimise the time delay (and therefore variation) from reagent addition to read time. Note that a time delay still existed within columns. Plates 1, 3, 4, 6, and 7 were rerun due to inadequate Z’factor scores due to highly variable 0.1% DMSO controls. Plates 2, 5, 8, 9 and 10 were not rerun.

All values were normalised to 0.1% DMSO controls, all ratios were divided by the average of 0.1% DMSO ratios (ratios were determined by dividing the Renilla luciferase reading by the Firefly luciferase reading for that well).

2.13.4. Hit determination

Potential hits were determined according to the following criteria:
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1. *Renilla*/Firefly ratio greater than 3 x standard deviations from the mean of the 0.1% DMSO control.

2. Those with elevated raw Firefly counts skewing the ratio were excluded (greater than 1 x standard deviation from the mean of 0.1% DMSO only Firefly count and where the *Renilla* count was not below at least 1 x standard deviation from the mean of the 0.1% DMSO only *Renilla* count).

3. Those which exhibited general toxicity were excluded where both *Renilla* and Firefly counts were negatively affected, except where the ratio of the two still fell greater than 3 x standard deviations from the 0.1% DMSO control as per criteria 1.

4. Those compounds where the decrease in *Renilla* was more than 1 x standard deviation below the average *Renilla* counts for 0.1% DMSO only and raw Firefly counts fell within the range of raw 0.1% DMSO counts +/- 1 standard deviation were tentatively noted.

2.14. Validating hit compounds

2.14.1. RT qPCR to determine microRNA-7 upregulation

To confirm compounds identified in the initial and repeat screens upregulate miR-7, miR-7 levels were directly measured by TaqMan RT qPCR. First, 3 x 10^5 HT29 psiCHECK-2 (6mer +NeoR) stable cells were seeded in a total volume of 2 mL in each well of a 6-well plate. The following day compounds were added at a final concentration of 10 µM (0.1% DMSO) in triplicate. Following treatment for 24 h RNA was extracted according to the protocol in section 2.6 and TaqMan RT qPCR was performed according to the protocol outlined in section 2.7.

2.14.2. Compound dose-response

To determine whether the compounds affected luciferase activity, and therefore induced miR-7 upregulation in a dose-responsive manner 60 µL of cells at 1.5 x 10^5 cells/mL were seeded in a white 96-well plate in phenol red-free media. Cells were treated in triplicate with each compound in a total volume of 15 µL to give a final
concentration of 0 (0.2% DMSO only), 1, 2.5, 5, 10 and 20 μM. Cells transfected with 10 nM miR-7 were also included as a positive control. Twenty-four hours following treatment cells were assayed for luciferase expression using the Dual-Glo luciferase assay system (Promega) according to the manufacturer’s instructions.

2.14.3. Compound EC_{50} cell viability assay

To determine the half-maximal effective concentration (EC_{50}) of cantharidin (Sigma-Aldrich) and 5-fluorouridine (5-FUrd; Sigma-Aldrich) on the three cell lines HT29, HCT116 and Huh7 an MTT assay was performed. Cells were seeded in a 96-well plate and treated the following day with various concentrations of cantharidin (0 – 50 μM) and 5-FUrd (0 – 10 μM). In each well, 2.5 × 10^3 HT29 cells, 2.5 × 10^3 HCT116 cells and 5 × 10^3 Huh7 cells were seeded in a total volume of 150 μL of a 96-well plate. Six replicates of each treatment at a final concentration of 0, 0.001, 0.01, 0.1, 1, 2.5, 5, 10, 20, 50 μM for cantharidin and 0, 0.00001, 0.0001, 0.001, 0.01, 0.1, 1, 2.5, 5, 10 μM for 5-FUrd were included. Cells were incubated at 37°C and 5% CO₂ for 72 h.

To measure cell viability, media was first removed from each well and replaced with 180 μL of media containing 1% FBS (Gibco) and 20 μL of Cell Titer 96 Aqueous One Solution (Promega). The plates were incubated at 37°C and 5% CO₂ for 1 – 4 h. Colour development was monitored and plates were read when control wells appeared to have developed to completion.

The EC_{50} values were calculated using GraphPad Prism software (version 5.04).

2.14.4. Compound treatment for Western blot

To determine the effect of cantharidin (Sigma-Aldrich) and 5-FUrd (Sigma-Aldrich) on miR-7 targets Akt, pAkt, EGFR and predicted target survivin, cells were plated in a total volume of 2 mL in a 6-well plate. HT29 and HCT116 cells were plated at 3 × 10^5 cells/well and Huh7 cells were plated at 4 × 10^4 cells/well. Cells were treated the following day with various concentrations of drug. These concentrations were optimised so that there were enough surviving cells for protein harvest 24 h later. HT29 cells were treated with
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10 μM cantharidin and 10 μM 5-FUrd and Huh7 cells were treated at 1 μM cantharidin and 10 μM 5-FUrd. To harvest protein, 100 μL CEB lysis buffer (10 mM HEPES pH 7.1, 3 mM MgCl$_2$, 14 mM KCl, 5% glycerol, 0.2% NP40 (IPEGAL)) with one complete Mini EDTA-free Protease Inhibitor cocktail tablet (Roche) and one Phos STOP Phosphatase Inhibitor cocktail tablet (Roche) per 10 mL of CEB was added to each well. Cells were scraped using a cell scraper and collected. Lysates were then subjected to one freeze-thaw cycle at -80°C to further aid cell lysis. Lysates were cleared by centrifugation at 13 000 x g for 5 min and the supernatant collected. Protein was quantified as per section 2.10.3 and the Western blot carried out as outlined in section 2.10.

2.14.5. Cantharidin time course Western blot

To determine the effect of cantharidin (Sigma-Aldrich) on the miR-7 targets Akt, pAkt and EGFR over the course of 4 h, HT29 cells were first plated at 3 x 10$^5$ cells/well in a total volume of 2 mL in a 6-well plate. Cells were treated the following day with 10 μM cantharidin and 0.001% DMSO at minus 4 h and minus 1 h time points. To harvest protein, 100 μL CEB lysis buffer (10 mM HEPES pH 7.1, 3 mM MgCl$_2$, 14 mM KCl, 5% glycerol, 0.2% NP40 (IPEGAL)) with one complete Mini EDTA-free Protease Inhibitor cocktail tablet (Roche) and one Phos STOP Phosphatase Inhibitor cocktail tablet (Roche) per 10 mL of CEB was added to each well. Cells were scraped using a cell scraper and collected. Lysates were then subjected to one freeze-thaw cycle at -80°C to further aid cell lysis. Lysates were cleared by centrifugation at 13 000 x g for 5 min and the supernatant collected. Protein was quantified as per section 2.10.3 and the Western blot carried out as outlined in section 2.10.

2.14.6. Anti-microRNA-7 rescue

To determine whether a reduction in miR-7 target proteins by hit compounds is due to their capacity for miR-7 upregulation cells were treated with compounds in conjunction with anti-miRNA mimics. First, HT29 cells were plated at 2 x 10$^5$ cells/well in a total volume of 1.5 mL in a 6-well plate and reverse transfected with anti-miR-7 (Anti-miR™ miRNA Inhibitor, Thermo Fisher Scientific, AM17000) or a negative control anti-miRNA
(Anti-miR™ miRNA Inhibitor, Thermo Fisher Scientific, AM17010) in a volume of 0.5 mL. Reverse transfections of miR-7 were performed using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. A Lipofectamine 2000 only control was included. Transfection mix (per well) contained 8.33 µL of Lipofectamine 2000 and either miR-7 or miR-NC at a final concentration of 30 nM per well. Cells were incubated at 37°C and 5% CO₂. Twenty-four hours post-transfection cells were treated with cantharidin (10 µM), 5-FUrd (10 µM) or DMSO only. Protein was then harvested 24 h post-treatment with the drug (48 h post-transfection).

2.14.7. Caspase- Glo apoptosis assay

To determine if cantharidin and 5-FUrd were inducing apoptosis in HT29 and Huh7 cell lines, a Caspase-Glo 3/7 assay (Promega) was employed. Cells were plated at 5 x 10³ cells/well in a total volume of 50 µL in a clear bottomed white 96-well plate. The following day drug was added at various concentrations in triplicate in a total volume of 50 µL. Cantharidin (Sigma-Aldrich) was added at 2.5, 5 and 10 µM, 5-FUrd (Sigma-Aldrich) was added at 0.001, 1 and 5 µM. Camptothecin (Sigma-Aldrich; 10 µM) was used as a positive control for HT29 cells, ABT737 (Sigma-Aldrich; 10 µM) was used as a positive control for Huh7 cells and DMSO (Sigma-Aldrich; 0.1%) was a negative control for both cell lines. Twenty-four hours post-treatment cells were assayed for caspase activity following the manufacturer’s instructions.
PART I
Nanoparticle-mediated microRNA delivery
3. MicroRNA-7 delivery via polymeric nanoparticles

This chapter examines the efficacy of polymeric nanoparticles as delivery vehicles for miR-7. It includes preliminary experiments to examine nanoparticle uptake, miR-7 delivery to cells in vitro and subsequent effects on protein target expression.

3.1. Introduction

Part I of the project was to deliver exogenous miR-7 to cancer tissues via novel polymeric nanoparticles. It was envisaged that this polymeric nanoparticle would result in miR-7 uptake into CRC cells with knockdown of key target genes including EGFR and pAkt. Nanoparticles sourced from the Iyer lab at UWA were examined for their potential as delivery vehicles for miR-7. Studies herein assessed the nanoparticle construct for efficient miR-7 delivery into CRC cells, and protein target knockdown.

3.1.1. Nanoparticles as delivery vesicles

Alternatives to viral vector systems are now being considered for gene delivery as viral-mediated gene delivery is limited due to safety concerns regarding their immunogenicity and insertional mutagenesis (Muthiah, Park et al. 2013). Currently, nonviral vector systems such as cationic polymer nanoparticles are potential candidates for miRNA transportation in the body. Nonviral systems are safer and elicit lower immunogenicity than their viral counterparts and protect the RNA cargo from nuclease degradation (Zhang, Wang et al. 2013). Nanoparticles are small ultra-fine particles that range in size with those proposed for drug delivery systems ranging from 5 – 250 nm in diameter (Alexis, Pridgen et al. 2008) and may be composed of diverse materials from inorganic and metallic particles such as gold and iron oxide to lipids and cationic polymers with various compositions and conformations. They are amenable to surface modifications with such agents as ligands and polymers to increase cellular uptake, enhance transfection efficiency, biocompatibility, targeting efficacy and even to cross the blood-brain barrier. Further, their immunogenicity may be controlled by altering their size (Mottram, Leong et al. 2007), charge and hydrophobicity/hydrophilicity (Zolnik,
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Gonzalez-Fernandez et al. 2010, Muthiah, Park et al. 2013). Further, surface properties of nanoparticles can extend circulation time of a particle in the body, aid in cellular uptake, and decrease toxicity and interactions with blood components (Byrne, Betancourt et al. 2008, Guo and Huang 2012). Nanoparticles accumulate passively at the site of the tumour via the enhanced permeability and retention effect, an effect where nanoparticles accumulate due to permeable tumour blood vessels (Guo and Huang 2012). Thus, nanoparticles that circulate in the body for a shorter period have lower delivery efficiency. The addition of surface modifications can enhance circulation time and transfection efficiency. For example, the addition of polyethylene glycol (PEG) or PEGylation to the surface is widely accepted and used to allow a greater circulation time of nanomaterials in vivo. It has low toxicity and reduces clearance by the reticuloendothelial system via binding a specific composition of proteins to the surface (Butcher, Mortimer et al. 2016, Schottler, Becker et al. 2016). Further, the addition of polyethyleneimine (PEI) (used in nanoparticles here), a cationic polymer, enhances transfection efficiency through binding to the anionic cell surface and allowing for electrostatic loading of miRNA to the surface of the nanoparticles (Draz, Fang et al. 2014, Tangudu, Verma et al. 2015). Further surface modifications such as the addition of cell-penetrating peptides e.g. TAT peptide derived from HIV-1 TAT protein can enhance cellular uptake (Zhang, Wang et al. 2016). One advantage of using nanoparticles is that multiple different miRNAs could be simultaneously loaded into the particle, and then targeted to tumour sites (Chen, Zhu et al. 2010). Figure 5 illustrates several of the main types of nanoparticle formulations and some of their potential surface modifications.

3.1.2. Nanoparticle-mediated microRNA delivery

There have been a number of studies which have successfully delivered miRNA and siRNA to cancer cells. Nanoparticles synthesised from \( N^4,N^{11} \)-bisethynorspermine (BENSpm), termed DSS-BEN nanoparticles, were used to deliver miR-34a mimics to HCT116 CRC cells. These nanoparticles are induced by the antioxidant glutathione to self-disassemble in the cytoplasm, thereby releasing their miRNA cargo. The result was a subsequent elevation in miR-34a expression and downregulation of Bcl-2 a miR-34a target (Xie, Murray-Stewart et al. 2017). Further, gold nanoparticles have been used to
Results chapter: MicroRNA-7 delivery via polymeric nanoparticles deliver miR-130b to multiple myeloma cells. Increasing gold nanoparticle-miRNA conjugate concentration increased inhibition of a luciferase reporter (Crew, Tessel et al. 2012). Targeted gold nanoparticles embedded in a hydrogel have been used to locally deliver miR-96 and miR-182 in combination with cisplatin to breast cancer *in vivo* reducing metastasis (Gilam, Conde et al. 2016). In addition, poly(amidoamine) (PAMAM) has been used to deliver miR-7 to glioma *in vitro* and *in vivo* reducing miR-7 target proteins (Liu, Li et al. 2013). Short hairpin RNA (shRNA) and siRNA have been delivered to MCF-7 breast cancer cells in two studies. In one, the authors used alkyl modified PEI coated PGLA (poly (lactic-co-glycolic acid)) nanoparticles to simultaneously deliver doxorubicin and a shRNA against Bcl-xL (Ebrahimian, Taghavi et al. 2017). In the other, carbon nanotubes conjugated with a PEI derivative and targeted to cell surface proteins with an aptamer were used to deliver siRNA against BCL9I which decreased target protein levels and induced apoptosis (Mohammadi, Salmasi et al. 2015). Furthermore, PEG-PEI nanoparticles have been used to deliver siRNA targeting CD44v6 in gastric carcinoma cells (Wu, Wang et al. 2010). Finally, Huschka *et al.* demonstrated on-demand laser light-induced release of siRNA against GFP using gold nanoshell based nanoparticles with a poly-L-lysine peptide surface layer and achieved a 49% reduction in GFP expression (Huschka, Barhoumi et al. 2012).

There are several nano-based formulations already approved for clinical use in cancer that deliver anti-cancer drugs, the majority of which are lipid-based (Leonard, Williams et al. 2009, Miele, Spinelli et al. 2009, Ando, Mori et al. 2011, Barenholz 2012, Silverman and Deitcher 2013, Carnevale and Ko 2016). While several clinical trials have been conducted for siRNA/miRNA nanoparticle formulations, many of which are also lipid-based, none have yet been approved for clinical use (Ribas, Kalinoski et al. 2010, Tabernero, Shapiro et al. 2013, Schultheis, Strumberg et al. 2014, Tolcher, Papadopoulos et al. 2015, Anselmo and Mitragotri 2016, Chakraborty, Sharma et al. 2017).

However, there are still limitations to the technology and the field continues to work through some level of immunogenicity and toxicity elicited by the nanoparticles, as well as achieving greater distribution and overcoming clearance by the reticuloendothelial system (Miele, Spinelli et al. 2012, Xin, Huang et al. 2017).
Figure 5. Types of nanoparticles used in drug delivery and their possible surface modifications.

A) Nanoparticles designed for drug delivery are composed of diverse materials and miRNAs may be physically bound, encapsulated or associated with the particle via electrostatic interaction. B) Nanoparticles are amenable to surface modifications which can be used for tracking/imaging (e.g. fluorescent dyes), improving circulation time (e.g. polymers such as PEG) or for targeting cell surface receptors (e.g. peptides and antibodies).
3.1.3. PGMA – PEI nanoparticles created by the Iyer Lab at UWA

Nanoparticles from the Iyer lab at UWA are synthesised from polyglycidal methacrylate (PGMA) as a core component with the addition of PEI chains to the surface. The PEI coating of this particle is positively charged and wraps around negatively charged miRNA binding them to the surface via electrostatic interaction. PEI also facilitates entry across the negatively charged cell membrane. Once the nanoparticles are internalised into endosomes, the PEI constituent of the particle acts as a proton sponge. This buffers the natural lowering of pH and in turn leads to osmotic swelling, rupture of the vesicle and release of the nanoparticle-miRNA complexes into the cytoplasm. However, PEI has been shown to be inherently toxic and the incorporation of PGMA helps to reduce this (Tangudu, Verma et al. 2015). Smaller iron (III) oxide (Fe$_2$O$_3$) nanoparticles are encapsulated by the core PGMA sphere allowing for MRI. The nanoparticles are further modified by the incorporation of Rhodamine B fluorescent dye for optical imaging. The nanoparticles had a diameter ranging between 100 – 150 nm. An illustration of these particles is given in Figure 6.
Figure 6. PGMA-PEI nanoparticles.
PGMA-PEI nanoparticles used in this study were generated by the Iyer Lab and are composed of PEI chains bound to a PGMA core component. Rhodamine B which allows for fluorescent imaging is linked to the PGMA polymer (not illustrated). Iron oxide particles are contained within the PGMA core allowing for MRI.
3.1.4. Chapter aims

The specific aims of this chapter are to:

1) Ensure nanoparticles enter CRC cells in culture, and further are not hindered by the addition of miR-7 to the surface.

2) Measure the relative level of miR-7 delivery by nanoparticles to cells and compare efficiency with Lipofectamine 2000.

3) Demonstrate that miR-7 is active upon delivery through downregulation of miR-7 target proteins.
3.2. Results

3.2.1. Time-course study of PGMA-PEI nanoparticle uptake

To qualitatively examine the uptake of PGMA-PEI nanoparticles by HCT116 and HT29 cells, they were incubated with the nanoparticles and imaged using fluorescence microscopy at various time points over the course of 24 h (Figure 7). While there were very few cells containing nanoparticles at 30 min, most cells contained nanoparticles after 2 h. Nanoparticles are labelled with Rhodamine B and are observed as red.

3.2.2. Flow cytometry

Flow cytometry was used to confirm the uptake of nanoparticles by HCT116 cells after 24 h (Figure 8). Figure 8 shows very clear separation of untransfected cells vs those that have been incubated with nanoparticles. All cells incubated with nanoparticles retained them with a relatively narrow range of intensity, indicating an even distribution of nanoparticles within cells. Flow cytometry was further used to confirm the uptake of nanoparticles was not affected when coupled with miR-7 (Figure 9).
Figure 7. Fluorescence microscopy to observe nanoparticle uptake in HCT116 and HT29 cells.
HCT116 and HT29 cells were treated with nanoparticles in a 6-well plate and nanoparticle uptake was monitored over a 24 h period by fluorescence microscopy. Cells were imaged using the 200 x objective and the yellow bar in the bottom right of the images represents 100 µm. The result shown is representative of three independent experiments.
Figure 8. Flow cytometry to assess nanoparticle uptake in HCT116 cells. HCT116 cells were incubated with/without PGMA-PEI nanoparticles for 24 h before the percentage of cells that had successfully taken up nanoparticles (99.7%) was determined by flow cytometry. Data for the negative control (cells only) is displayed in blue and data for the nanoparticle transfected cells is given in red. The result shown is representative of two independent experiments.
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Figure 9. Flow cytometry to assess uptake of nanoparticles coupled with miRNA in HCT116 cells.

PGMA-PEI nanoparticles (NPs) were first coupled with miR-7. HCT116 cells were then subject to incubation without NPs (negative control), NPs only and NPs coupled with miR-7 (NP + miR-7) for 24 h before the portion of cells that had taken up nanoparticles was determined by flow cytometry. The result shown is representative of two independent experiments.
3.2.2.1. **Intracellular imaging of nanoparticles**

To confirm that nanoparticles were inside the cells rather than attached to the cell surface, leftover aliquots from those used for (but not subjected to) flow cytometry were fixed and stained with DAPI and imaged on a DeltaVision Elite Imaging System and Softworx software (GE Healthcare). **Figure 10** shows the Rhodamine B labelled nanoparticles (red) and DAPI stained cell nuclei (blue). It was evident that the nanoparticles were not associated with the cell nucleus upon focal length adjustment and appear to cluster to one region of the cytoplasm.

3.2.3. **MicroRNA-7 delivery using nanoparticles**

miR-7 was transfected into HCT116 cells over the course of 24 h using nanoparticles (NP) and Lipofectamine 2000 (LF) reagent. Relative miR-7 expression was measured by RT qPCR (**Figure 11**). Values are normalised to Lipofectamine 2000 only transfected cells. When miR-7 was transfected with Lipofectamine 2000 (LF-miR-7) cells showed much greater miR-7 expression compared to when miR-7 was transfected with nanoparticles (NP-miR-7). Upon transfection with NP-miR-7, miR-7 levels were highly variable between replicate treatments (performed in triplicate) ranging from 7 to 60-fold, with an average of 27-fold and upregulation was relatively small when compared to LF-miR-7 transfected cells (3296-fold increase).
Figure 10. Fluorescence microscopy to assess the intracellular distribution of rhodamine-B labelled PGMA-PEI nanoparticles. HCT116 cells were incubated with PGMA-PEI nanoparticles for 24 h prior to being fixed and visualised. Cell nuclei are stained with DAPI (blue) and rhodamine-B labelled nanoparticles appear red. The result shown is representative of a single experiment.
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Figure 11. RT qPCR for miR-7 expression following transfection using Lipofectamine 2000 when compared with miR-7 delivered by nanoparticles in HCT116 cells.

RT qPCR analysis of miR-7 expression was performed in HCT116 cells following transfection of miR-7 using Lipofectamine 2000 (LF) and nanoparticles (NP) for 24 h. miR-7 expression was normalised to U44 expression and is shown as a ratio to LF only transfected cells (±SD) using the $2^{-\Delta\Delta Ct}$ method. A significant difference in miR-7 expression of miR-7 delivered by Lipofectamine 2000 (LF-miR-7) and miR-7 delivered by nanoparticles (NP-miR-7), was observed from LF and NP only transfected cells, respectively. The result shown is representative of two independent experiments. ****, $p < 0.0001$; *, $p < 0.05$. 
3.2.4. Assessing EGFR, Akt and pAkt downregulation

To assess whether the quantity of miR-7 delivered by the nanoparticles was enough to have a biological effect, EGFR, Akt and pAkt expression were measured by Western blot. Western Blot analysis was conducted at 72 h (as opposed to 24 h for nanoparticle uptake and miR-7 expression experiments) to allow time for the miRNA to associate with RISC, repress target protein expression and for this effect to be reflected following protein turnover. The effect of miR-7 when delivered using Lipofectamine 2000 in HCT116 (Figure 12) and HT29 cells (Figure 13) was first confirmed. This was then compared to when miR-7 was delivered using nanoparticles in HCT116 (Figure 14) and HT29 cells (Figure 15). The concentration of miR-7-nanoparticle conjugates delivered to HCT116 cells was then doubled to test whether this would have any further effect on protein expression (Figure 16). While EGFR and pAkt were downregulated in HCT116 and HT29 cells when treated with Lipofectamine 2000 delivered miR-7, no effect was observed for nanoparticle-delivered miR-7. EGFR mRNA was also measured by RT qPCR for comparison between the two delivery methods in HCT116 (Figure 17) and no downregulation of EGFR mRNA was observed in NP-miR-7 treated cells.
Figure 12. Western blot and densitometry analysis of miR-7 target proteins following miR-7 transfection using Lipofectamine 2000 in HCT116 cells.

Western blot and densitometry analysis in duplicate of EGFR, Akt and pAkt levels in HCT116 cells treated for 72 h with Lipofectamine 2000 only (LF), miR-NC (30 nM) or miR-7 (30 nM). β-actin is included as a loading control. The result shown is representative of three independent experiments. Densitometry is normalised to β-actin and expressed relative to LF. *, p < 0.05.
Figure 13. Western blot and densitometry analysis of miR-7 target proteins following miR-7 transfection using Lipofectamine 2000 in HT29 cells.

Western blot and densitometry analysis in duplicate of EGFR, Akt and pAkt levels in HT29 cells treated for 72 h with Lipofectamine 2000 only (LF), miR-NC (30 nM) or miR-7 (30 nM). β-actin is included as a loading control. The result shown is representative of three independent experiments. Densitometry is normalised to β-actin and expressed relative to LF. **, p < 0.01, given relative to LF.
Figure 14. Western blot and densitometry analysis of miR-7 target proteins following miR-7 transfection using nanoparticles in HCT116 cells.

Western blot and densitometry analysis in duplicate of EGFR, Akt and pAkt levels in HCT116 cells treated for 72 h with nanoparticles coupled with miR-7 (NP + miR-7) (30 nM), miR-NC (NP + miR-NC) (30 nM) or on their own (NP only). β-actin is included as a loading control. The result shown is representative of two independent experiments. Densitometry is normalised to β-actin and expressed relative to NP only.
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Figure 15. Western blot and densitometry analysis of miR-7 target proteins following miR-7 transfection using nanoparticles in HT29 cells.

Western blot and densitometry analysis in duplicate of EGFR, Akt and pAkt levels in HT29 cells treated for 72 h with nanoparticles coupled with miR-7 (NP + miR-7) (30 nM), miR-NC (NP + miR-NC) (30 nM) or on their own (NP only). β-actin is included as a loading control. The result shown is representative of two independent experiments. Densitometry is normalised to β-actin and expressed relative to NP only.
Figure 16. Western blot and densitometry analysis of miR-7 target proteins following miR-7 transfection using twice the concentration (60 nM) of miR-7 delivered by nanoparticles in HCT116 cells.

Western blot and densitometry analysis in duplicate of EGFR, Akt and pAkt levels in HCT116 cells treated for 72 h with nanoparticles coupled with miR-7 (NP + miR-7) (60 nM), miR-NC (NP+miR-NC) (60 nM) or on their own (NP only). β-actin is included as a loading control. The result shown is representative of a single experiment. Densitometry is normalised to β-actin and expressed relative to NP only.
Figure 17. RT qPCR for EGFR mRNA expression following transfection of miR-7 delivered using Lipofectamine 2000 when compared with miR-7 delivered by nanoparticles.

A RT qPCR for EGFR expression was performed in HCT116 cells following treatment with miR-7 (30 nM) or miR-NC (30 nM) delivered using either Lipofectamine 2000 (LF) or nanoparticles (NP) for 24 h. Data are normalised to GAPDH expression and expressed relative to LF only treated cells. The result shown is representative of a single experiment. **, p < 0.01.
3.3. Discussion

3.3.1. Efficiency of nanoparticle uptake by cells

The uptake of nanoparticles into CRC cells was highly efficient. Nanoparticles appeared to enter cells relatively rapidly (30 min – 2 h incubation) (Figure 7) and accumulate in distinct regions of the cytoplasm (Figure 10). It is suggested however that confocal microscopy be used to ascertain the internal distribution of nanoparticles in future studies. A near 100% transfection efficiency was observed by flow cytometry with some variability in the intensity of fluorescence present in the cells (100-fold range) (Figure 8) and this was near identical for nanoparticles that were coupled with miR-7 indicating uptake was unaffected by the presence of miR-7 (Figure 9).

3.3.2. Nanoparticle-mediated microRNA-7 delivery and action

Despite efficient uptake of nanoparticles into the cells, when the nanoparticles were coupled with miR-7 there was a significant but highly variable increase in detectable miR-7 in the cells (27-fold). However, this was a relatively small increase when compared to miR-7 delivered using Lipofectamine 2000 reagent (Figure 11). Given the same quantity of miR-7 (30 nM) was coupled with both nanoparticles and Lipofectamine 2000 this suggests that the nanoparticles are not as effective at either A) associating/coupling with miR-7 prior to transfection or B) if miR-7 couples just as effectively to the nanoparticles as it does the Lipofectamine, then the nanoparticles may not take miR-7 across the cell membrane.

Further, to investigate whether the miR-7 increase detected by nanoparticle delivery was enough to elicit a biological effect, known miR-7 targets EGFR, Akt and pAkt were subjected to Western blot analysis. It was important to first confirm miR-7 action on target proteins as delivered by Lipofectamine 2000 in HCT116 (Figure 12) and HT29 cells (Figure 13) for comparison to nanoparticle-mediated delivery. Downregulation of EGFR and pAkt was evident in both lines. Unfortunately, there was no observable decrease in protein expression upon nanoparticle-mediated miR-7 delivery in both HCT116 cells (Figure 14) and HT29 cells (Figure 15). The result was the same even when the amount of nanoparticles conjugated with miR-7 delivered to HCT116 cells was doubled (Figure 16).
Results chapter: MicroRNA-7 delivery via polymeric nanoparticles. This was confirmed by RT qPCR analysis of EGFR transcript levels in HCT116 (Figure 17). These results suggest that despite some miR-7 delivery by the nanoparticles, the fold increase is not sufficient for a significant response. This could be due to an insufficient fold increase but could also be the result of ineffective release of the miR in the cell that would prevent the miR from binding to its targets. Further modifications could be made to the nanoparticles to improve the amount of miR-7 being coupled to the particle, entering the cells and becoming active in the cells. Several experiments would first need to be conducted to identify the primary cause. Firstly, RT qPCR could be used to determine the relative amount of miR-7 coupled to the nanoparticles when compared to Lipofectamine 2000 (following wash steps to remove unbound miRNA from the nanoparticle), this may confirm why less miRNA was detected in the cells following transfection given equal amounts were provided in each transfection mix (Figure 11). Second, following transfection with nanoparticles and Lipofectamine 2000 RT qPCR could be used to determine miR-7 levels in media commonly removed prior to isolation of the cells themselves to determine relative levels of miR-7 that have not entered cells. This may confirm that the issue is a lack of successful entry into the cell rather than miRNA coupling to the particle. If this were the case, physically attaching the miRNA (for example, via a disulphide bond (Crew, Tessel et al. 2012) to the nanoparticle may improve movement of the miRNA together with the particle across the cell membrane as the miRNA is associated with the nanoparticles here via electrostatic interaction only. Thirdly, miRNA may simply be binding to the outside of the cell and are not efficiently removed during wash steps prior to RNA isolation. Cells could be treated with miRNA only to determine if those levels seen in Figure 11 are residual levels only. Fourthly, assuming some miRNA enters cells but is not enough to elicit a response, miR-7 delivered by Lipofectamine 2000 could be titrated down to see if a similar effect (no effect) on target proteins is observed at comparable miR-7 levels (i.e. 27-fold as detected by RT qPCR for NP-miR-7). Modifications such as EGFR targeted aptamers or polypeptides (as illustrated in Figure 5B) may improve nanoparticle uptake by enhancing internalisation by endocytosis (Gao, Yang et al. 2013). Fifth, if the miRNA is not being released by the nanoparticle or the particle is being sequestered into an organelle, fluorescent miRNA labelling could be used to determine its relative position in the cell. Reports have indicated that attaching a fluorophore to one or both strands of a miRNA does not affect its ability to bind to AGO or silencing activity (Chandradoss, Schirle et al. 2012).
Results chapter: MicroRNA-7 delivery via polymeric nanoparticles (Pitchiaya, Heinicke et al. 2015, 2017). Finally, of general interest would be time-lapse fluorescence microscopy to determine the disposal of the nanoparticles by the cell.

Despite successful miR-7 delivery by the nanoparticles, there was consistently very large variability observed for miR-7 delivery by nanoparticles (Figure 11) and this may also attribute to the lack of an effect seen in the subsequent Western blots (Figure 14 through Figure 16). Although the results appeared promising from this first part of the project, in parallel the small molecule screening was progressing well, so a decision was made to focus on the latter for the rest of the thesis.
PART II
A screen for small molecule modifiers of microRNA-7 expression
4. Generating a screening platform

This chapter outlines the processes of developing a luminescent cell-based assay platform for detecting miR-7 activity in a small molecule screen. It includes details of the vector construction, choice of cell line, and pre-screening transient assays. Finally, it covers the generation of a stable cell line and validation of the screening platform.

4.1. Drug discovery assay platforms

Drug discovery strategies can largely be generalised into two distinct approaches; classical and reverse pharmacology. Classical pharmacology approaches, also known as phenotypic drug discovery, identify drug candidates based on a therapeutic phenotypic response. Conversely, reverse pharmacology is hypothesis-driven and starts with the identification of a therapeutic molecular target for a disorder and compounds are screened to identify modulators of this target to deliver a therapeutic response. To this end, the development of a suitable assay platform must be suited to the molecular target of interest and requires several important considerations (Takenaka 2001). Assay platforms can be generalised as being either biochemical (cell-free) or cell-based. Cell-based assays have the advantage of offering greater biological relevance, as they provide some indication of cell toxicity, efficacy and membrane permeability. In addition, time, cost, sensitivity and reproducibility are major factors to consider prior to assay development (Hughes, Rees et al. 2011). These factors have been considered in the design of a cell-based assay platform for detecting changes in miR-7 expression using a dual-bioluminescent system, which is described in this chapter. A generalised overview of the basis of the platform developed here is given in Figure 18.

4.1.1. Small molecule-mediated microRNA regulation

As mentioned in the thesis introduction, using small molecules to regulate miRNA expression is an alternative strategy to exogenous miRNA delivery for disease therapy which is currently hindered by a lack of in vivo delivery systems. Small molecule inhibitors and enhancers of the RNA interference (RNAi) pathway have been previously
identified using both luminescent and fluorescent-based assays. For example, a small molecule inhibitor of miR-21, an oncogenic miRNA linked to several human cancers (Krichevsky and Gabriely 2009), was identified using a cell-based luciferase screen (Gumireddy, Young et al. 2008). This screen identified a compound, diazobenzene that specifically regulated miR-21 and was hypothesised to act transcriptionally. Further analysis of the structure-activity relationship of the molecule led to the development of a new azobenzene core compound with increased activity. Subsequently, two inhibitors and an activator of miR-122 transcription were found by the same lab using the psiCHECK-2 luciferase system (Young, Connelly et al. 2010). They demonstrated that the inhibitors had therapeutic potential in HCC, preventing hepatitis C viral replication in Huh7 cells and reducing HepG2 cell viability (Young, Connelly et al. 2010). Similarly, a small molecule termed Rubone, capable of upregulating the tumour suppressor miRNA, miR-34a, was also found using a cell-based luciferase assay. Rubone was shown to preferentially upregulate miR-34a in HCC in a p53-dependent manner and subsequently downregulated miR-34a targets (Xiao, Li et al. 2014). Further, Lee et al. conducted a small-scale screen using RT qPCR. An approach which was only viable given the small-scale of the library and it successfully identified the miR-182 activator Kenpaullone (Lee, Lee et al. 2016). In another study, a screen of 15 aminoglycosides using a luciferase-based vector in MCF-7 cells for inhibitors of miR-21 identified streptomycin. By directly binding to the precursor (pre-miR-21) streptomycin inhibited processing by Dicer and the authors noted semi-specific action with the downregulation of only one other miRNA out of nine tested (Bose, Jayaraj et al. 2012). The screening platform described in this thesis is similar in design to the psiCHECK-2 vector system previously utilised in miR-122 studies by Connelly et al. (Connelly, Thomas et al. 2012).

In addition, molecules have been identified that affect global miRNA expression. For example, Enoxacin, a fluoroquinolone which is used as an antibacterial compound, was identified in a cell-based fluorescence assay to globally enhance miRNA expression, including miR-7, via enhanced RISC loading (Shan, Li et al. 2008). Enoxacin was shown to have anti-proliferative effects in a broad range of cancer cell lines (Melo, Villanueva et al. 2011). Further, curcumin has been reported to significantly upregulate miR-7 expression, among other miRNAs in pancreatic cancer resulting in reduced migration, invasion and cell growth, and induced apoptosis (Ma, Fang et al. 2014, Bi, Shen et al.
Similarly, curcumin upregulated miR-7 in nasopharyngeal carcinoma resulting in Skp2 downregulation and subsequently inhibiting cell growth and triggering apoptosis (Feng, Wang et al. 2017). Trichostatin A was found to upregulate miR-7, possibly through transcriptional upregulation of the miR-7-3 host gene PGSF1a, and was shown to subsequently downregulate EGFR expression in MDA-MB-231 breast cancer cells (Tu, Chen et al. 2014). Other reports describe Trichostatin A as both up and downregulating multiple miRNAs in MCF-7TN-R breast cancer cells, however, miR-7 was not identified in these studies (Rhodes, Nitschke et al. 2012). A recent study published during the final phase of this thesis identified that Breviscapine could upregulate miR-7 in NSCLC. The relatively high dose of 100 μM induced a ~7-fold increase in miR-7 expression in A549 cells (Zeng and Cai 2017). As no other miRNAs were looked at and there are limited miRNA-based studies on Breviscapine, it is unknown whether Breviscapine is specific for miR-7 upregulation. Similarly, Oleic acid was shown to induce miR-7 expression in HeLa cells. As oleic acid inhibited the binding of negative miR-7 regulators HuR and MSI2 to the pri-miR-7 transcript, it restored miR-7 processing (Kumar, Velasco et al. 2017).

Experimentally validated small molecules (and supporting information) that have been identified to regulate miRNA expression are searchable in the database ‘SM2miR’ (http://bioinfo.hrbmu.edu.cn/SM2miR) (Liu, Wang et al. 2013). There exist other databases that encompass confirmed and computationally predicted small molecule modulators of miRNA, such as, miREnvironment a database which includes experimentally supported miRNA perturbations as a result of environmental factors, including compound treatments (Yang, Qiu et al. 2011), and Psmir, a database for predicted associations between small molecules and miRNAs based on the similarity of transcriptional responses (Meng, Wang et al. 2016). Meanwhile, others have proposed novel methods of predicting small molecule-miRNA interactions such as functional similarities of perturbed genes (Wang, Meng et al. 2016). Whilst a number of compounds have been identified to regulate miR-7, compounds which specifically upregulate miR-7 have not been reported previously and formed the basis for this investigation.
4.1.2. Luminescence vs fluorescence-based assays

For cell-based reporter gene assays such as the one developed here, fluorescent and luminescent reporter genes are most commonly used. A luciferase reporter assay is based on the expression of the luciferase enzyme, which, when provided with its substrate will undergo an enzymatic reaction, generating a measurable light output in the process. There are many sources of luciferase with *Renilla* (sourced from the sea pansy *Renilla reniformis*) and Firefly (sourced from the beetle *Photinus pyralis*) luciferases being the most commonly used forms. Both *Renilla* and Firefly luciferase require different substrates and cofactors and produce different wavelengths of light making the measurement of their activity in the same sample feasible.

On the other hand, fluorescent assays require an external source of photons to excite a fluorophore which produces a signal that is detected. This method has higher background than luciferase assays and therefore reduced signal-to-noise ratio and sensitivity. In addition, the slow turnover of fluorescent proteins such as GFP means that assay sensitivity is further compromised. However, this issue can be somewhat circumvented by including destabilisation sequences in the reporter construct (Li, Zhao et al. 1998). Furthermore, specificity of fluorescence-based assays is limited as some compounds emit fluorescence (Fan and Wood 2007). Therefore, luminescence-based assays are the preferable method for cell-based drug screening.

4.1.3. Screening for compounds that modify microRNA-7 expression

The overarching aim of Part II of this thesis was to identify small molecule modifiers of miR-7 expression and validate them in cancer cell systems. In this chapter a cell-based reporter system is generated for the purpose of screening 800 naturally occurring small molecules from the NPL-800 library (TimTec) to identify those that upregulate endogenous miR-7 *in vitro*.

In this project, the psiCHECK-2 vector, a plasmid encoding both Firefly and *Renilla* luciferase was used as the backbone for the reporter system. Vectors were generated with a concatemer of either three (3mer) or six (6mer) perfect miR-7 target sites cloned into the 3’UTR of the *Renilla* luciferase gene. By compounding the perfect target
sequence the likelihood of at least one binding event is increased (Doench, Petersen et al. 2003). Earlier studies have shown that when target sequences are compounded, upregulated miRNAs are not sequestered (Brown, Gentner et al. 2007, Kato, Miyaki et al. 2009). It is suggested that this is due to the high catalytic rate achieved when using a perfectly complementary system (Brown, Gentner et al. 2007). The advantage of a dual-luciferase system where both luciferase genes are encoded on the same plasmid, such as psiCHECK-2, is that Firefly luminescence may be used for normalisation of the Renilla luciferase reporter expression, accounting for variation in transfection efficiency and cell viability. Small molecules which result in increased levels of miR-7 will reduce the Renilla luciferase signal via binding to one of the sites in the 3’UTR, as illustrated in Figure 18. Once normalised to Firefly expression, the ratio of active compounds will be lower, the extent of which will be determined by their potency, than inactive compounds. Conversely, a reduction in miR-7 expression will theoretically result in an increased ratio where Renilla luciferase expression is increased relative to Firefly luciferase due to a lack of transcriptional inhibition by endogenous miR-7. While in cancer treatment it can be argued that identification of miR-7 activators are of greater interest, compound inhibitors of miR-7 may be of therapeutic value in other diseases such as diabetes, where downregulation of miR-7 is associated with improved insulin secretion (Latreille, Hausser et al. 2014). While they are not looked at here, this platform could, in theory, be used for this purpose.

In addition to assessing the sensitivity of psiCHECK-2 vector constructs with either three or six miR-7 target sites, three different CRC cell lines (HCT116, and HT29, SW620) are assessed for their suitability as hosts. CRC is currently treated with targeted EGFR inhibitors such as cetuximab (Chong and Janne 2013) making it a potential target for treatment with miR-7 activators. In addition, miR-7 is downregulated in a number of CRC cell lines when compared to normal colon tissue and ectopic miR-7 expression has been shown to inhibit cell proliferation and induce apoptosis (Zhang, Li et al. 2013, Xu, Chen et al. 2014, Suto, Yokobori et al. 2015).
4.1.4. Use of the Z’factor

A Z’factor is a statistical parameter used in high throughput screening to evaluate an assay platform which takes into account the signal-to-noise ratio and assay variability (Zhang, Chung et al. 1999). An assay with the recommended signal range and level of variation is generally accepted as having a Z’factor ≥ 0.4 and this criterion was used when assessing the screening platform during development. The equation for the Z’factor can be found in section 2.9.1. Minimal data variability and a large difference in the mean values of controls are required to achieve an adequate separation band suitable for screening. Figure 19 illustrates the relationship between these parameters.

4.1.5. Chapter aims

The specific aims of this chapter are to:

1) Characterise the action of miR-7 in the CRC cell lines HCT116, HT29 and SW620.
2) Generate a dual-luciferase reporter vector sensitive to miR-7 regulation.
3) Select the most appropriate cell line based on responsiveness to miR-7 action and sensitivity of the vector platform in the cells.
4) Generate a cell line stably expressing the dual-luciferase reporter system and test its effectiveness as a screening tool.
Small molecule activator

Figure 18. Schematic of the cell-based dual-luciferase reporter system for detecting activation of miR-7 expression.
Six perfect miR-7 target sites were cloned downstream of the Renilla luciferase gene and miR-7 upregulation will result in inhibition of Renilla luciferase expression. Small molecule activators of miR-7 expression will be detected by a reduction in Renilla luciferase signal relative to Firefly luciferase.

Figure 19. An illustration of how the Z’factor is used to evaluate the signal window of an assay.
The Z’factor is used to evaluate the separation band and is a ratio of the data variability (where σ is the standard deviation) and the dynamic range (where μ is the mean) of the assay. The dynamic range is a measure of the difference between the positive and negative controls. This figure has been reprinted from (Zhang, Chung et al. 1999).
4.2. Results

4.2.1. Cell line selection

To select an appropriate CRC cell line as the host line for conducting the cell-based screen the three CRC cell lines HT29, HCT116 and SW620 were compared. It is important that basal miR-7 levels in these cell lines are low and that miR-7 expression can be activated to ensure miR-7 processing pathways are active. RT qPCR was used to confirm this. Further, Western blot was used to confirm miR-7 inhibits known miR-7 target proteins in these cell lines as this will be used to indicate that hit compounds which upregulate miR-7 have a functional biological effect. Finally, the three cell lines were compared in pilot transient assays using the screening vector to select the cell line with the most sensitive response to miR-7 upregulation.

4.2.1.1. Comparative basal miR-7 levels

To compare basal levels of miR-7 expression in SW620, HCT116 and HT29 RT qPCR analysis was performed as greater levels of endogenous miR-7 may negatively affect the dynamic range of the assay. The relative level of miR-7 expression is given in Figure 20 and no significant difference was observed between the cell lines.

4.2.1.2. MicroRNA-7 upregulation by HOXD10

As miR-7 expression is reduced in several CRC cell lines compared to normal colon tissue (Zhang, Li et al. 2013, Xu, Chen et al. 2014, Suto, Yokobori et al. 2015), it was necessary to verify that miR-7 processing pathways are active and inducible via endogenous mechanisms. HOXD10 is a transcription factor which has been shown to upregulate miR-7 at the transcriptional level (see Figure 4 herein) (Reddy, Ohshiro et al. 2008). To test miR-7 was induced in response to HOXD10 overexpression SW620, HCT116 and HT29 CRC cells along with Huh7 HCC cells (included to demonstrate effect was not tissue-specific) were transiently transfected with either empty vector (pcDNA3) or pcDNA3-HOXD10. RNA from the transfected cells was harvested for RT qPCR analysis. The relative level of miR-7 expression as a result of HOXD10 upregulation was 2.1-fold in SW620,
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59.5-fold in HCT116, 2.6-fold in HT29 and 5.0-fold in Huh7 as shown in Figure 21. All cell lines showed inducible miR-7 expression confirming that the miR-7 processing pathway is active in these cells and therefore they represent suitable hosts for the drug screening platform.
Figure 20. RT qPCR analysis of basal miR-7 expression levels in SW620, HT29 and HCT116 cell lines.

miR-7 expression was normalised to U44 expression and is shown as a ratio to SW620 cells ± SD using the $2^{\Delta\Delta CT}$ method. The result shown is representative of two independent experiments.
Figure 21. RT qPCR analysis of HOXD10 induced miR-7 expression in SW620, HCT116, HT29 and Huh7 cells.
Cells were transfected with either pcDNA3 control or pcDNA3-HOXD10 using Lipofectamine 2000 and RNA was extracted 24 h later. miR-7 expression was normalised to U44 and is expressed relative to pcDNA3 only transfected cells ± SD using the 2^-ΔΔCt method (Livak and Schmittgen 2001). The result shown is representative of two independent experiments. *, p < 0.05; ****, p < 0.0001.
4.2.1.3. Basal EGFR, Akt and phospho-Akt

As miR-7 directly targets EGFR and downregulates downstream Akt signalling (pAkt), the endogenous levels of these proteins may affect the way that each of the cell lines responds to miR-7 regulation. A Western blot was performed to compare basal levels of EGFR, Akt and pAkt between SW620, HCT116 and HT29 cells to help determine their suitability for the drug screening platform. The results in Figure 22 show variable and inversely correlated endogenous expression of Akt and active pAkt across the cell lines. No EGFR expression was observed in SW620 and this was then found to be consistent with previous literature (Hughes, Rees et al. 2011) and suggests that this cell line would not be an optimal model to investigate miR-7 regulation as EGFR downregulation will be used to indicate successful miR-7 upregulation by hit compounds.

4.2.1.4. Effect of miR-7 on EGFR, Akt and pAkt protein expression

miR-7 has previously been shown to directly inhibit EGFR expression and indirectly inhibit Akt activity in other cancer types including head and neck (Kalinowski, Giles et al. 2012), prostate, breast and lung (Webster, Giles et al. 2009), and glioblastoma (Kefas, Godlewski et al. 2008). To confirm miR-7 could inhibit these proteins in CRC as well, a Western blot was performed to determine the effect of miR-7 on the expression of the known miR-7 target, EGFR and downstream Akt signalling in HCT116, HT29 and SW620 cell lines. Transfection with miR-7 (30 nM) resulted in a decrease in EGFR expression in HCT116 and HT29 cells (SW620 has no detectable level of EGFR to begin with) as well as a decrease in pAkt in all three cell lines (Figure 23 through Figure 25). Western Blot analysis was conducted at 72 h (as opposed to 24 h for miR-7 expression RT qPCR experiments) to allow time for the miRNA to associate with RISC, repress target protein expression and for this effect to be reflected following protein turnover.

4.2.1.5. Cell viability dose-response assay

A cell titer assay was performed to assess the dose-response effect of miR-7 on cell viability. SW620, HCT116 and HT29 cells were transfected with miR-7 or miR-NC over a
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range of concentrations (0 – 60 nM). GraphPad Prism software (version 5.04) was used to determine the half maximal effective value (EC$_{50}$) to compare sensitivity. SW620 (Figure 26) and HCT116 (Figure 27) cells were sensitive to miR-7 in the low nanomolar range. However, HT29 cells (Figure 28) were less sensitive to miR-7 with greater than 50% of cells remaining viable when treated with the highest concentration of miR-7 (60 nM). However, both HT29 and HCT116 also display sensitivity to miR-NC. The sequence of miR-NC is proprietary information and so the reason for this can only be speculated.

If modifications made to miR-NC by the supplier are sequence-based and do not affect RISC binding, high concentrations of miR-NC could saturate RISC. This would result in miR-NC competing with endogenous miRNAs which have regulatory roles in the cell, thereby affecting cell viability (Singh, Narang et al. 2011). While miR-NC affected both HCT116 and HT29 cells, the effect of miR-NC was greater than that of miR-7 in HT29 cells. This is believed to be due to cell specific off-target effects.
Figure 22. Western blot and densitometry analysis of basal EGFR, Akt and pAkt levels in HT29, HCT116 and SW620 cells.

Western blot and densitometry analysis comparing basal EGFR, Akt and pAkt in HT29, HCT116 and SW620 cells. β-actin is included as a loading control. The result is representative of two independent experiments, duplicates are biological replicates. Densitometry is normalised to β-actin and expressed relative to HT29. *, p < 0.05, **, p < 0.01, ***, p < 0.001.
Figure 23. Western blot and densitometry analysis of EGFR, Akt and pAkt levels in HT29 cells following transfection with miR-7.

Western blot and densitometry analysis of EGFR, Akt and pAkt levels in HT29 cells lines when treated with miR-7. Protein was harvested 72 h post-transfection with miR-7 (30 nM), miR-NC (30 nM) or Lipofectamine 2000 (LF) only. β-actin is included as a loading control. The result is representative of three independent experiments. Densitometry is normalised to β-actin and expressed relative to LF. *, p < 0.05, **, p < 0.01, given relative to LF.
Figure 24. Western blot and densitometry analysis of EGFR, Akt and pAkt levels in HCT116 cells following transfection with miR-7.

Western blot and densitometry analysis of EGFR, Akt and pAkt levels in HCT116 cell lines when treated with miR-7 (30 nM), miR-NC (30 nM) or Lipofectamine 2000 (LF) only. β-actin is included as a loading control. The result is representative of three independent experiments. Densitometry is normalised to β-actin and expressed relative to LF. *, p < 0.05, **, p < 0.01, given relative to LF.
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Figure 25. Western blot analysis of EGFR, Akt and pAkt levels in SW620 cells following transfection with miR-7.

Western blot analysis of EGFR, Akt and pAkt levels in SW620 cells when treated with miR-7. Protein was harvested 72 h post-transfection with miR-7 (30 nM), miR-NC (30 nM) or Lipofectamine 2000 (LF) only. β-actin is included as a loading control. The result is representative of two independent experiments. Densitometry is normalised to β-actin and expressed relative to LF.
Figure 26. EC₅₀ of miR-7 in SW620 cells.
SW620 cells were transfected with varying concentrations of miR-7 (0.03, 0.3, 1.5, 3, 6, 12, 30, 60 nM). The half maximal effective concentration (EC₅₀) of miR-7 was determined after measurement of the relative number of viable cells by cell titre assay 72 h after transfection. Data are normalised to the lowest concentration of miR-7. Error bars represent SDs. Data are representative of three independent experiments.

Figure 27. EC₅₀ of miR-7 in HCT116 cells.
HCT116 cells were transfected with varying concentrations of miR-7 (0.03, 0.3, 1.5, 3, 6, 12, 30, 60 nM). The half maximal effective concentration (EC₅₀) of miR-7 was determined after measurement of the relative number of viable cells by cell titre assay 72 h after transfection. Data are normalised to the lowest concentration of miR-7. Error bars represent SDs. Data are representative of three independent experiments.
Figure 28. EC$_{50}$ of miR-7 in HT29 cells.
HT29 cells were transfected with varying concentrations of miR-7 (0.03, 0.3, 1.5, 3, 6, 12, 30, 60 nM). The half maximal effective concentration (EC$_{50}$) of miR-7 was determined after measurement of the relative number of viable cells by cell titre assay 72 h after transfection. Data are normalised to the lowest concentration of miR-7. Error bars represent SDs. An EC$_{50}$ value for HT29 could not be determined. Data are representative of three independent experiments.
4.2.2. Vector construction

4.2.2.1. Generating psiCHECK-2 miR-7 target reporter plasmids

The psiCHECK-2 vector is a dual-luciferase reporter with a multiple cloning region in the 3’UTR of the Renilla luciferase gene. Two vectors containing either three or six consensus miR-7 target site repeats cloned into the 3’UTR of the Renilla luciferase gene in the psiCHECK-2 vector were generated. These vectors are referred to as psiCHECK-2 (3mer) and psiCHECK-2 (6mer) respectively. Mutant (MT) versions of both vectors were generated with three point mutations in the seed region of the miR-7 consensus sequence to prevent miR-7 binding. An illustration of the consensus and mutant target sites is shown in Figure 29. An illustration of cloning steps to generate both the vector harbouring 3 target sites and six target sites is shown in Figure 30. A vector map of psiCHECK-2 (6mer) is given in Figure 31. All vector maps may be found in the Appendix.
Figure 29. Consensus and mutant miR-7 target sites used in psiCHECK-2 vector construction shown aligned to the hsa-miR-7 sequence.

The miR-7 consensus target site perfectly aligns with the has-miR-7 sequence. Mutant target sequence contained three point mutations in the seed region (seed region shown in red and mutated based are shown in orange). Full-length oligonucleotide sequences harbouring three mutant or consensus miR-7 target sequences cloned downstream of Renilla luciferase in psiCHECK-2 vectors can be found in the methods section.
Figure 30. Concatemerisation strategy used to clone miR-7 target sites into psiCHECK-2 vectors.
An oligomer of three miR-7 target sites was cloned into the XhoI and NotI restriction sites downstream of Renilla luciferase in psiCHECK-2. To insert further miR-7 target sites this vector was further digested with SalI and NotI and the same oligomer (with XhoI and NotI overhangs) was inserted into this site. This is possible due to SalI overhangs being complementary to XhoI. The vector can continue to be concatemerised via repeating the second cloning step.
Figure 31. psiCHECK-2 (6mer) vector map.
A concatemer of six consensus miR-7 target sequences was cloned into Xhol and NotI sites in the 3’UTR of the Renilla luciferase gene (hRluc) in the psiCHECK-2 vector. All vector maps may be found in the appendix.
4.2.2.2. **Specificity of miR-7 target site**

To confirm the specificity of the miR-7 reporter construct for detecting miR-7, the consensus psiCHECK-2 (6mer) vector or control containing mutant target sites (MT) was transfected into HCT116, HT29 and SW620 cells along with either miR-7, miR-NC, pcDNA3-HOXD10 or pcDNA3 vector only. As shown in Figure 32 A) *Renilla* levels in mutant vector-transfected cells were not significantly affected by the presence of miR-7. Figure 32 B) compares the effect of miR-7 on the consensus vector only and shows HT29 and HCT116 cell lines are more responsive to elevated miR-7 levels whether by HOXD10-mediated activation of miR-7 transcription or transfection of miR-7 mimic. Thus, the reporter shows specificity for miR-7.

4.2.2.3. **Three and six target site concatemer sensitivity**

Next, to determine if the sensitivity of the psiCHECK-2 vector is increased by increasing the number of consensus sites, vectors containing either three or six repeats of the miR-7 target site were co-transfected into HT29 and HCT116 cell lines with either miR-7, miR-NC, pDNA3-HOXD10 or vector control and subsequently assayed. The psiCHECK-2 (6mer) vector showed a small but significant improvement in assay sensitivity for HOXD10 induced miR-7 expression when compared with the vector containing three target sites in both cell lines, but not with miR-7 mimic (Figure 33). Therefore, the psiCHECK-2 (6mer) was chosen for subsequent experiments.
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A)

Figure 32. A luciferase reporter assay comparing the specificity and sensitivity of the psiCHECK-2 (6mer MT) vector or psiCHECK-2 (6mer) consensus vectors in HCT116, HT29 and SW620 cells. 

A) Consensus (Cons) or mutant (MT) psiCHECK-2 vectors were co-transfected with either miR-NC, pcDNA3-HOXD10, miR-7 or psiCHECK-2 (6mer) consensus/psiCHECK-2 (6mer) MT only (vector only). Luciferase activity was measured 24 h post-transfection. Renilla luciferase activity was normalised to Firefly luciferase activity. Data are expressed as a ratio of psiCHECK-2 (6mer MT) cells treated with vector only ± SD for each cell type. 

B) The same data as in A) of cells transfected with the consensus vector expressed relative to cells treated with consensus vector only ± SD for each cell type to highlight the sensitivity of the consensus vector. The result shown is the average of two independent experiments for HCT116 and HT29 and a single experiment for SW620. *, p < 0.05; ****, p < 0.0001.
Figure 33. A luciferase reporter assay comparing the sensitivity of psiCHECK-2 (3mer) vs psiCHECK-2 (6mer) consensus vectors. psiCHECK-2 (3mer) and (6mer) vectors were co-transfected with either miR-NC, pcDNA3-HOXD10, miR-7 or psiCHECK-2 (3mer)/psiCHECK-2 (6mer) only (vector only) in HCT116 and HT29 cells. Luciferase activity was measured 24 h post-transfection. Renilla luciferase activity was normalised to Firefly luciferase activity. Data are expressed as a ratio of cells treated with vector only ± SD for each cell type and vector. Data are the average of two independent experiments. *, p < 0.05 (HOXD10).
4.2.3. Stable cell line construction

Having confirmed the suitability of HT29 cells and vector construct sensitivity a stable cell line was constructed. Stable cell-based assay platforms offer improved assay sensitivity, are less labour intensive once generated and reduce the cost of transfection reagents. To generate a stable HT29 psiCHECK-2 (6mer) cell line attempts were made at co-transfection of the psiCHECK-2 (6mer) vector with pBABE-puro followed by puromycin selection with little success. To increase the probability of generating a stable line a Neomycin resistance cassette was cloned into the BamHI site of psiCHECK-2 (6mer) to generate the psiCHECK-2 (6mer + NeoR) pictured in Figure 34. The psiCHECK-2 (6mer) vector was compared with the newly generated psiCHECK-2 (6mer + NeoR) vector via luciferase assay. Both vectors responded to the addition of miR-7 and HOXD10 and little to no change was seen in vector sensitivity as a result of the Neomycin resistance cassette (Figure 35). Therefore, the psiCHECK-2 (6mer + NeoR) vector was deemed suitable for generation of a stable cell line.

4.2.3.1. Selection of stable HT29 psiCHECK-2 (6mer + NeoR) reporter cell line

To generate a stable cell line bearing the psiCHECK-2 (6mer + NeoR) vector, HT29 cells were transfected with the linearised plasmid and subsequently cultured in media containing a final concentration of 500 μg/mL G418/Geneticin. This concentration was determined as the point at which over 80% cell death occurred after 72 h and complete cell death was observed after two weeks. Prior to isolating single clones, the mixed population was assayed for luciferase expression to ensure some level of stable integration of the plasmid. One month post-transfection single clones were isolated and of these, the clone most responsive to miR-7 and which displayed the least amount of variation (Z’factor calculation) was chosen (data not shown).
Figure 34. The psiCHECK-2 (6mer + NeoR) vector map.
A Neomycin resistance cassette was cloned into the BamHI site of the psiCHECK-2 (6mer) vector. All vector maps may be found in the appendix.
A luciferase reporter assay in HT29 cells transfected with either psICHECK-2 (6mer) or psICHECK-2 (6mer + NeoR) vectors using Lipofectamine 2000. psICHECK-2 vectors were co-transfected with either miR-NC, pcDNA3-HOXD10, miR-7 or psICHECK-2 (6mer)/psICHECK-2 (6mer + NeoR) only (vector only). Luciferase activity was measured 24 h post-transfection. Renilla luciferase activity was normalised to Firefly luciferase activity. Data are expressed as a ratio of vector-transfected cells treated with LF only ± SD for each vector. Data are representative of a single experiment. ****, p < 0.0001.
4.2.4. Stable assay platform

Figure 36 shows the assay performance in stable HT29 psiCHECK-2 (6mer + NeoR) cells. The Z’factor was 0.5 using 1 nM miR-7 and 0.8 using 10 nM miR-7 when compared to the 0.1% DMSO control in Z’factor calculations. Both Z’factors were considered to be excellent, with ≥ 0.5 deemed ‘excellent’. Treatments were conducted in triplicate and the percent coefficient of variation (%CV) for these treatments is given in Table 6. The standard deviation of samples has a great impact on the Z’factor calculation. The advantage of a stable line is that there will be less variability, and more reproducible and consistent results (as demonstrated by the small error bars in Figure 36). Therefore, the HT29 psiCHECK-2 (6mer + NeoR) stable line is the most optimal cell line for drug screening due to reduced variability, cost and labour.

4.2.5. Dose-response

To gauge the sensitivity of the stable platform for detecting variations in miR-7 levels a dose-response assay for transfected miR-7 was carried out. As the concentration of miR-7 transfected increased, the relative Renilla signal was diminished. However, the relationship was not linear with small quantities of miR-7 e.g. 1 nM, having a significant effect (Figure 37) suggesting that even a small upregulation of miR-7 could be detected using this system.
Figure 36. Assessing the sensitivity of the HT29 psiCHECK-2 (6mer + NeoR) stable assay platform.

HT29 psiCHECK-2 (6mer + NeoR) stable cell line treated with 0.1% DMSO, Lipofectamine 2000 (LF) or transfected with either miR-7 or miR-NC. Treatments were conducted in triplicate. Luciferase activity was measured 24 h post-transfection. Renilla luciferase activity was normalised to Firefly luciferase activity. Data are expressed as a ratio of cells treated with 0.1% DMSO ± SD. Data are representative of a single experiment. **, p < 0.01; ****, p < 0.0001.

Table 6. Percent coefficient of variation (%CV) for the pilot HT29 psiCHECK-2 (6mer + NeoR) stable assay platform (Figure 36).

As treatments were conducted in triplicate, three wells have been used to calculate %CV.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Coefficient of variation (CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO (0.1%)</td>
<td>2</td>
</tr>
<tr>
<td>LF</td>
<td>2.7</td>
</tr>
<tr>
<td>miR-NC (1 nM)</td>
<td>9.7</td>
</tr>
<tr>
<td>miR-7 (1 nM)</td>
<td>8.5</td>
</tr>
<tr>
<td>miR-NC (10 nM)</td>
<td>5.3</td>
</tr>
<tr>
<td>miR-7 (10 nM)</td>
<td>4.3</td>
</tr>
<tr>
<td>miR-NC (30 nM)</td>
<td>8.8</td>
</tr>
<tr>
<td>miR-7 (30 nM)</td>
<td>8.6</td>
</tr>
</tbody>
</table>
Figure 37. A luciferase reporter assay to assess the dose-response of HT29 psiCHECK-2 (6mer + NeoR) stable cell line to variable miR-7 levels.
Cells were transfected with either Lipofectamine 2000 only (0 nM) or various concentrations of either miR-7 or miR-NC (up to 30 nM). Luciferase activity was measured 24 h post-transfection. Data are representative of a single experiment. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
4.3. Discussion

This chapter describes the generation of a miR-7 screening platform comprised of HT29 CRC cells stably transfected with a dual-luciferase vector modified to contain six consensus miR-7 target sites.

4.3.1. Choosing an appropriate host cell line

In order to identify the cell line most suited to hosting the screen, three CRC cell lines were compared (SW620, HT29 and HCT116). As mentioned previously, miR-7 expression is downregulated in CRC compared to normal tissue and it was predicted that miR-7 activators would be clinically useful for these cancers (Zhang, Li et al. 2013, Xu, Chen et al. 2014, Suto, Yokobori et al. 2015). In determining the most appropriate of these cell lines it was crucial that the ability to upregulate miR-7 was confirmed and that the assay was sensitive to miR-7 upregulation in screening pilots. Further, low endogenous levels of miR-7 are preferable as this would improve the dynamic range and sensitivity of the screen by reducing basal inhibition of the reporter by endogenous miR-7. Endogenous levels of miR-7 were measured by RT qPCR in HCT116, HT29 and SW620 (Figure 20) with no significant difference observed in miR-7 expression levels between the cell lines. These findings differ from those published previously by Zhang et al., who reported SW620 to have ~ 10-fold more endogenous miR-7 than HCT116 or HT29 (Zhang, Li et al. 2013). This could be due to a difference in reference gene used (Zhang et al. measured miR-7 relative to U6 while here miR-7 was measured relative to U44). There is also the possibility of normal genetic drift of the cell lines, however, all cell lines were STR typed and all cells lines were between a 93% and 100% match to ATCC repository cell lines. Endogenous miR-7 in the SW620 cell line is more than 5 times lower than in normal colonic mucosa. However, HT29 and HCT116 cell lines were not examined in this study (Xu, Chen et al. 2014). Given the similarity in levels detected between SW620, HCT116 and HT29 this low level of miR-7 expression is ideal for use in the screening platform.

The effect of endogenous miR-7 on the screening platform was evident in pilot experiments conducted with mutant and consensus psiCHECK-2 (6mer) vectors (Figure 32). The ratio of luciferase expression of cells transfected with the psiCHECK-2 (6mer) vector only is significantly reduced in comparison to cells transfected with the psiCHECK-
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2 (6mer MT) vector only. However, there was very little effect on the dynamic range of pilot screens using consensus vectors in all three cell lines (Figure 32 B). Of note, the effect of miR-7 transfection was marginally but significantly greater in HT29 cells as opposed to both HCT116 and SW620.

Defects in miRNA processing pathways have previously been noted in cancer cell lines, for example, Melo et al. showed the CRC cell line Co115 harboured a mutation in the TARBP2 gene. This lead to diminished TRBP expression, a core protein in Dicer processing thereby impairing miRNA processing from pre-miRNA to mature miRNA (Melo, Ropero et al. 2009). The transcription factor HOXD10, known to induce miR-7 expression (Reddy, Ohshiro et al. 2008), was used to confirm miR-7 upregulation in the cell lines used here, verifying miR-7 processing pathways are active. All cell lines showed significant upregulation of miR-7 by HOXD10 (Figure 21). HCT116 showed the greatest increase in miR-7 expression (approximately 60-fold) while other cell lines displayed more modest but significant upregulation. It is important to note that this experiment was performed by transient transfection of the pcDNA3-HOXD10 plasmid using Lipofectamine 2000. Transfection efficiency using Lipofectamine 2000 is markedly affected by a number of factors including the cell type, viability and confluency. The distributing company, Thermo Fisher Scientific, report a less than 10% transfection efficiency for SW620 cells using Lipofectamine 2000 (Thermo Fisher Scientific, https://www.thermofisher.com/au/en/home/references/gibco-cell-culture-basics/transfection-basics/factors-influencing-transfection-efficiency.html). Therefore, the level of miR-7 upregulation upon pcDNA3-HOXD10 transfection is not directly comparable between cell lines. In addition, as only a subpopulation of cells will be successfully transfected, the level of miR-7 upregulation observed here is expected to be an underrepresentation. Encouragingly, despite only modest upregulation in HT29, the effect of HOXD10 transfection in psiCHECK-2 (6mer) luciferase pilots in this cell line was significant (Figure 32B). While previous efforts have shown HOXD10 can upregulate miR-7 in the breast cancer cell line MCF10DCIS and human embryonic kidney cells HEK293 (Reddy, Ohshiro et al. 2008), the results shown here further confirm the action of this transcription factor in both CRC and HCC cell lines.

It is undesirable for miR-7 to negatively affect the viability of the host cell line as this could affect the identification of positive hit compounds that promote miR-7
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overexpression i.e. in dead/dying cells both the internal control (Firefly) and miR-7 reporter (Renilla) would be affected thereby disturbing the derived ratios. EC\textsubscript{50} data indicated that SW620 was moderately sensitive to miR-7 (EC\textsubscript{50} = 3.88 nM) when compared to HCT116 (EC\textsubscript{50} of 1.59 nM). There was no significant inhibition of the proliferation of HT29 cells with increasing concentrations of miR-7. This is supported by the findings of Suto et al. who, during the course of this PhD, published their findings on the effect of miR-7 in HCT116, SW480 and HT29 cells (Suto, Yokobori et al. 2015). SW480 and SW620 cell lines are derived from primary and secondary tumours, respectively, from the same patient, however, are phenotypically different (Hewitt, McMarlin et al. 2000). Suto et al. showed miR-7 transfection both significantly inhibited the proliferation of HCT116 and SW480 cells and the response was mitigated when transfected with a miR-7 inhibitor. However, the proliferation of HT29 cells was not significantly affected by miR-7 transfection or inhibition. It was suggested that this was due to the KRAS mutation harboured by HCT116 and SW480 cells as opposed to the BRAF affected HT29 cells (Suto, Yokobori et al. 2015). Signalling by cells with a KRAS mutation occurs via RAF-1, also a miR-7 target (Webster, Giles et al. 2009, Kalinowski, Giles et al. 2012), which is not involved in cells with BRAF mutations. In line with what was found by Suto and colleagues, Western blot analysis confirmed miR-7 directed downregulation of EGFR and pAkt protein levels in HCT116 and HT29, as well as pAkt levels in SW620 (Figure 23 through Figure 25). This suggests that miR-7 is functionally active in CRC cells and that a decrease in EGFR and pAkt levels could be used as sensitive biomarkers to validate compound modulation of miR-7. Suto et al. further showed that inhibiting miR-7 reversed this effect and subsequently observed increased protein expression (Suto, Yokobori et al. 2015). Since EGFR and pAkt are downregulated in all HT29 (BRAF mutant), HCT116 and SW620 (KRAS mutants) cells, however miR-7 directed inhibition of proliferation is only evident in the KRAS mutants, this may suggest that inhibition of the miR-7 target RAF-1 downstream of EGFR is responsible and a more potent target in the KRAS mutants. Alternatively, there may be another miR-7 target, which is absent in HT29 cells, that allows for cell viability despite downregulated EGFR and pAkt. The findings by Suto et al. support the findings here regarding HT29 sensitivity and choice of the HT29 cell line to be used as a screening host for miR-7 upregulation, given its level of effect on cell viability.
In summary, in each of the CRC cell lines tested there was little difference in endogenous miR-7 expression, miR-7 expression was inducible by the known miR-7 regulator HOXD10, miR-7 downregulated known direct and indirect targets EGFR and pAkt, EC$_{50}$ data indicated HT29 cells are viable in the range of miR-7 concentrations tested here, and pilot transient luciferase assays showed little difference in the detectability of miR-7 expression between cell lines. Therefore, cell line choice was not a limiting factor in the generation of this screen, however, the HT29 cell line was preferable given the lack of miR-7 directed growth inhibition and marginally but significantly greater luciferase inhibition by exogenous miR-7 in pilot transient luciferase screening assays. The next stage in the development of the screening platform was to generate a miR-7 responsive reporter system and assess its capability for miR-7 detection.

### 4.3.2. Target concatemerisation

Increasing the number of miR-7 target sites in the vector from three to six gave a small but significant increase in the sensitivity of the assay when comparing HOXD10 upregulation of miR-7 ([Figure 33](#)). However, there was no significant difference in vector sensitivity between miR-7 transfected cells. This is largely accounted for by the near complete Renilla luciferase knockdown (luciferase counts near to background levels by miR-7 transfection in both 3 and 6-target vector systems). For this reason, the vector containing 6 miR-7 target sites was chosen for the screen. It was considered that further concatemerisation would not greatly improve assay sensitivity and so additional target sites were not added to the vector. However, it is appreciated that increasing the number of binding sites could lead to small improvements in luciferase inhibition. In a study by Doench *et al.* that examined concatemerisation of target sites containing a central bulge and target sites with perfect complementarity, it was found that increasing the number of perfect sites from one to three improved target inhibition. These target sites functioned independently of one another and increasing their number increased the likelihood of a single miRNA binding event which would result in mRNA cleavage. However, when target sites containing a central bulge were increased from two to six they found that inhibition was more than additive, suggesting that increasing the number of imperfect binding sites could result in a cooperative effect (Doench, Petersen *et al.* 2003). An additional concern is that by further increasing the number of target sites...
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sites available miR-7/RISC complex may be sequestered and act as a sponge. When Brown et al. introduced a high copy number plasmid containing multiple targets, they found that perfectly complementary target sites did not result in miRNA/RISC sequestration, due to the nature of regulation of perfect target sites by miRNAs (target cleavage). However, vectors harbouring imperfect target sites did sequester the miRNA/RISC at high copy number (Brown, Gentner et al. 2007). These findings support the use of perfect miR-7 target sites in this assay system and that by increasing the number of perfect target sites, it is likely there will be improved target inhibition without the risk of depleting available miRNA.

4.3.3. Statistical analysis and assay sensitivity

As mentioned in the introduction of this chapter, the Z’factor is a measure of assay sensitivity, taking into account the signal window (separation of means of the positive and negative control) as well as the variation. A negative Z’factor is given if the signal from positive and negative controls overlap. A Z’factor of between 0 and 0.4 is considered marginal, while an assay with a Z’factor of greater than 0.4 is adequate and a score that falls between 0.5 and 1 would be considered excellent. The Z’factor was used here as a guide to assay performance. The Z’factor calculated in initial transient pilot assays were all considered ‘excellent’ i.e. ≥ 0.5. Later, the Z’factor calculated for the stably generated assay system was 0.8 and this is discussed in the following section.

4.3.4. Stable vs transient assay platform

Cell-based screens may be configured as either stable or transient systems and there are advantages and disadvantages to each method. Generating a stable system can be time-consuming and further, developing a stable system that reliably expresses the protein of interest is challenging as the expression of a transgene in a stable system may diminish over time (Sharma, Dutta et al. 2014). On the other hand, transient systems tend to have higher variability due to transfection efficiency affecting reproducibility and significance of results (Zhang 2012). Due to the nature of transient transfection, there will be a great number of copies of vector in a single cell and suboptimal transfection
efficiency will result in variability of cells expressing plasmid across both the plate and well (i.e. different portions of cells may not receive any plasmid at all). Those that receive a large number of vector copies results in some cells expressing large quantities of mRNA transcripts that need to be inhibited by small molecule-induced miR-7, therefore making the transient platform less sensitive to small changes in miR-7 than a stable line where fewer copies of the transcript are expressed in all cells in a well and across a plate. Therefore, a stable system will be more sensitive to small changes in miRNA activity.

Previous pilot experiments have been conducted using co-transfected miR-7 or HOXD10 vector. This means a cell will simultaneously receive psiCHECK-2 vector and miR-7/HOXD10 (Figure 35) explaining the near complete knockdown of luciferase signal observed for miR-7 mimic transfected cells. Further, due to their increased tendency for variability, transient assay platforms may need to be screened in larger well formats as signal-to-noise ratios can be a challenge for smaller well screening formats, this, in turn, can increase the cost of the screen by increasing reagent consumption.

Stable lines generally increase assay sensitivity, reduce replicate variability and the overall cost of screening (Connelly, Thomas et al. 2012) which was achieved in the stable line generated here (Figure 36). Further, increasing the concentration of miR-7 transfected into the stable system (Figure 37) increased the assay response, therefore, the relative degree of signal inhibition will be indicative of a compounds potency.

4.3.5. Assay limitations

One major limitation of this screening platform was the lack of an appropriate small molecule positive control. Whilst alternatives were sought, such as the compound Enoxacin which has been documented to cause the upregulation of a number of miRNAs including miR-7, albeit only by ~ 2-fold in HCT116 cells (Melo, Villanueva et al. 2011) and Nicorandil reported to upregulate miR-7 expression in astrocytes (glial cells) (Dong, Chen et al. 2016), these controls were unsuccessful here (data not shown). Other possible miR-7 regulating compounds mentioned in the thesis introduction that could have been trialled here were published after this screen was conducted. For Z’factor calculations, positive and negative controls provide the maximum detectable range or signal window.
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For the Z’factors calculated in this thesis the signal decrease upon miR-7 transfection was used as a positive control. The use of miR-7 mimic is not an ideal positive control as it is transfected into the cells using Lipofectamine 2000 which has limited transfection efficiency as mentioned previously. For this reason, it is difficult to compare the stable assay platform to the transient system for sensitivity to miR-7 mimic transfection. In prior transient pilot experiments, the vector and miRNA have been co-transfected therefore eliciting the greatest possible miR-7 driven luciferase inhibition. However, when comparing this assay performance to that of a stable system using transfected miR-7 as a positive control there is one outstanding limitation. In the stable system, all cells express a steady state of luciferase as each contains the vector, upon transfection only a small subset of these cells will receive miR-7. The level of luciferase inhibition is therefore diluted by those untransfected luciferase-expressing cells. As the effect of a compound would not have limited cell targeting efficiency, it is not expected that this will be a limiting factor in determining hit compounds. Therefore, Z’factors here were used as a guide only and not strictly adhered to.

A further limitation is that by requiring a decrease in signal, this assay system is subject to false positives by compounds which affect signal production. Cell death due to cytotoxicity may also affect the Renilla to Firefly ratio further increasing the likelihood that these compounds may appear to be miR-7 regulators. It is a common phenomenon of antagonist or inhibitor assays to have a higher ‘hit rate’ than agonist assay platforms (Zhang, Chung et al. 1999).

In summary, a stable assay platform was developed in HT29 cells using the psiCHECK-2 (6mer + NeoR) vector. The Z’factor calculated in pilot assays with this system was 0.8, indicating an ‘excellent’ separation between signals with minimal overlap. Given the data, this platform was used to screen the Natural Products Library (TimTec) of 800 compounds which is the focus of the next chapter.
5. Screening the library

This chapter describes the screening of the NPL-800 library using the stable psiCHECK-2 (6mer + NeoR) reporter cell line developed in the previous chapter. It outlines compounds identified as hits as well as other compounds of interest and the triage process to select the strongest candidates. These compounds undergo a validation process in the subsequent chapter.

5.1. Introduction

The NPL-800 library, a library of 800 pure naturally occurring compounds, was screened using the stable HT29 psiCHECK-2 (6mer + NeoR) cell line. Hit compounds were identified by a reduction in the ratio of Renilla luciferase (reporter) to Firefly luciferase (internal control) when compared to controls. During the development of this screen there were many general technical considerations to be addressed such as; maximising the signal, edge effect, appropriate inter and intraplate controls, compound library management, inclusion or exclusion of replicates, available luciferase systems and reagents, and choice of compound library.

5.1.1. Maximising the luciferase signal

Several assay conditions can affect the quality of luminescence detection such as the compound solvent, media and plate type. Compound libraries are commonly provided in the solvent DMSO. DMSO is reported to have little or no effect on the luminescence signal when using the Dual-Glo luciferase assay system (Technical Manual, Dual-Glo luciferase assay system, Promega (revised September 2015)). On the other hand, the presence of phenol red in media can adversely affect the luminescence signal strength by absorbing light. Luciferase assays of this nature should be carried out in phenol red-free media. In addition, the colour and opacity of the microtiter plate will affect luminescence readings. Opaque white plates are used in luminescence assays for a couple of reasons; their reflective surface maximises the signal, prevents light from escaping the well and also prevents cross-talk between wells (Technical Manual, Dual-Glo luciferase assay system, Promega (revised September 2015)).
5.1.2. Edge effect

An edge effect or position-related bias may occur due to uneven heat distribution across a plate resulting in uneven evaporation and thereby affecting cell growth. To minimise the impact of this the use of a dual-luciferase system will account for variability in cell number through normalisation of the reporter signal to the internal control. However, a further consideration is the arrangement of compound vs control wells on a plate. While it is possible to customise the format of compound libraries, standard formats include empty wells located in the first and last columns for controls (columns 1 and 12 for a 96-well plate). This position-related bias will affect all data across the plate when compound activity is expressed relative to control wells. To will minimise (but not circumvent) this issue, equal distribution of positive and negative controls in columns 1 and 12 in an interleaved pattern is recommended. This will ensure even distribution of controls across rows as shown in Figure 38 (Malo, Hanley et al. 2006).
Figure 38. Suggested placement of compounds and controls in a 96-well plate. An interleaved pattern of positive and negative controls in the first and last columns of a 96-well plate minimises position related bias and drift across a plate once compound readings are normalised to controls. This figure has been adapted from (Malo, Hanley et al. 2006).
5.1.3. Inter and intraplate controls

Intraplate controls should be included in each plate to ensure responsiveness of the assay system. These control wells should contain treatments that give the maximum and minimum signal range of the screen. A Z’factor may then be calculated for each plate to ensure an adequate screening window. The Z’factor should be ≥ 0.4 for plate acceptance. To monitor the variation in signal between plates, interplate controls (generally vehicle only) should be included and monitored for any fold change (Iversen, Beck et al. 2004).

5.1.4. Compound management

Ensuring proper compound management is crucial for the integrity of the screen. Compounds that undergo many freeze/thaw cycles, as well as prolonged storage, may degrade or precipitate. It is common for researchers to notice changed compound activity over time (Hughes, Rees et al. 2011). In addition, DMSO can absorb water thereby changing the concentration (Chimote, Sreenivasan et al. 2014). In order to minimise this, the stock compound library may be serially diluted to generate working plates which are used and kept only for a short period of time. In the screen presented here stock plates and intermediate plates were stored at -80°C while the working plates were stored at -20°C for a maximum of one week.

5.1.5. Singlicate vs triplicate

While screening a compound library with replicates can have several advantages, it significantly increases the time and cost associated with the screen. On the other hand, screening in singlicate increases the potential for both false negatives and false positives which are also a problem as this can increase the amount of downstream work. Provided an adequate Z’factor is achieved, compound screening is commonly conducted in singlicate. To further minimise costs, in high-throughput screens in which there are thousands of compounds it is sometimes appropriate to ‘pool’ compounds. In other words, mixtures of compounds are tested in one well as opposed to one compound per well. There are obvious potential limitations of this approach, namely, the potential for
interactions between compounds resulting in additivity, synergy, or antagonism (Kainkaryam and Woolf 2009). As just 800 compounds were tested in the screen presented here, compounds were tested individually.

5.1.6. Flash vs glow luminescence

In this screen a Dual-Glo luciferase assay system was used. There are several advantages of a glow-based luciferase system over the alternative ‘flash’ luminescence. Using glow luminescence eliminates the need for a machine with internal injectors as the signal is stable for a number of hours. Further, there is no need for lysate preparation of individual compound treatments, as is the case for flash luminescence, as the substrate reagents also contain a detergent. This again reduces protocol steps providing a much simpler assay format suitable for high-throughput 96-well screens. However, one disadvantage to the glow assay is that it may be subject to ‘drift’ in readings if reagents are added at the same time across wells but have a delay in reading time. Incorporating a time-delay in reagent addition may minimise this.

5.1.7. Further assay considerations

There are many further considerations when planning and conducting a luciferase-based compound screen, including the temperature of luciferase reagents. Luciferase substrates are sensitive to freeze-thaw cycles and gradually degrade over time. In addition, the temperature of these substrates during testing may affect the assay as the temperature will continue to equilibrate during plate reading. Other considerations include the potential for compound precipitation upon dilution into cell culture media, desired compound concentration for screening and interval time. Further, white microtiter plates phosphoresce when exposed to white light. This will affect luciferase readings by giving higher count values that may decrease during the time taken to read the plate, further contributing to plate ‘drift’. To avoid this, plates may be dark adapted prior to being read in the microplate reader. The use of automation where possible will further minimise variability in results which can be crucial for attaining appropriate Z’factors.
5.1.8. Hit compounds

Assays based on the inhibition of signal such as here are commonly prone to higher hit rates than agonist assays as they also detect compounds that may simply interfere with signal generation (Hughes, Rees et al. 2011). In order for compounds to be considered leads, they require further validation in a secondary assay system which is different from the first (Zhang, Chung et al. 1999). Secondary assays are conducted and discussed in the subsequent chapter. Hit compounds identified in this chapter were compounds that sufficiently inhibited reporter expression (greater than three standard deviations from the negative control) and which were confirmed upon re-testing in the same system.

5.1.9. The NPL-800 library by TimTec

The Natural Product Library – 800 (NPL-800) from TimTec is a compound library which contains 800 pure naturally occurring compounds. The compounds have been selected as lead identifying material sourced primarily from plant materials, however, a minority of the compounds are sourced from bacteria, fungus and animals. A major advantage of this library lies in its broad diversity of compounds, which enables screening of a large chemical space. Further, TimTec do not hold intellectual property rights for the compounds (www.timtec.net). The library has been successfully screened for a range of applications (Shaneyfelt, Burke et al. 2006, Bisson, Koch et al. 2009, Nielsen, Kristensen et al. 2012, Luo, Reid et al. 2015). Most importantly, this library has been previously screened by Xiao et al. in 2014 and Rubone was identified to specifically upregulate miR-34a in HCC (Xiao, Li et al. 2014). The library contained 640 compounds at the time and has since been expanded. There are two main advantages to screening natural products: 1) They are proven sources of lead compounds and it is estimated that more than 60% of current anticancer drugs are derived from natural sources (Demain and Vaishnav 2011) and 2) they have great chemical/structural diversity which have been evolved to interact with biological molecules (Hong 2011).
5.1.10. Chapter aims

The specific aims of this chapter are to:

1) Screen the NPL-800 library using the screening platform generated in the previous chapter.

2) Identify and select hit compounds for further validation.
5.2. Results

5.2.1. The effect of DMSO on the screening platform

To confirm that the compound library solvent DMSO does not affect luciferase reporter readings in the assay, the stable psiCHECK-2 (6mer + NeoR) cells were subjected to concentrations of DMSO from 0 – 0.2% for 24 h before luciferase readings were made (Figure 39). There was no significant change in luciferase readings for all DMSO concentrations.
Figure 39. The effect of DMSO on the stable HT29 psiCHECK-2 (6mer + NeoR) cells. Stable HT29 psiCHECK-2 (6mer + NeoR) cells were treated with various concentrations of DMSO (0 – 0.2%) and luciferase readings were made after 24 h. The result shown is representative of a single experiment and error bars represent SDs.
5.2.2. Plate data from the initial screen

Progressing now to the formal screen, compounds were screened in 96-well format and cells were treated for 24 h prior to measurement of luciferase readings. A workflow is provided in Figure 40. Plates that did not meet the Z’-factor cut-off of ≥ 0.4 were screened again. In Figure 41 through Figure 50, a ‘(2)’ following the plate number indicates that the data displayed is that of a rerun due to a poor initial Z’-factor (< 0.4). Three plates (plates 3, 4 and 7) failed to meet the Z’-factor cut-off again in the second run. For these plates, compound hits were compared between the two data sets to look for consistent activity. Several compounds were identified from the library to be potential miR-7 regulators that inhibited luciferase greater than 3 x standard deviations from the DMSO control. However, several compounds were eliminated and not included in further screening upon closer inspection of their raw luciferase counts, which showed many did not exhibit a genuine decrease in Renilla luciferase but instead a substantial increase in Firefly luciferase, distorting the ratio. Compounds selected to be taken forward after the initial screen are highlighted in pink in the following figures (Figure 41 through Figure 50), however, those compounds that were eliminated for the aforementioned reason remain grey, despite appearing below the dotted line (3 x standard deviations of 0.1% DMSO controls) and were not further pursued.
Figure 40. Workflow of the compound screen.
Cells were initially seeded in a white 96-well plate and incubated at 37°C and 5% CO₂ overnight to allow cells to adhere. The following day the cells were either transfected with 10 nM miR-7 or miR-NC, treated with 0.1% DMSO or 10 µM compound. Twenty-four hours following treatment the cells were assayed for luciferase activity.
Figure 41. Screen plate 1.
The average of miR-7 treated wells is given by the solid blue line, the average of miR-NC treated wells is given by the orange solid line and the average of the 0.1% DMSO control is given by the black solid line. Three SDs below the average of the 0.1% DMSO control is given by the black dotted line. Points below this line were considered for the repeat primary screen, those in pink were included in the repeat primary screen. This graph was produced as a result of screening a second ‘(2)’ time as the initial Z’factor was inadequate. The Z’factor of this plate was 0.48. This graph does not include points which lie outside the axis limit of 1.5 (1 point). Technical issues with column 1 of this plate meant that control values in this column were removed and not included in data analysis. For this reason, there are less control data points in this iteration of this plate.
Figure 42. Screen plate 2.
The average of miR-7 treated wells is given by the solid blue line, the average of miR-NC treated wells is given by the orange solid line and the average of the 0.1% DMSO control is given by the black solid line. Three SDs below the average of the 0.1% DMSO control is given by the black dotted line. Points below this line were considered for the repeat primary screen, those in pink were included in the repeat primary screen. The Z’factor of this plate was 0.69. This graph does not include points which lie outside the axis limit of 1.5 (2 points). Compounds falling below the black dotted line but still displayed in grey were eliminated upon closer inspection of raw luciferase counts.
Figure 43. Screen plate 3.
The average of miR-7 treated wells is given by the solid blue line, the average of miR-NC treated wells is given by the orange solid line and the average of the 0.1% DMSO control is given by the black solid line. Three SDs below the average of the 0.1% DMSO control is given by the black dotted line. Points below this line were considered for the repeat primary screen but were eliminated upon closer inspection of raw luciferase counts. The Z’ factor of this plate was 0.03. This graph was produced as a result of screening a second ‘(2)’ time as the initial Z’factor was inadequate. However, upon rescreening the Z’factor was still below 0.4, therefore some points above the dotted line were considered, but none selected. This graph does not include points which lie outside the axis limit of 1.5 (4 points).
**Figure 44. Screen plate 4.**

The average of miR-7 treated wells is given by the solid blue line, the average of miR-NC treated wells is given by the orange solid line and the average of the 0.1% DMSO control is given by the black solid line. Three SDs below the average of the 0.1% DMSO control is given by the black dotted line. Points below this line were considered for the repeat primary screen, those in pink were included in the repeat primary screen. The Z’factor of this plate was -0.22. This graph was produced as a result of screening a second ‘(2)’ time as the initial Z’factor was inadequate. However, upon rescreening the Z’factor was still below 0.4, therefore some points above the dotted line were considered, but none selected. Despite the Z’factor being below 0.4 on both occasions, some compounds below the line were chosen due to having a similar effect on luciferase activity in both runs of plate 4. This graph does not include any points which lie outside the axis limit of 1.5 (1 point).
**Figure 45. Screen plate 5.**
The average of miR-7 treated wells is given by the solid blue line, the average of miR-NC treated wells is given by the orange solid line and the average of the 0.1% DMSO control is given by the black solid line. Three SDs below the average of the 0.1% DMSO control is given by the black dotted line. Points below this line were considered for the repeat primary screen, those in pink were included in the repeat primary screen. The Z’factor of this plate was 0.46. This graph does not include points which lie outside the axis limit of 1.5 (1 point). Compounds falling below the black dotted line but still displayed in grey were eliminated upon closer inspection of raw luciferase counts.
Figure 46. Screen plate 6.
The average of miR-7 treated wells is given by the solid blue line, the average of miR-NC treated wells is given by the orange solid line and the average of the 0.1% DMSO control is given by the black solid line. Three SDs below the average of the 0.1% DMSO control is given by the black dotted line. Points below this line were considered for the repeat primary screen, those in pink were included in the repeat primary screen. This graph was produced as a result of screening a second ‘(2)’ time as the initial Z’factor was inadequate. The Z’factor of this plate was 0.46. This graph does not include points which lie outside the axis limit of 1.5 (3 points). Compounds falling below the black dotted line but still displayed in grey were eliminated upon closer inspection of raw luciferase counts.
Figure 47. Screen plate 7.
The average of miR-7 treated wells is given by the solid blue line, the average of miR-NC treated wells is given by the orange solid line and the average of the 0.1% DMSO control is given by the black solid line. Three SDs below the average of the 0.1% DMSO control is given by the black dotted line. The point below this line was considered for the repeat primary screen but was eliminated upon closer inspection of raw luciferase counts. As the Z' factor of this plate was 0.18, some points above the dotted line were considered. No potential hits were identified. This graph was produced as a result of screening a second ‘(2)’ time as the initial Z'factor was inadequate. However, upon rescreening Z'factor was still below 0.4. This graph does not include points which lie outside the axis limit of 1.5 (2 points).
Figure 48. Screen plate 8.
The average of miR-7 treated wells is given by the solid blue line, the average of miR-NC treated wells is given by the orange solid line and the average of the 0.1% DMSO control is given by the black solid line. Three SDs below the average of the 0.1% DMSO control is given by the black dotted line. Points below this line were considered for the repeat primary screen, those in pink were included in the repeat primary screen. The Z’factor of this plate was 0.46. This graph does not include points which lie outside the axis limit of 1.5 (2 points). Compounds falling below the black dotted line but still displayed in grey were eliminated upon closer inspection of raw luciferase counts.
Figure 49. Screen plate 9.
The average of miR-7 treated wells is given by the solid blue line, the average of miR-NC treated wells is given by the orange solid line and the average of the 0.1% DMSO control is given by the black solid line. Three SDs below the average of the 0.1% DMSO control is given by the black dotted line. Points below this line were considered for the repeat primary screen but were eliminated upon closer inspection of raw luciferase counts. No potential hits were identified. The Z’factor of this plate was marginal, however, upon rescreening the Z’factor was not improved. The Z’factor of this plate was 0.39. There are no points on this plate which lie outside of the axis limit.
Figure 50. Screen plate 10.
The average of miR-7 treated wells is given by the solid blue line, the average of miR-NC treated wells is given by the orange solid line and the average of the 0.1% DMSO control is given by the black solid line. Three SDs below the average of the 0.1% DMSO control is given by the black dotted line. As there were no points below this line, no potential hits were identified. The Z’factor of this plate was 0.51. This graph does not include points which lie outside the axis limit of 1.5 (1 point).
5.2.3. Z’factor

During screening, the Z’factor of each plate was calculated to ensure there was a sufficient signal window. The Z’factor of all plates is shown in **Figure 51**. After repeating any plates that did not meet a Z’factor of 0.4 the first time, seven plates met an acceptable Z’factor of 0.4 while three plates did not meet the cut-off. Despite both plate 4 runs failing to meet the acceptable Z’factor of ≥ 0.4, some compounds were still selected. These were chosen based on careful consideration of the raw data and their similar effect on *Renilla* luciferase activity in both runs. There is the risk that these compounds are false positives due to too much variability in control wells but three compounds were chosen despite this.

The initial screen was conducted of the course of a single day. The repeat screen for plates with poor initial Z’factors was similarly conducted over one day and in the same week as the initial screen. Z’factor, mean and percent coefficient of variation (%CV) values for DMSO and miR-7 controls are given in **Table 7**. There appeared to be no position related bias to the variation of controls.

5.2.4. Repeat primary screen

Potential hits identified in initial screening runs were validated a second time on the one plate to ensure that their activity in the primary screen was reproducible (**Figure 52**). This also allowed individual compound ratios to be comparable to one another. At this stage, several more compounds were eliminated from further testing, which is discussed below.
Results chapter: Screening the library

Figure 51. Plate Z’factors.
Plates that generated a Z’factor below 0.4 (indicated by the red solid line) in the initial screen (blue circles) were rescreened (orange circles). However, upon rescreening in three cases (plates 3, 4 and 7) adequate Z’factors were not achieved. These plates were not further repeated.

Table 7. Z’factor, mean and percent coefficient of variation (%CV) values for controls.
Four DMSO control wells and six miR-7 control wells were used to calculate values. ‘NA’ (not applicable) is given where a plate was not included in the repeat screen.

<table>
<thead>
<tr>
<th>Plate</th>
<th>Initial screen</th>
<th>Repeat screen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Z’</td>
<td>DMSO</td>
</tr>
<tr>
<td>1</td>
<td>-0.25</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>0.68</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>-0.17</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>-0.75</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>0.46</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>-0.01</td>
<td>1.0</td>
</tr>
<tr>
<td>7</td>
<td>0.18</td>
<td>1.0</td>
</tr>
<tr>
<td>8</td>
<td>0.46</td>
<td>1.0</td>
</tr>
<tr>
<td>9</td>
<td>0.39</td>
<td>1.0</td>
</tr>
<tr>
<td>10</td>
<td>0.51</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Figure 52. Repeat primary screen of compounds identified with potential from initial screening (plates 1-10).
The average of miR-7 treated wells is given by the solid blue line, the average of miR-NC treated wells is given by the orange solid line and the average of the 0.1% DMSO control is given by the black solid line. Three SDs below the average of the 0.1% DMSO control is given by the black dotted line. The Z’factor of this plate was -0.21 however, this plate was not rescreened.
5.2.5. Tentative hit compound data analysis and triaging

Hit compounds were those compounds identified in the initial screen and where activity was confirmed upon retesting in the repeat primary screen. All compounds that were identified in the initial screen are given in Table 8.

The compounds were then triaged based on several criteria; the ratio of *Renilla*/*Firefly* was reduced more than 3 x standard deviations from DMSO controls in primary and repeat screens, raw Firefly luciferase values were not greater than 1 x standard deviation of raw DMSO Firefly values and raw *Renilla* counts were less than 1 x standard deviation of raw DMSO *Renilla* counts. These are outlined for all compounds included in the primary repeat screen in Table 9. Six compounds were selected and taken forward for further validation based on their performance in both screens. These compounds are listed in Table 10.
Table 8. Potential hit compounds and their Firefly/Renilla ratio in initial and repeat screens.

Raw ratio values for the initial screen (Ratio 1), rerun (if conducted due to an inadequate Z’factor, Ratio 2) and the repeat primary screen (Ratio 3). Plates were either repeated due to variability in the first screen or not screened (NS) a second time as Z’factor was acceptable and hits should have been confidently detected.

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Plate</th>
<th>Well</th>
<th>Ratio 1</th>
<th>Ratio 2</th>
<th>Ratio 3</th>
<th>&lt; 3xSD in Screen 3?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin</td>
<td>5</td>
<td>D3</td>
<td>0.71</td>
<td>NS</td>
<td>0.69</td>
<td>Y</td>
</tr>
<tr>
<td>5-Fluorouridine (5-FUrd)</td>
<td>4</td>
<td>E2</td>
<td>0.19</td>
<td>0.30</td>
<td>0.24</td>
<td>Y</td>
</tr>
<tr>
<td>Digitonin</td>
<td>6</td>
<td>F5</td>
<td>1.17</td>
<td>0.80</td>
<td>0.70</td>
<td>Y</td>
</tr>
<tr>
<td>Harmine</td>
<td>6</td>
<td>H4</td>
<td>0.67</td>
<td>0.57</td>
<td>0.51</td>
<td>Y</td>
</tr>
<tr>
<td>6-Benzylaminopurine riboside</td>
<td>4</td>
<td>E4</td>
<td>0.72</td>
<td>0.58</td>
<td>0.48</td>
<td>Y</td>
</tr>
<tr>
<td>Cantharidin</td>
<td>5</td>
<td>A6</td>
<td>0.45</td>
<td>NS</td>
<td>0.51</td>
<td>Y</td>
</tr>
<tr>
<td>N-Alllyadenosine</td>
<td>4</td>
<td>G5</td>
<td>0.46</td>
<td>0.40</td>
<td>0.45</td>
<td>Y</td>
</tr>
<tr>
<td>Harmol hydrochloride dehydrate</td>
<td>5</td>
<td>A9</td>
<td>0.77</td>
<td>NS</td>
<td>0.83</td>
<td>N</td>
</tr>
<tr>
<td>Chemical transformation of Dehydroglaucine</td>
<td>5</td>
<td>B8</td>
<td>0.61</td>
<td>NS</td>
<td>0.85</td>
<td>N</td>
</tr>
<tr>
<td>7-Benzoxygramine</td>
<td>8</td>
<td>A4</td>
<td>0.65</td>
<td>NS</td>
<td>0.76</td>
<td>N</td>
</tr>
<tr>
<td>n-Butyl gallate</td>
<td>1</td>
<td>B2</td>
<td>1.10</td>
<td>0.63</td>
<td>1.19</td>
<td>N</td>
</tr>
<tr>
<td>3-formyl Rifamycin SV</td>
<td>2</td>
<td>D8</td>
<td>0.75</td>
<td>NS</td>
<td>0.95</td>
<td>N</td>
</tr>
<tr>
<td>Dehydroglaucine</td>
<td>2</td>
<td>C8</td>
<td>0.70</td>
<td>NS</td>
<td>1.41</td>
<td>N</td>
</tr>
<tr>
<td>Pinocembrine; 5,7-Dihydroxyflavanone</td>
<td>1</td>
<td>A11</td>
<td>0.86</td>
<td>0.78</td>
<td>1.14</td>
<td>N</td>
</tr>
</tbody>
</table>
Table 9. Compound selection via triage.
Compounds were ranked based on raw Firefly (F), raw Renilla (R) and standard deviation (SD) from control (0.1% DMSO) values to identify the strongest hits. Raw data were examined for both primary (Pri) and repeat screens (Rep). Compounds locations are given by the plate number followed by the well number.

<table>
<thead>
<tr>
<th>Rank</th>
<th>Location</th>
<th>Compound name</th>
<th>Ratio &lt; DMSO 3xSD</th>
<th>Raw F &lt; DMSO + 1 x SD</th>
<th>Raw R &lt; DMSO - 1 x SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 D3</td>
<td>Rifampicin</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>4 E2</td>
<td>5-Fluorouridine (5-FUrd)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>6 F5</td>
<td>Digitonin</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>2</td>
<td>6 H4</td>
<td>Harmine</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>4 E4</td>
<td>6-Benzylaminopurine riboside</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>3</td>
<td>5 A6</td>
<td>Cantharidin</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>4 G5</td>
<td>N-Allyladenosine</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>4</td>
<td>5 A9</td>
<td>Harmol hydrochloride dihydrate</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>5</td>
<td>5 B8</td>
<td>Chemical transformation of Dehydroglaucine</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>6</td>
<td>8 A4</td>
<td>7-Benzylxgramine</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>7</td>
<td>1 B2</td>
<td>n-Butyl gallate</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>2 D8</td>
<td>3-formyl Rifamycin SV</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>2 C8</td>
<td>Dehydroglaucine</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>8</td>
<td>1 A11</td>
<td>Pinocembrine; 5,7-Dihydroxyflavanone</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>
Table 10. Compounds selected for further validation.
These compounds ranked 3 and above in Table 9. Digitonin was excluded as it is a detergent.

<table>
<thead>
<tr>
<th>Number</th>
<th>Compounds taken forward</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rifampicin</td>
</tr>
<tr>
<td>2</td>
<td>5-Fluorouridine</td>
</tr>
<tr>
<td>3</td>
<td>Harmine</td>
</tr>
<tr>
<td>4</td>
<td>6-Benzylaminopurine riboside</td>
</tr>
<tr>
<td>5</td>
<td>Cantharidin</td>
</tr>
<tr>
<td>6</td>
<td>N-Allyladenosine</td>
</tr>
</tbody>
</table>
5.2.6. Compound ‘drug-likeness’

There are several properties that may be used to assess a compound’s ‘drug-likeness’. The Lipinski rule of five which is used to predict the likelihood a compound is orally active (Lipinski 2004) has four criteria; molecular weight (MW) less than 500 Daltons, number of hydrogen bond acceptors (≤ 10), number of hydrogen bond donors (≤ 5) and lipophilicity (LogP) (<5). The number of Lipinski criteria met, as well as the MW, LogP, compound structure and chemical abstracts service (CAS) identification number are outlined in Table 11.
Table 11. Hit compound structure, CAS number (numerical identifiers), molecular weight (MW), lipophilicity (LogP), Lipinski score (evaluation of ‘drug-likeness’) and source.

Information is as described by the library provider TimTec.

<table>
<thead>
<tr>
<th>Compound and source</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Name:</strong> Rifampicin</td>
<td><img src="image" alt="Rifampicin structure" /></td>
</tr>
<tr>
<td><strong>CAS:</strong> 13292-46-1</td>
<td></td>
</tr>
<tr>
<td><strong>MW:</strong> 822.95</td>
<td></td>
</tr>
<tr>
<td><strong>LogP:</strong> 4.20</td>
<td></td>
</tr>
<tr>
<td><strong>Lipinski:</strong> 1</td>
<td></td>
</tr>
<tr>
<td><strong>Source:</strong> Semisynthetic antibiotic obtained by reacting 3-formylrifamycin SV with 1-amino-4-methylpiperazine in tetrahydrofuran</td>
<td></td>
</tr>
</tbody>
</table>

<p>| <strong>Name:</strong> 5-Fluorouridine | <img src="image" alt="5-Fluorouridine structure" /> |
| <strong>CAS:</strong> 316-46-1 | |
| <strong>MW:</strong> 262.19 | |
| <strong>LogP:</strong> -3.17 | |
| <strong>Lipinski:</strong> 4 | |
| <strong>Source:</strong> Nucleoside analogue | |</p>
<table>
<thead>
<tr>
<th>Name:</th>
<th>Harmine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CAS:</strong></td>
<td>442-51-3</td>
</tr>
<tr>
<td><strong>MW:</strong></td>
<td>212.25</td>
</tr>
<tr>
<td><strong>LogP:</strong></td>
<td>2.96</td>
</tr>
<tr>
<td><strong>Lipinski:</strong></td>
<td>4</td>
</tr>
<tr>
<td><strong>Source:</strong></td>
<td>From seeds of Peganum harmala L., Zygophyllaceae; from Banisteria caapi Spruce, Malpighiaceae; from Banisteriopsis inebrians Morton, Malpighiaceae</td>
</tr>
</tbody>
</table>

![Harmine](image1)

<table>
<thead>
<tr>
<th>Name:</th>
<th>6-Benzylaminopurine riboside</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CAS:</strong></td>
<td>4294-16-0</td>
</tr>
<tr>
<td><strong>MW:</strong></td>
<td>357.37</td>
</tr>
<tr>
<td><strong>LogP:</strong></td>
<td>-0.34</td>
</tr>
<tr>
<td><strong>Lipinski:</strong></td>
<td>4</td>
</tr>
<tr>
<td><strong>Source:</strong></td>
<td>N6-bezyl derivative of adenosine (nucleoside)</td>
</tr>
</tbody>
</table>

![6-Benzylaminopurine riboside](image2)

<table>
<thead>
<tr>
<th>Name:</th>
<th>Cantharidin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CAS:</strong></td>
<td>56-25-7</td>
</tr>
<tr>
<td><strong>MW:</strong></td>
<td>196.20</td>
</tr>
<tr>
<td><strong>LogP:</strong></td>
<td>0.19</td>
</tr>
<tr>
<td><strong>Lipinski:</strong></td>
<td>4</td>
</tr>
<tr>
<td><strong>Source:</strong></td>
<td>Active principle of cantharides and other insects; Spanish Fly aphrodisiac</td>
</tr>
</tbody>
</table>

![Cantharidin](image3)
<table>
<thead>
<tr>
<th><strong>Name:</strong> N-Allyladenosine</th>
<th>![Chemical Structure]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CAS:</strong> 15763-12-9</td>
<td></td>
</tr>
<tr>
<td><strong>MW:</strong> 307.31</td>
<td></td>
</tr>
<tr>
<td><strong>LogP:</strong> -1.44</td>
<td></td>
</tr>
<tr>
<td><strong>Lipinski:</strong> 4</td>
<td></td>
</tr>
<tr>
<td><strong>Source:</strong> Adenosine analog</td>
<td></td>
</tr>
</tbody>
</table>
5.2.7. Generally cytotoxic compounds

During the screen, several compounds were identified to reduce both Firefly and *Renilla* expression. This may indicate that the compounds are generally toxic. Such compounds are not the focus of this thesis; however, they are listed here as they may be of future interest. These compounds have been divided into two tables, those which appeared to be toxic are given in Table 12 (Firefly luciferase and *Renilla* luciferase counts were below 25% of the average 0.1% DMSO counts for that plate) and those which appeared to have milder toxicity are given in Table 13 (Firefly luciferase and *Renilla* luciferase counts were below 60% of the average 0.1% DMSO treated counts for that plate). It is important to note these results are gathered at one particular concentration and time interval and confirmation of these compound’s cytotoxic activity would need to be made in follow up experiments as this is a simple observation of reduced reporter and control luciferase activity.
Table 12. Potentially cytotoxic compounds.
Compounds where Firefly luciferase and Renilla luciferase counts were below 25% of the average 0.1% DMSO treated counts for that plate. Compound location is given by plate number and then well number.

<table>
<thead>
<tr>
<th>Location</th>
<th>Compound name</th>
<th>Firefly luciferase relative to DMSO</th>
<th>Renilla luciferase relative to DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 C10</td>
<td>Nebularine</td>
<td>6%</td>
<td>15%</td>
</tr>
<tr>
<td>3 D9</td>
<td>(+)-Emetine dihydrochloride hydrate</td>
<td>8%</td>
<td>5%</td>
</tr>
<tr>
<td>3 H7</td>
<td>(-)-Ouabain hydrate</td>
<td>16%</td>
<td>21%</td>
</tr>
<tr>
<td>5 C4</td>
<td>(S,S)-(+) Tetrandrine</td>
<td>15%</td>
<td>14%</td>
</tr>
<tr>
<td>6 E8</td>
<td>Plumbagin</td>
<td>2%</td>
<td>1%</td>
</tr>
<tr>
<td>6 C7</td>
<td>Digoxin</td>
<td>17%</td>
<td>25%</td>
</tr>
<tr>
<td>8 A2</td>
<td>Piperlongumine</td>
<td>7%</td>
<td>7%</td>
</tr>
<tr>
<td>10 B7</td>
<td>Anisomycin</td>
<td>10%</td>
<td>12%</td>
</tr>
<tr>
<td>10 G6</td>
<td>Brefeldin A</td>
<td>6%</td>
<td>8%</td>
</tr>
</tbody>
</table>

Table 13. Potentially moderately cytotoxic compounds.
Compounds where Firefly luciferase and Renilla luciferase counts were below 60% of the average 0.1% DMSO treated counts for that plate. Compound location is given by plate number and then well number. 5-Fluorouridine highlighted in blue was included in the repeat primary screen based on ratios in the initial screen and identified as a potential hit.

<table>
<thead>
<tr>
<th>Location</th>
<th>Compound name</th>
<th>Firefly luciferase relative to DMSO</th>
<th>Renilla luciferase relative to DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 E7</td>
<td>Camptothecin</td>
<td>39.5%</td>
<td>54%</td>
</tr>
<tr>
<td>4 E2</td>
<td>5-Fluorouridine, (5-FUrd)</td>
<td>54.4%</td>
<td>11%</td>
</tr>
<tr>
<td>5 B4</td>
<td>Berbamine dihydrochloride</td>
<td>40%</td>
<td>40%</td>
</tr>
<tr>
<td>6 C11</td>
<td>Digitoxin</td>
<td>19.9%</td>
<td>31%</td>
</tr>
<tr>
<td>6 F5</td>
<td>Digitonin</td>
<td>37.8%</td>
<td>30%</td>
</tr>
<tr>
<td>7 A7</td>
<td>proscillaridin A</td>
<td>20%</td>
<td>46%</td>
</tr>
<tr>
<td>7 G6</td>
<td>lanatoside C</td>
<td>23.6%</td>
<td>66%</td>
</tr>
<tr>
<td>8 F6</td>
<td>Ouabain octahydrate</td>
<td>19%</td>
<td>48%</td>
</tr>
<tr>
<td>10 G11</td>
<td>Artesunate</td>
<td>39.6%</td>
<td>44%</td>
</tr>
</tbody>
</table>
5.3. Discussion

5.3.1. Screening performance

While the screening platform was able to determine several hit compounds for further validation, there were some shortfalls, primarily in achieving adequate Z’factors and assay variability. As shown in Figure 51, many plates did not meet the Z’factor cut-off during screening, and for some plates they failed again upon being repeated. This is due to large variation in the 0.1% DMSO controls. The 0.1% DMSO controls were placed in all four corners of the 96-well plate as illustrated in Figure 40. This was to account for as much variability as possible as the corners of the plate will be subjected to the largest potential ‘drift’ in assay readings. Provided the Z’factor for that plate was met, this would ensure the greatest possible opportunity for genuine hit compounds to be captured. If 0.1% DMSO controls were placed in adjacent wells therefore minimising any variation caused by drift, the Z’factor is more likely to be calculated as acceptable, despite the rest of the plate succumbing to large potential drift.

Further, the Z’factor is not only dependent on the variability of the controls but also on the difference between the means of the controls. In the repeat primary screen a fresh dilution of miR-7 was used. In this run the difference between the means of miR-7 and 0.1% DMSO was smaller and there was larger variation in the effect of miR-7 on the luciferase signal, resulting in an inadequate Z’factor (Figure 52). Repeating this screen with a fresh dilution of more concentrated miR-7 may have rectified the issue with the Z’factor for this plate, however, it was thought that this would not affect the compound data and so raw data of all compounds with ratios less than the mean of the 0.1% DMSO control were considered. The quality of controls is crucial to obtaining adequate Z’factors. In this instance, transfection of miR-7 was the best control available.

Steps were taken to minimise drift during the screening process. As the assay was set up so that each plate was read by column, the luciferase reagent was added using a multichannel pipette with delayed timing across the plate in line with the time required for the reader to read one column. In other words, a column of luciferase reagent was dispensed every 17 seconds as the reader returned to the start of each column every 17 seconds. The readings are still subject to drift as the plate reader moves down a column, however, the effect is minimised across the plate.
Overall the screening platform performed adequately and hit compounds were identified for follow up validation. However, the assay platform could be improved with adjustments made to minimise variation in controls and to maximise the signal window. A better positive control should it become available, such as a small molecule, may aid this.

### 5.3.2. Data analysis and compound selection

Defining the threshold of luciferase reduction that identifies a hit will affect the number of false positives and false negatives. A less stringent threshold will result in a greater portion of false-positives and reduce the potential for false-negatives. The opposite will be true if a more stringent threshold is applied. A standard-activity based approach was used for this screen whereby a reduction in the ratio of Renilla/Firefly luciferase readings of more than 3 x standard deviations from the DMSO control was considered a hit. This is a commonly used approach for hit determination (McFadyen 2005). At this stage upon analysis of raw data some compounds were eliminated despite reducing the ratio of luciferase more than 3 x standard deviations of the DMSO control. These compounds either exhibited a significant increase in Firefly luciferase and no reduction in Renilla luciferase, as was the case for the exclusion of most compounds, for example H10 on Plate 2 (Figure 42), or completely reduced both Renilla and Firefly luciferase signal-to-noise levels, for example compound E8 on plate 6 (Figure 46) which is subsequently listed as a potential cytotoxic compound (Table 12). Fourteen compounds were rescreened for activity in the stable assay system (Figure 52). These compounds and their relative raw luciferase counts and ratios in the primary and repeat screens can be found in Table 9. Upon rescreening six compounds were selected for further validation (Table 10).

### 5.3.3. LogP, molecular weight and the Lipinski rule of five

There are a number of properties that provide information about the suitability of a molecule as a potential drug candidate. These parameters can affect a drugs absorption and distribution. The LogP of a compound is a measure of its lipophilicity. This can affect
the transport of the small molecule through the cell membrane as well as its metabolism. The Lipinski rule of five is defined by four parameters of compound chemistry (MW ≤ 500, log P ≤ 5, H-bond donors ≤ 5, H-bond acceptors ≤ 10). Compounds that satisfy all of these parameters are thought to have greater oral activity, bioavailability and absorption, however it is important to note that the Lipinski rules apply to passive diffusion and not active uptake of the compound (Leeson 2012). These parameters and compound structures are given for tentative hit compounds in (Table 11). A molecular weight of less than 350 Da and LogP value of less than 3 is common in currently marketed drugs (Hughes, Rees et al. 2011). All tentative hits with the exception of rifampicin met all four Lipinski rules.

5.3.4. Cytotoxic compounds

A number of compounds that caused a reduction in both Renilla and Firefly luciferase were identified in the screen and are outlined in Table 12 and Table 13. It is thought that these compounds may be generally toxic. While it is possible that some of these compounds could be general luciferase inhibitors, it is unlikely that these compounds are inhibitors of both Renilla and Firefly luciferase due to their differences in ATP dependency. Firefly luciferase is ATP dependent while Renilla luciferase is not (Auld D. S. 2016). Such compounds are not the focus of this thesis and so these compounds were not further studied, nor screened a second time, except one, 5-fluorouridine (5 FUrd).

However, given some of the compounds noted are currently used in chemotherapy, such as camptothecin or closely related to current chemotherapies such as 5-FUrd (related to the chemotherapy 5-fluorouracil (5-FU)), these compounds were listed here. Camptothecin was identified when it was included in a natural products screen for anti-cancer drugs (Wall 1966). Since then two improved molecules have been derived from camptothecin (topotecan and irinotecan) which are currently used to treat ovarian, cervical and certain types of lung cancer (topotecan) and CRC (irinotecan). 5-FU is used to treat several cancer types, in particular CRC. 5-FUrd was flagged as a potential miR-7 inducer given its further reduction in Renilla luciferase relative to firefly and therefore adequate ratio despite its apparent cytotoxic activity. The compounds were divided into two categories, those being more toxic (counts were below 25% of the counts observed control Firefly counts) and mildly toxic (where Firefly counts were below 60% of the
Results chapter: Screening the library observed Firefly counts of the controls on the relevant plate). It is important to note that these compounds may simply affect luciferase expression and EC$_{50}$ data as well as further characterisation is required. As these compounds may be of future interest the data has been included here as a guide only.

5.3.5. Summary

In this chapter, a screen of 800 natural product compounds was completed using the stable cell line generated in chapter 4. The screening assay worked relatively well, with controls providing confidence on the quality of the data. After several stages of data analysis and stratification, the compounds identified as hits in this chapter were; rifampicin, 5-FUrd, harmine, 6-benzylaminopurine riboside, cantharidin and N-allyladenosine. Digitonin was eliminated as it is a detergent, solubilising cell membranes and therefore is an undesirable drug candidate. The next stage of the drug discovery pipeline is known as the ‘hit to lead’ stage. Hit compounds undergo ‘secondary screening’ whereby they are screened in a secondary assay platform. Here TaqMan RT qPCR was used to confirm miR-7 upregulation and these results form part of the next chapter.
6. Hit compound validation

In this chapter compounds that were identified as hits in the previous chapter undergo validation. miR-7 upregulation is first confirmed and their action is characterised through a series of molecular assays.

6.1. Drug discovery process

The drug discovery process is a costly venture, in 2012 the cost was estimated to be 9.2 million dollars and taking an estimated 8.5 years in an academic setting to go from target identification through to clinical proof of concept (Strovel, Sittampalam et al. 2012). While others estimate the cost is into the billions to go from an original idea right through to the bedside in 12 – 15 years (Hughes, Rees et al. 2011). Either way, cost is a major factor in the progression of the drug discovery process from one stage to the next, especially given the success rate of a compound from phase I clinical trials through to approval is just 11.8% (DiMasi, Grabowski et al. 2016).

The drug discovery process begins with the identification of a target and its validation in disease models. This is followed by the development of an assay platform and screening of compound libraries for potential hit compounds. Hit compounds are those which show reproducible activity in the screening assay platform and then undergo several stages of testing before clinical drug development (Hughes, Rees et al. 2011, Strovel, Sittampalam et al. 2012). Following the identification of a series of hits that are ideally ‘drug-like’ (i.e. conform to the Lipinski rule of five), they are then examined in order to triage, refine and identify those worth pursuing. If there are several hits that display structural similarity, it is recommended to ensure that structural diversity is maintained in further testing to cast the widest possible net. Compounds that may be promiscuous ‘frequent hitters’ or those that upon closer examination of raw data do not appear to be actioning the target as desired (e.g. here, increasing Firefly luciferase) should be eliminated. Selected compounds are then validated in a secondary assay which is different from the first, to independently confirm their activity. They are subjected to dose-response assays to ensure competitive behaviour and EC50 data generated to compare potency. Additional validation assays may take place to ensure the desired biological effect and further characterise the compound’s behaviour. In these validation
stages, fresh compounds are used to ensure compounds from the screening library have not since become degraded (Hughes, Rees et al. 2011, Strovel, Sittampalam et al. 2012). The remaining stages of the drug development pipeline and those not addressed in this thesis include; identification of the structure-activity relationship, generation of analogues and subsequent testing for increased potency, reduced toxicity, and improved stability and bioavailability. Finally, lead compounds from these further stages enter pre-clinical and clinical drug development stages (Hughes, Rees et al. 2011, Strovel, Sittampalam et al. 2012).

6.1.1. Compound validation stages in this chapter

Secondary assays should be biologically relevant and confirm the action of the drug upon its target (Hughes, Rees et al. 2011, Strovel, Sittampalam et al. 2012). The secondary assay conducted here, TaqMan RT qPCR, provides a direct and biologically relevant method of determining the relative level of miR-7 upregulation upon compound treatment.

Dose-response assays are then carried out to ensure that compound action is concentration dependent. Compounds with an ‘all or nothing’ effect are undesirable drug candidates due to safety concerns (Hughes, Rees et al. 2011). In addition, concentration-dependent inhibition is generally associated with genuine activity (Thorne, Auld et al. 2010). Here, dose-response curves were generated using the stable psiCHECK-2 (6mer + NeoR) in the same way they the primary screen was conducted. However, inferring genuine activity in this way is challenging given some false-positive compounds can inhibit luciferase dose-dependently. Therefore, dose-response studies using the primary assay platform were carried out only after TaqMan RT qPCR confirmation of miR-7 upregulation. Dose-response assays in the primary screen can also provide important information with regard to the maximal response of a compound (Thorne, Auld et al. 2010). In another type of dose-dependent assay, the half maximal inhibitory concentration or EC_{50} is determined. This gives an indication of compound potency and can be used to compare compounds. Finally, further functional validation is carried out to ensure that the desired biological effect is achieved. Here Western blot is used to ensure knockdown of miR-7 target proteins in line with miR-7 upregulation.
Results Chapter: Hit compound validation

being achieved by compound treatment. Further, the compounds are assessed for their potential to induce apoptosis. For a general overview of the screening and validation process conducted here see Figure 53.

Lead compounds are only determined after a hit compound is found to have reproducible activity in both primary and secondary assays. Further considerations in lead compound development are a compound’s pharmacokinetic profile (i.e. how the drug behaves in the body regarding absorption, distribution, metabolism, and excretion), efficacy, specificity and selectivity as well as it’s amenability to modification in order to define its structure-activity relationship.

6.1.2. Chapter aims

The specific aims of this chapter are to:

1) Confirm miR-7 upregulation by hit compounds using RT qPCR in CRC cell lines.
2) Confirm similar action in human HCC, Huh7 cells.
3) Demonstrate downregulation of miR-7 targets by confirmed compound hits.
4) Further characterise compound activity.
Figure 53. General overview of key screening and validation stages.
Compounds initially identified in the primary screen were rescreened by the same process to ensure their action was reproducible. Those compounds that were successfully identified in the repeat screen underwent a secondary screen (TaqMan RT qPCR) to confirm miR-7 upregulation. Finally, compound action was validated by Western blot to examine their effect on miR-7 target proteins.
6.2. Results

6.2.1. MicroRNA extraction for RT qPCR validation

Initially, all RNA extracted from compound-treated cells was extracted using the Bioline Isolate II RNA mini kit (BIO-52073). However, the cycle threshold (Ct) values for miR-7 expression were an average of 38. It is generally accepted that Ct values of greater than 35 cycles are unreliable. This issue was resolved upon reverting to RNA extraction using the Trizol method and average Ct values were reduced to below 35 cycles (Figure 54). This data lead to an investigation into RNA extraction methods and miRNA recovery in tissues conducted by myself and colleagues. As was identified here, it was found that the Bioline extraction kit was unsuitable for the recovery of miRNA and Trizol or other column based kits trialled such as miRvana outperformed Bioline (Brown, Epis et al. 2018).
Figure 54. RT qPCR comparing Trizol vs column-based Bioline Isolate II kit extraction of miRNA.
Ct values for miR-7 in DMSO treated cells extracted using Bioline Isolate II RNA mini kit and Trizol. Data are normalised to U6 expression. The result shown is representative of Ct values for 6 compounds tested in three independent experiments per cell line. ****, p < 0.0001.
6.2.2. TaqMan RT qPCR validation of hit compounds in CRC cells

To confirm that compounds upregulate miR-7, cells were treated with compounds for 24 h and miR-7 levels were measured by TaqMan RT qPCR. The list of compounds to be validated in HT29 and HCT116 cell lines can be found in Table 10. Compounds were tested at 10 µM in HT29 cells. In HCT116 however, these concentrations caused a great amount of cell death, limiting the RNA yield. Therefore, the concentration was appropriately reduced and HCT116 cells were treated using 4 µM cantharidin and 0.01 µM 5-FUrd. While several compounds were validated in the screening cell line HT29 (Figure 55), none could be confirmed in HCT116 (Figure 56). The experiment was conducted several times and in some cases, gave highly variable results. As Figure 55 and Figure 56 are representative, the range of miR-7 fold upregulation for each compound as observed in HT29 cells was: 6-benzylaminopurine riboside, 1.6 – 4.9; N-allyladenosine, 0.9 – 1.2; rifampicin, 1.6 – 3.3; cantharidin, 2.8 – 14.8; 5-fluorouridine, 1.5 – 8.4; harmine, 2.0 – 4.8. The range of miR-7 fold upregulation for each compound in HCT116 cells as observed was: 6-benzylaminopurine riboside, 1.4 – 1.6; N-allyladenosine, 0.7 – 1.0; rifampicin, 0.9 – 1.1; cantharidin, 1.2 – 1.5; 5-fluorouridine, 1.0 – 2.0; harmine, 0.9 – 1.1.
Figure 55. RT qPCR for miR-7 upregulation by hit compounds in HT29 cells. HT29 cells were treated with 10 µM of each compound for 24 h before miR-7 expression was measured by RT qPCR. U6 was used for normalisation. Data are representative of three independent experiments per compound. Data are expressed relative to DMSO treatment and error bars represent SDs. **, p < 0.01; ****, p < 0.0001.
Figure 56. RT qPCR for miR-7 upregulation by hit compounds in HCT116 cells.
HCT116 cells were treated with 10 µM of each compound (except for cantharidin (4 µM) and 5-fluorouridine (0.01 µM) due to toxicity) for 24 h before miR-7 expression was measured by RT qPCR. U6 was used for normalisation. Data are representative of three independent experiments per compound. Data are expressed relative to DMSO treatment and error bars represent SDs.
6.2.3. Dose-response luciferase assay in HT29 psiCHECK-2 (6mer + NeoR) stable cells

To confirm that compounds acted in a dose-responsive manner, stable HT29 (psiCHECK-2 6mer NeoR) cells were treated with various concentrations of compound (0 – 50 µM) for 24 h before luciferase readings were made. It is assumed that dose-dependent inhibition of luciferase would equate to dose-dependent upregulation of miR-7, however, this is an assumption and there may be other factors affecting luciferase expression. Dose-response data for cantharidin, 5-FUrd, harmine and 6-benzylaminopurine riboside are given in Figure 57 through Figure 60. Dose-response data is not included for rifampicin as in initial experiments conducted using a smaller range of doses (1 – 30 µM) there appeared to be no dose-dependent inhibition. This compound was therefore eliminated at this stage and was not followed up upon revalidating dose-response data across a larger range of concentrations as shown below (0 – 50 µM), including concentrations below 1 µM required to generate IC$_{50}$ values.
Figure 57. Cantharidin dose-response luciferase assay in HT29 psiCHECK-2 (6mer + NeoR) cells.

HT29 psiCHECK-2 (6mer + NeoR) cells were treated with various concentrations of cantharidin (0 – 50 µM) for 24 h. Luciferase activity is expressed relative to the lowest concentration of drug. The data represents the average of three independent experiments and error bars represent SDs. The IC\textsubscript{50} is 3.49 µM.
Figure 58. 5-Fluorouridine dose-response luciferase assay in HT29 psiCHECK-2 (6mer + NeoR) cells.
HT29 psiCHECK-2 (6mer + NeoR) cells were treated with various concentrations of 5-fluorouridine (0 – 50 µM) for 24 h. Luciferase activity is expressed relative to the lowest concentration of drug. The data represents the average of three independent experiments and error bars represent SDs. The IC<sub>50</sub> is 2.48 µM.
Figure 59. Harmine dose-response luciferase assay in HT29 psiCHECK-2 (6mer + NeoR) cells.
HT29 psiCHECK-2 (6mer + NeoR) cells were treated with various concentrations of harmine (0 – 50 µM) for 24 h. Luciferase activity is expressed relative to the lowest concentration of drug. The data represents the average of three independent experiments and error bars represent SDs. The IC₅₀ is 1.67 µM.
Figure 60. 6-Benzylaminopurine riboside dose-response luciferase assay in HT29 psiCHECK-2 (6mer + NeoR) cells. HT29 psiCHECK-2 (6mer + NeoR) cells were treated with various concentrations of 6-benzylaminopurine (0 – 50 µM) for 24 h. Luciferase activity is expressed relative to the lowest concentration of drug. The data represents the average of three independent experiments and error bars represent SDs. The IC50 is 2.27 μM.
6.2.4. RT qPCR validation of cantharidin and 5-FUrd in Huh7

Compounds which upregulated miR-7 in either HCT116 or HT29 cell lines were to be further tested in the HCC cell line Huh7 to ensure the results were not specific to one cell line/cancer type. As no compounds were confirmed in the second CRC cell line HCT116, at this stage just cantharidin and 5-FUrd were chosen to follow up in Huh7. Despite upregulation of several compounds in HT29 cells, cantharidin and 5-FUrd gave the greatest fold upregulation in the RT qPCR studies conducted (cantharidin; 2.8 – 14.8, 5-FUrd; 1.5 – 8.4) when compared to the fold change of other compounds (6-benzylaminopurine riboside, 1.6 – 4.9; N-allyladenosine, 1.2; rifampicin, 1.6 – 3.3; harmine; 2.0 – 4.8). For these experiments, the concentration of cantharidin needed to be adjusted in Huh7 cells to 1 µM as the standard 10 µM concentration caused a great amount of cell death within 24 h, limiting the RNA yield. Cantharidin was confirmed to upregulate miR-7 in Huh7 cells (Figure 61). The range of miR-7 fold upregulation for each compound in Huh7 cells across the two experiments was; cantharidin, 1.8 – 10.3; 5-FUrd, 0.8 – 1.0.
Figure 61. RT qPCR for miR-7 upregulation by 5-fluorouridine and cantharidin in Huh7 HCC cells.

Huh7 cells were treated for 24 h with DMSO (0.01%), 5-fluorouridine (10 μM) or cantharidin (1 μM) before miR-7 expression was measured by RT qPCR. U6 was used for normalisation. Data are representative of two independent experiments. Data are expressed relative to DMSO treatment and error bars represent SDs. ****, p < 0.0001.
6.2.5. Compound EC$_{50}$

To complement the data generated above using the luciferase reporter assays, EC$_{50}$ concentrations were determined for cantharidin and 5-FUrd in HCT116, HT29 and Huh7 cells (Figure 62 through Figure 67). The EC$_{50}$ is related to a drug’s potency and those that are more potent (or lower EC$_{50}$) are generally considered as more desirable drug candidates. Despite no upregulation of miR-7 detected in HCT116 cells by cantharidin or 5-FUrd, they were included in EC$_{50}$ studies to further compare potency.
Figure 62. Cell titer analysis of cantharidin sensitivity of HT29 cells.
HT29 cells were seeded in 96-well plates and treated with cantharidin (0.001, 0.01, 0.1, 1, 2.5, 5, 10, 20, 50 µM), and the half maximal effective concentration (EC$_{50}$) was determined after 72 h. Data are normalised to the lowest concentration of cantharidin. Error bars represent SDs. The EC$_{50}$ was 3.47 µM. Data are representative of three independent experiments.

Figure 63. Cell titer analysis of 5-fluorouridine sensitivity of HT29 cells.
HT29 cells were seeded in 96-well plates and treated with 5-fluorouridine (0.00001, 0.0001, 0.001, 0.01, 0.1, 1, 2.5, 5, 10 µM), and the half maximal effective concentration (EC$_{50}$) was determined after 72 h. Data are normalised to the lowest concentration of 5-fluorouridine. Error bars represent SDs. The EC$_{50}$ was 0.0095 µM. Data are representative of three independent experiments.
Figure 64. Cell titer analysis of cantharidin sensitivity of HCT116 cells.
HCT116 cells were seeded in 96-well plates and treated with cantharidin (0.001, 0.01, 0.1, 1, 2.5, 5, 10, 20, 50 µM), and the half maximal effective concentration (EC$_{50}$) was determined after 72 h. Data are normalised to the lowest concentration of cantharidin. Error bars represent SDs. The EC$_{50}$ was 2.89 µM. Data are representative of three independent experiments.

Figure 65. Cell titer analysis of 5-fluorouridine sensitivity of HCT116 cells.
HCT116 cells were seeded in 96-well plates and treated with 5-fluorouridine (0.00001, 0.0001, 0.001, 0.01, 0.1, 2.5, 5, 10 µM), and the half maximal effective concentration (EC$_{50}$) was determined after 72 h. Data are normalised to the lowest concentration of 5-fluorouridine. Error bars represent SDs. The EC$_{50}$ was 0.0041 µM. Data are representative of three independent experiments.
Figure 66. Cell titer analysis of cantharidin sensitivity of Huh7 cells. Huh7 cells were seeded in 96-well plates and treated with cantharidin (0.001, 0.01, 0.1, 1, 2.5, 5, 10, 20, 50 µM), and the half maximal effective concentration (EC₅₀) was determined after 72 h. Data are normalised to the lowest concentration of cantharidin. Error bars represent SDs. The EC₅₀ was 1.42 µM. Data are representative of three independent experiments.

Figure 67. Cell titer analysis of 5-fluorouridine sensitivity of Huh7 cells. Huh7 cells were seeded in 96-well plates and treated with 5-fluorouridine (0.00001, 0.0001, 0.001, 0.01, 0.1, 1, 2.5, 5, 10 µM), and the half maximal effective concentration (EC₅₀) was determined after 72 h. Data are normalised to the lowest concentration of 5-fluorouridine. Error bars represent SDs. The EC₅₀ was 0.056 µM. Data are representative of three independent experiments.
6.2.6. Western blot analysis of the effect of compound addition on microRNA-7 target proteins in HT29 and Huh7

Western blots were performed to examine the effect of compounds on the miR-7 target proteins EGFR, Akt and pAkt. Survivin was included in initial experiments in HT29 cells as it is a predicted miR-7 target (TargetScan) (Agarwal, Bell et al. 2015, Agarwal, Bell et al. 2016). However, due to the discontinuation of the antibody by the supplier and no suitable replacement despite considerable efforts, it was not included in further studies in Huh7 cells or in subsequent parts of this chapter. Yet the result for survivin in HT29 cells is still included here given the striking downregulation observed as a result of cantharidin treatment. 5-FUrd inhibited EGFR and Akt and cantharidin inhibited Akt, pAkt and survivin (Figure 68).

As responsiveness of these proteins to miR-7 transfection was demonstrated in 4.2.1 for HT29 and HCT116 cells, it is here shown for Huh7 cells (Figure 69). The effect of cantharidin and 5-FUrd on EGFR, Akt and pAkt in Huh7 is then assessed. The concentration of cantharidin was reduced from 10 µM to 1 µM in Huh7 cells as was done for RT qPCR studies. Both cantharidin and 5FUrd downregulated pAkt in this cell line (Figure 70). pAkt downregulation by 5-FUrd is inconsistent with a lack of miR-7 upregulation in this cell line (as per Figure 61). However, given 5-FUrd does not downregulate pAkt in HT29 (Figure 68) where miR-7 upregulation is confirmed (Figure 55), this effect is likely irrespective of miR-7 action.
Figure 68. Western blot and densitometry analysis of miR-7 target protein expression in HT29 cells when treated with 5-fluorouridine or cantharidin.

Western blot and densitometry analysis of EGFR, Akt, pAkt and survivin levels in HT29 cells 24 h post-treatment with 5-fluorouridine (10 µM), cantharidin (10 µM), and DMSO (0.001%). β-actin is included as a loading control. The result shown is representative of two independent experiments, duplicates are biological replicates. Densitometry is normalised to β-actin and expressed relative to DMSO. *, p < 0.05, **, p < 0.01.
Figure 69. Western blot and densitometry analysis of miR-7 target protein expression in Huh7 cells lines when treated with miR-7.

Western blot and densitometry analysis of miR-7 target protein expression in Huh7 cells lines when treated with miR-7. Protein from Huh7 cells was harvested 72 h post-transfection with miR-7 (30 nM), miR-NC (30 nM) or Lipofectamine 2000 (LF) only. β-actin is included as a loading control. This figure was generated by Clarissa Ganda and reproduced here with permission. Data are representative of three independent experiments. Densitometry is normalised to β-actin and expressed relative to LF.
Western blot and densitometry analysis of miR-7 target protein expression in Huh7 cells when treated with cantharidin or 5-fluorouridine.

Western blot and densitometry analysis of EGFR, Akt and pAkt levels in Huh7 cells 24 h post-treatment with 5-fluorouridine (10 μM), cantharidin (1 μM), and DMSO (0.001%). β-actin is included as a loading control. The result shown is representative of two independent experiments, duplicates are biological replicates. Densitometry is normalised to β-actin and expressed relative to DMSO. *, p < 0.05.
6.2.7. Cantharidin time course

As cantharidin downregulated both Akt and pAkt in HT29 cells (Figure 68) a short time course was carried out to determine how rapidly these proteins were inhibited as a result of cantharidin treatment (Figure 71). The downregulation of pAkt but not total Akt can be seen in just 4 h suggesting cantharidin affects Akt activation in this time frame and not total Akt protein as observed after 24 h (Figure 68).
Figure 71. Western blot and densitometry analysis of miR-7 target protein expression over 4 h in HT29 cells when treated with cantharidin.

Western blot and densitometry analysis of EGFR, Akt and pAkt levels in HT29 cells 1 h and 4 h post-treatment with cantharidin (10 μM), and DMSO (0.001%). β-actin is included as a loading control. Data are representative of a single experiment. Densitometry is normalised to β-actin and expressed relative to DMSO 1 h.
6.2.8. Anti-microRNA-7 rescue of microRNA-7 target proteins with cantharidin addition

Cantharidin was shown to downregulate Akt and pAkt in HT29 cells. To identify whether this can be attributed in part or in entirety to the upregulation of miR-7 the cells were concurrently treated with an anti-miR-7 mimic. Anti-miR-7 was used to inhibit miR-7 and thereby counteract the effect of cantharidin, preventing the downregulation of miR-7 target proteins due to induced miR-7 expression. As it is not possible to both transfected the cells with anti-miRNA and treat the cells with drug at the same time due to a great loss in cell viability, cells were first treated with anti-miRNA and treated with drug 24 h post-transfection. Cells were then harvested 24 h post treatment with the drug. This means that the total time of transfection was 48 h (shorter than 72 h as conducted in previous miRNA transfection experiments for protein analysis) and is due to the fact that the action of anti-miR-7 is expected to be more rapid than miR-7 transfections. Here, anti-miR-7 binds to endogenous miR-7 that is associated with the RISC complex and inhibits mRNA target recognition and association. The partial return of Akt and pAkt levels indicate that downregulation of these proteins could be due to cantharidin-induced upregulation of miR-7 (Figure 72). However, it must be noted that treatment with miR-NC also shows some return of Akt and pAkt proteins, to a lesser extent.
Figure 72. Western blot and densitometry analysis of miR-7 target protein expression when treated with cantharidin and anti-miR-7 mimic in HT29 cells. 
HT29 cells were transfected with anti-miR-7 (30 nM), anti-miR-NC (30 nM) or Lipofectamine 2000 (LF) only. Twenty-four hours following transfection cells were treated with cantharidin (10 µM) or DMSO (0.001%). Protein was harvested 24 h post-treatment with cantharidin or DMSO. β-actin is included as a loading control. The result shown is representative of two independent experiments. Densitometry is normalised to β-actin and expressed relative to LF + DMSO treated cells.
6.2.9. Apoptosis assays

6.2.9.1. MicroRNA-7 induces caspase 3/7 activity

As there is some evidence that miR-7 induces apoptosis (Xu, Chen et al. 2014), it was thought that cantharidin and 5-FUrd may similarly induce apoptosis. First, it was confirmed that miR-7 does indeed induce apoptosis in HT29 (Figure 73) and Huh7 (Figure 74) cells via the Caspase-Glo assay. Caspases 3 and 7 are executioner caspases responsible for degrading cellular components in the apoptosis pathway (McIlwain, Berger et al. 2013) and the Caspase-Glo assay detects their activation.
Figure 73. Caspase 3/7 activity in HT29 cells following treatment with miR-7.

HT29 cells were transfected with miR-7 (30 nM), miR-NC (30 nM) or Lipofectamine 2000 only (LF) for 72 h. Caspase 3/7 activity was measured 72 h after transfection. Data are representative of three independent experiments and is expressed as mean ± SD relative to LF only. Camptothecin was used as a positive control and is expressed relative to DMSO. This figure was generated by Rikki Brown for the purpose of this thesis and is included here with permission. *, p < 0.05.
Figure 74. Caspase 3/7 activity in Huh7 cells following treatment with miR-7.
Huh7 cells were transfected with miR-7 (30 nM), miR-NC (30 nM) or Lipofectamine 2000 only (LF) for 72 h. Caspase 3/7 activity was measured 72 h after transfection. Data are representative of three independent experiments and is expressed as mean ± SD relative to LF only. ABT737 (10 µM) was used as a positive control and is expressed relative to DMSO. This figure was generated by Clarissa Ganda and is reproduced here with permission. ****, p < 0.0001.


6.2.9.2. Caspase 3/7 activity in HT29 and Huh7 following treatment with cantharidin and 5-FUrd

Since caspase activity was confirmed for both HT29 and Huh7 cell lines upon miR-7 transfection, these cell lines were treated with various concentrations of cantharidin and 5-FUrd in order to assess whether they similarly induce caspase activity. Caspase activity was confirmed for cantharidin in Huh7 (Figure 76), however, was not confirmed for HT29 (Figure 75). Data for HT29 was unreliable with 2 out of 4 runs showing that cantharidin did indeed induce caspase activity and 2 out of 4 runs where no activity was detected. Note that all data for these runs are not included and Figure 75 is representative of an experiment where no caspase activity was induced. 5-FUrd showed no caspase activity in either cell line.
Figure 75. Caspase 3/7 activity in HT29 cells following treatment with cantharidin and 5-fluorouridine.

HT29 cells were treated with various concentrations of cantharidin (2.5 – 10 µM) and 5-fluorouridine (0.001 – 5 µM) or DMSO only (0.1%). Caspase activity was measured 24 h following treatment. Camptothecin was used as a positive control. The result shown is representative of two independent experiments. *** p < 0.001.
Figure 76. Caspase 3/7 activity in Huh7 cells following treatment with cantharidin and 5-fluorouridine.

Huh7 cells were treated with various concentrations of cantharidin (2.5 – 10 µM) and 5-fluorouridine (0.001 – 5 µM) or DMSO only (0.1%). Caspase activity was measured 24 h following treatment. ABT737 was used as a positive control. The result shown is representative of two independent experiments. **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.
6.2.9.3. **MicroRNA-7 induces cleaved PARP**

As at least two assays are recommended to confirm apoptosis. HT29 cells and Huh7 cells were again transfected with miR-7 to first ensure that miR-7 induces cleaved poly (ADP-ribose) polymerase (PARP), an indicator of apoptosis, in these cell lines. PARP is involved in DNA damage repair among other cellular functions and is cleaved by caspases 3 and 7 during the apoptosis signalling cascade (Chaitanya, Steven et al. 2010). Western Blot analysis was conducted at 72 h to allow time for miR-7 action i.e. to associate with RISC, repress target protein expression, for this effect to be reflected following protein turnover and possibly the induction of apoptosis. An increase in cleaved PARP upon miR-7 transfection is shown for HT29 (Figure 77) and Huh7 (Figure 78).
**Figure 77. Western blot and densitometry analysis of cleaved PARP upon miR-7 transfection in HT29 cells.**

HT29 cells were transfected with miR-7 (30 nM), miR-NC (30 nM) or Lipofectamine 2000 (LF) only. Protein was harvested 72 h post-treatment. Western blot analysis of cleaved PARP levels was performed. β-actin is included as a loading control. The result shown here is representative of two independent experiments. Densitometry is normalised to β-actin and expressed relative to LF.
Figure 78. Western blot and densitometry analysis of cleaved PARP upon miR-7 transfection in Huh7 cells.

Huh7 cells were transfected with miR-7 (30 nM), miR-NC (30 nM) or Lipofectamine 2000 (LF) only. Protein was harvested 72 h post-treatment. Western blot analysis of cleaved PARP levels was performed. β-actin is included as a loading control. This figure was generated by Clarissa Ganda and is reproduced here with permission. The result shown here is representative of two independent experiments. Densitometry is normalised to β-actin and expressed relative to LF.
6.2.9.4. **Western blot for cleaved PARP as a result of cantharidin and 5-FUrd treatment in HT29 and Huh7 cells**

Given miR-7 transfection resulted in cleaved PARP in both HT29 and Huh7 cells, Western blots were performed to assess whether treatment with either cantharidin or 5-FUrd would also result in the presence of cleaved PARP. Treatment with cantharidin was found to result in cleaved PARP in both HT29 (Figure 79) and Huh7 (Figure 80) cells lines and is consistent with the result of the Caspase-Glo assay for Huh7 cells. However, for cantharidin treated HT29 cells although the caspase 3/7 activity was inconclusive (Figure 75) the presence of cleaved PARP supports apoptosis. As these compounds are highly toxic, assays for cleaved PARP were conducted at 24 h to ensure sufficient yield of cells required for Western blot analysis. Cleaved PARP was not detected following 5-FUrd treatment in either cell line, consistent with what was found in the Caspase-Glo assay.
Figure 79. Western blot and densitometry analysis of cleaved PARP following treatment with cantharidin or 5-fluorouridine in HT29 cells.

HT29 cells were treated with cantharidin (10 µM), 5-fluorouridine (10 µM) or DMSO only (0.01%). Protein was harvested 24 h post-treatment. Western blot analysis of cleaved PARP levels was performed. β-actin is included as a loading control. The result shown here is representative of two independent experiments. Densitometry is normalised to β-actin and expressed relative to DMSO.
Figure 80. Western blot and densitometry analysis of cleaved PARP following treatment with cantharidin or 5-fluorouridine in Huh7 cells.

Huh7 cells were treated with cantharidin (1 µM), 5-fluorouridine (10 µM) or DMSO only (0.01%). Protein was harvested 24 h post-treatment. Western blot analysis of cleaved PARP levels was performed. β-actin is included as a loading control. The result shown here is representative of two independent experiments. Densitometry is normalised to β-actin and expressed relative to DMSO.
6.3. Discussion

6.3.1. RNA extraction methods: Column vs Trizol

Aside from the main focus of this thesis, it was found that the column RNA extraction method first employed here (Bioline Isolate II RNA mini kit) was unsuitable for miRNA extraction due to unreliable Ct values in RT qPCR analysis (Figure 54). This generated interest in performing a comparison of several RNA extraction methods and their ability to isolate both long (mRNA and long non-coding RNA) and short (miRNA) RNA, or ‘total RNA’. While we focused mainly on the extraction of RNA from mouse organs, we found discrepancies in the yield, quality and composition of different RNA species. The conclusion of this study was that the Bioline total RNA kit was unsuitable for miRNA extraction and both Trizol and other column based kits tested proved suitable and comparable for miRNA detection (Brown, Epis et al. 2018).

6.3.2. Compound validation

Six hit compounds were selected for further validation after iterative elimination of several other hits believed to be false positives based on the analysis of raw luciferase counts and also a triaging process of those compounds that remained. Of the six hit compounds identified in the screen, five were confirmed to upregulate miR-7 in HT29 cells by RT qPCR (Figure 55), with the sixth compound, N-allyladenosine, being unable to upregulate miR-7 in HT29 cells and was a false positive. The potential for independent luciferase regulation is discussed further below. Surprisingly none of the hit compounds were confirmed in the second CRC cell line HCT116 (Figure 56) suggesting cell line specificity. Two of the five confirmed miR-7 regulators in HT29 cells were chosen to undergo further investigation. These compounds, cantharidin and 5-FUrd, were chosen based on consistent and greater fold upregulation of miR-7 observed in HT29 cells than other validated compounds (section 6.2.2). This selection was made after one of the five compounds, rifampicin was eliminated based upon dose-response studies, whereby there was no dose-dependent inhibition of Renilla luciferase, while the other compounds appeared to inhibit Renilla luciferase in a dose-responsive manner (Figure 57 through Figure 60). Ensuring that a compound acts in a dose-responsive manner is
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important for further drug development. Compounds that give an ‘all or nothing’ response are not desirable, whereas those that act in a reversible manner are useful when considering patient withdrawal and safety (Hughes, Rees et al. 2011). When cantharidin and 5-FUrd were tested in Huh7 cells, cantharidin upregulated miR-7 in this cell line also (Figure 61). In the future, further analysis of all five confirmed hits could be conducted in more cell lines and cancer types given only two were pursued here. Also, other compounds identified in the primary screen (Table 9) but eliminated in the triaging process may be of interest. While some hits may only be active in some cell lines they could prove useful for certain cancers or cancer subtypes. A panel of cancer cell lines would provide a broader representation of compound action (Jaeger, Duran-Frigola et al. 2015). Further, analysis of compounds in non-tumour derived cell lines may provide an indication as to their cancer specificity (Kluwe 2016).

6.3.3. Predicted and literature based microRNA regulation by other identified hits

In 2016 a database was published online (Psmir) listing predicted associations between small molecules and miRNAs based on the computational comparison of miRNA perturbation and drug treatment in GEO datasets. Just 25 miRNAs are included in the database and interestingly, miR-7 is included. Several small molecule interactions are predicted for miR-7. Cantharidin is identified in this list. However, none of the other compounds confirmed here to upregulate miR-7 in HT29 are listed as predicted miR-7 regulators (Meng, Wang et al. 2016) (http://www.bio-bigdata.com/Psmir/).

The Psmir database also predicts associations between both harmine and rifampicin and other miRNAs. Rifampicin regulation of a number of miRNAs has been described in hepatocytes, however, miR-7 was not identified among these (Ramamoorthy, Liu et al. 2013, Takahashi, Tatsumi et al. 2014, Benson, Eadon et al. 2016). Psmir predicts interactions between harmine and miR-124 and miR-376a however, regulation of miRNAs by harmine has not been described to my knowledge. It is my understanding that there is no available literature showing any association between the other compounds selected after compound triaging (Table 10) and miRNA regulation.
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There has been limited literature exploring the anti-tumour effects of rifampicin (Shichiri and Tanaka 2010). Conversely there has been extensive research into harmine (Zhang, Sun et al. 2014, Sun, Tang et al. 2015, Yu, Sun et al. 2016, Zhang, Sun et al. 2016) and cantharidin as cancer therapies (Huang, Ko et al. 2011, Shen, Wu et al. 2015, Hsia, Yu et al. 2016) (Li, Xie et al. 2010, Huang, Ko et al. 2011, Li, Chen et al. 2011, Li, Chen et al. 2011, Shou, Zhang et al. 2013, Shen, Wu et al. 2015, Su, Liu et al. 2015, Tian, Zeng et al. 2015, Hsia, Yu et al. 2016, Le, Zhang et al. 2016). Cantharidin is discussed further in section 6.3.5.

Interestingly, there is research into compounds related to those identified by the screen as miRNA regulators. However, some of these were included in the compound library and were not identified as miR-7 regulators in the primary screen. For example, the compound harmaline, closely related to harmine has been shown to regulate miRNA expression (Rodrigues, Li et al. 2011). The compound harmaline hydrochloride dehydrate was screened in plate 1, well E8 and did not have any effect on Renilla luciferase. The Psmir database predicts an association between two other harmala alkaloids, harmol (plate 4, well B10) and harman (plate 1, well A6) with miR-7 (Meng, Wang et al. 2016) http://www.bio-bigdata.com/Psmir/), both of which were included in the screen but not identified as regulators of miR-7. Harmol did, however, reduce Renilla luciferase expression, just not enough to meet the threshold of 3 x standard deviations from control values. Another related compound harmol hydrochloride dehydrate (plate 5, well A9) was similar in this regard. In addition, 5-FU (which was not included in the screen and is related to 5-FUrd) has been shown to regulate miRNA expression in MCF-7 cells (Shah, Pan et al. 2011) and is currently used in cancer treatment, particularly in the treatment of CRC (Pardini, Kumar et al. 2011). Finally, there has been a great amount of research focused on norcantharidin, a demethylated version of cantharidin, as both a miRNA regulator and a cancer therapy and this is again further discussed in section 6.3.5. Norcantharidin was screened as part of the compound library (plate 5, well C2) but had no effect on Renilla luciferase despite cantharidin being the strongest hit identified.

In summary, several compounds identified here as miR-7 regulators along with their analogues have been shown to regulate the expression of other miRNAs and display anti-tumour activity.
6.3.4. False positives and general luciferase interference

As discussed in the previous chapter several compounds identified as hits in the primary screen were eliminated before the secondary screen based upon their raw luciferase counts. These compounds were believed to be either generally cytotoxic or have caused elevated Firefly luciferase counts thereby skewing the Renilla to Firefly luciferase ratio. Whilst there are several papers that comment on compound structures known to inhibit Firefly luciferase there are few that describe increased Firefly expression as a result of compound treatment. It is suggested that stabilisation of the Firefly luciferase protein by a compound may result in the accumulation of the Firefly protein resulting in increased luminescence detection (Thompson, Hayes et al. 1991, Thorne, Auld et al. 2010).

There was one compound, N-allyladenosine, which was not eliminated during the compound triaging process and failed the secondary screen. This compound was noted to have increased Firefly luciferase counts as they were greater than one standard deviation above the controls (Table 9). However, this was similar for cantharidin which proved to be the strongest hit and therefore the decision at this point not to eliminate it was justified. In addition, the Renilla luciferase counts appeared to be inhibited at the same time (reduced further than one standard deviation of the controls). An important point is that many compound libraries will contain compounds that generally inhibit luciferase signals and many compounds that inhibit Firefly, Renilla and other types of luciferase have been described (Auld, Southall et al. 2008, Herbst, Allen et al. 2009, Thorne, Auld et al. 2010, Auld D. S. 2016).

To confirm whether compounds are generally inhibiting luciferase they could be tested in a transient assay with empty and mutant psiCHECK-2 vectors (no stable cell line was generated for the mutant target site). If a compound inhibited Renilla luciferase in empty or mutant vectors it would indicate that the observed decrease in luciferase activity in the screen was due to general luciferase suppression rather than genuine miR-7 upregulation. Alternatively, a different format of assay using an alternative reporter, also known as an orthogonal assay, is sometimes used to confirm hits in the primary assay before proceeding to the secondary assay. However, given the small number of
identified hits, miR-7 upregulation was simply assessed directly by RT qPCR, enabling rapid confirmation of the nature of the hit.

In summary, false positive compounds were expected and eliminated through the validation phase. It is also important to remember that a compound which interferes with luciferase activity could have biological activity and may have been eliminated during the triaging process, in other words, a false negative.

6.3.5. Cantharidin

Cantharidin is a compound derived from blister beetles, also known as Cantharides or Spanish Fly, and has been used in traditional Chinese medicine for many medical applications including the treatment of cancer (Wang 1989). The anti-tumour activity of cantharidin has been described extensively in many cancers, including CRC and HCC (Li, Xie et al. 2010, Huang, Ko et al. 2011, Li, Chen et al. 2011, Li, Chen et al. 2011, Shou, Zhang et al. 2013, Shen, Wu et al. 2015, Tian, Zeng et al. 2015, Hsia, Yu et al. 2016, Le, Zhang et al. 2016). A few of its many described anti-tumour actions include several reports of G2/M phase cell cycle arrest and apoptosis (Li, Xie et al. 2010, Huang, Ko et al. 2011, Hsiao, Tsai et al. 2014, Zhang, Chen et al. 2014), inducing DNA damage and inhibiting DNA repair (Hsia, Lin et al. 2015, Kuo, Shih et al. 2015), and inhibiting invasion and migration (Gu, Xu et al. 2017). The action of cantharidin has been attributed to inhibiting invasion of pancreatic cells, in part, through post-transcriptional degradation of MMP2 (Shen, Wu et al. 2015). MMP2 is an enzyme which proteolytically degrades components of the extracellular matrix (Shah, Shukla et al. 2009). Suppression of MMP2 by cantharidin has been noted to inhibit migration and invasion in bladder cancer (Huang, Ni et al. 2013). Given MMP2 expression is also influenced by miR-7 (Wu, Wang et al. 2011, Zeng, Zhan et al. 2016) it would be interesting to explore whether the upregulation of miR-7 contributes in this context. Cantharidin has further been shown to downregulate FAK (Hsia, Yu et al. 2016) a direct miR-7 target (Cao, Mao et al. 2016) and investigating this relationship would also be of interest.

Cantharidin has been reported to both reverse the multi-drug resistance (MDR) phenotype (Zheng, Bao et al. 2008) and act despite the MDR phenotype (Rauh, Kahl et al. 2007). This observation is a great advantage when considering the next generation of
therapy. MDR is when cancers become resistant to single or classes of drug and show cross-resistance to other chemotypes (Gottesman, Fojo et al. 2002). Genes associated with sensitisation to oncotherapies are found to be upregulated as a result of cantharidin treatment in HL-60 promyeloid leukaemia cells (Zhang, Ying et al. 2004).

An additional advantage is the observation that cantharidin appears to be less toxic to normal pancreatic duct cells and liver cells when compared to pancreatic and HCC cells, respectively (Wang, Wu et al. 2000, Li, Xie et al. 2010), and does not cause myelosuppression (decrease in normal blood cells) in leukaemia (Liao, Su et al. 2007, Dorn, Kou et al. 2009). Work carried out with cantharidin analogues has further demonstrated selective toxicity in CRC cells relative to normal colon cells (Sakoff, Ackland et al. 2002). These attributes are desirable in potential drug candidates.

6.3.5.1. Cantharidin upregulates microRNA-7

Here cantharidin is identified and confirmed as a miR-7 regulator in HT29 and Huh7 cells. This supports previous studies carried out in MCF-7 breast cancer cells that identified a number of miRNAs upregulated as a result of cantharidin treatment, including miR-214 and miR-7 (Zhang and Yan 2015). Following a miRNA microarray which showed that cantharidin modulated the expression of miR-7 in MCF-7 cells, the authors confirmed miR-7 was upregulated approximately 2.5-fold after 48 h treatment with 1.75 µg/mL cantharidin (equivalent to 8.9 µM) and there was a dose-dependent inhibition of proliferation by cantharidin in this cell line (Zhang and Yan 2015). Here, 10 µM was used in HT29 cells and 1 µM was used in Huh7 cells to show upregulation by RT qPCR after 24 h (Figure 55 through Figure 61) and dose-dependent reduction in cell viability. The calculated EC₅₀ values were 3.49 µM for HT29 cells (Figure 62), 2.89 µM for HCT116 cells (Figure 64) and 1.42 µM for Huh7 cells (Figure 66) at 72 h, much lower than 8.9 µM which was the EC₅₀ determined for MCF-7 cells.

It is likely that similarly, cantharidin is not a specific miR-7 regulator in HT29 and Huh7, just as in MCF-7 cells. However, RT qPCR for several other miRNAs (those identified as upregulated in MCF-7 cells would be a good start) or a miRNA microarray could confirm
this. Nonetheless, cantharidin was further shown here to inhibit proteins known to be regulated by miR-7.

### 6.3.5.2. Cantharidin downregulates Akt and phospho-Akt

Cantharidin is a selective inhibitor of protein phosphatases PP2A and PP1 (Honkanen 1993). PP2A and PP1 modulate a large number of cellular pathways including apoptosis and cell cycle progression (Garcia, Cayla et al. 2003, Wlodarchak and Xing 2016) and inhibition of PP2A and PP1 has been attributed to the anti-cancer activity of cantharidin (Li, Xie et al. 2010). PP2A and PP1 is known to negatively regulate Akt activity by dephosphorylation of pAkt (Millward, Zolnierowicz et al. 1999, Ugi, Imamura et al. 2004). However, despite the fact that cantharidin inhibits PP2A and PP1, dephosphorylation of Akt following cantharidin treatment is evident in both HT29 (Figure 68) and Huh7 cells (Figure 70). However, it must be noted that the antibody used in this study recognises phosphorylation of the S473 residue and PP2A dephosphorylates T308. This may explain why, despite (predicted) cantharidin-mediated inhibition of PP2A, an expected increase in pAkt was not evident.

Although downregulation of total Akt in HT29 is evident, there is a complete loss of pAkt (Figure 68) which suggests the downregulation of pAkt is not simply due to a downregulation of total Akt expression. This has been noted in another study where cantharidin suppressed VEGF-induced phosphorylation of Akt in HMEC dermal microvascular endothelium cells (Wang, Liu et al. 2015). However, despite inhibition of Akt and pAkt, inhibition of the direct miR-7 target EGFR was not identified. This indicates that cantharidin’s mode of action is likely to be a combination of miR-7-dependent and miR-7-independent effects.

### 6.3.5.3. Cantharidin induces apoptosis?

Cantharidin was further shown to downregulate survivin in HT29 (Figure 68). Cantharidin has also previously been described to inhibit survivin in A549 cells and induce apoptosis (Zhang, Zhao et al. 2005). Survivin is considered an oncogene and is overexpressed in cancers where it promotes resistance to apoptotic pathways (Sah,
Survivin is believed to inhibit apoptosis through directly binding to caspases 3 and 7 (Tamm, Wang et al. 1998). However, both caspase-dependent and independent pathways have been described (Jaiswal, Goel et al. 2015). While there is convincing evidence for cantharidin-induced apoptosis in Huh7 cells via caspase 3/7 activity (Figure 76) and presence of cleaved PARP (Figure 78), due to the discontinuation of the survivin antibody confirmation of survivin downregulation in Huh7 cells was not able to be completed. On the other hand, survivin was clearly downregulated in HT29 cells but apoptosis via the Caspase-Glo assay was inconclusive (the assay was conducted 4 times which twice gave positive results and twice negative results). It is important to emphasise that survivin is merely a predicted miR-7 target (Agarwal, Bell et al. 2015, Agarwal, Bell et al. 2016). Several experiments would need to be conducted to establish whether the downregulation of survivin seen here is wholly or in part due to miR-7 upregulation by cantharidin, whether it subsequently plays a role in the observed apoptosis and finally, to confirm downregulation of survivin by cantharidin is not specific to HT29.

The upregulation of cleaved PARP in HT29 as a result of cantharidin treatment supports the data in favour of apoptosis. Keeping in mind that cleaved PARP may occur independent of caspase activity (Yang, Zhao et al. 2004) and also apoptosis may occur independent of caspase activity (Broker, Kruyt et al. 2005), may explain a negative result in Caspase-Glo assays for this cell line. As two assays are required as a minimum to confirm apoptosis, another assay, for example using an Annexin V assay, is also required. Annexin V staining detects the presence of phosphatidylserine of the outer surface of the cell membrane, another marker of imminent apoptosis (Crowley, Marfell et al. 2016). The Annexin V assay was carried out for HT29 and cantharidin with little success (data not shown), issues were solely technical preventing a conclusion from being drawn. In support of work conducted here, Huan et al. note apoptosis as a result of cantharidin treatment in HT29 CRC cells and T24 bladder cancer cells (Huan, Lee et al. 2006).

Apoptosis as a result of cantharidin treatment has been described in several cancer types including leukaemia via p38 and JNK MAPK pathways (Huh, Kang et al. 2004), CRC through downregulation of HSP70/BAG3 proteins resulting in the destabilisation of the BCL-2 family proteins (Kim, Kim et al. 2013), multiple myeloma via inhibiting the
JAK/STAT pathway (Sagawa, Nakazato et al. 2008) and pancreatic cancer via activation of the NF-κB and subsequent upregulation of pro-apoptotic genes (Li, Chen et al. 2011). Microarray analysis showed genes associated with apoptosis to be upregulated upon cantharidin treatment in leukaemia (Zhang, Ying et al. 2004) and in melanoma (Kadioglu, Kermani et al. 2014).

Apoptosis has also been described as a result of the cantharidin analogue, norcantharidin, in HT29 and HCT116 cells which was not identified as a miR-7 regulator. Suggesting that the mechanism of apoptosis in these cell lines and possibly Huh7 could be independent of miR-7 regulation (Peng, Wei et al. 2002). While this is likely the case, these findings support the findings here that indicate cantharidin induces apoptosis. In order to infer any link between miR-7 regulation by cantharidin and apoptosis in these cell lines anti-miR-7 could be used to determine if the offered any protection.

6.3.5.4. Cantharidin derivative, norcantharidin, was not identified as a miR-7 regulator

A major drawback of cantharidin is its systemic toxicity (Karras 1996, Cotovio, Silva et al. 2013) and therefore cantharidin derivatives have been the subject of much research. In recent times, there has been a focus on the demethylated version of cantharidin, norcantharidin. Norcantharidin has the advantage of reduced toxicity and urinary tract irritation whilst retaining PP2A and PP1 inhibitory activity and anti-tumourigenic effects (Massicot, Dutertre-Catella et al. 2005, Xie, Wu et al. 2015). Norcantharidin was included in the screen and was not identified as a hit (plate 5, well C2). This suggests that the anti-cancer activity of cantharidin may largely be independent of its regulation of miR-7. However, interestingly, while norcantharidin has similar anti-cancer properties to cantharidin, norcantharidin has little effect on pAkt (Zhang, Ji et al. 2013). Given cantharidin was identified by the screen as a miR-7 regulator where norcantharidin was not and cantharidin inhibits the indirect miR-7 target pAkt where norcantharidin does not, it is possible that some of this activity may be due to miR-7 activation. Findings in rescue experiments conducted in HT29 cells showed Akt and pAkt activity are rescued as a result of concurrent anti-miR-7 and cantharidin treatment (Figure 72). This indicates that the inhibition of Akt and pAkt by cantharidin could be in part due to its ability to
upregulate miR-7. However, treatment with anti-miR-NC showed some restoration of Akt and pAkt protein expression as well, albeit to a lesser extent. This may mean that the rescue of Akt and pAkt protein expression is somehow due to the introduction of an oligonucleotide and is independent of the sequence. To either confirm or eliminate this possibility the experiment could be repeated with a different anti-miRNA, where Akt and pAkt are not targets of the corresponding miRNA. Also, while miR-7 at a high concentration (30 nM) downregulates EGFR in HT29 cells Figure 23, cantharidin does not Figure 68. These findings suggest cantharidin action in relation to Akt and pAkt expression is likely not mediated by miR-7 regulation. A titration of EGFR expression relative to miR-7 concentration could be used to identify the lowest concentration of miR-7 required to observe a loss in EGFR protein expression.

In addition, the anti-cancer action of cantharidin beyond miR-7 upregulation would also explain why HT29 cells remain viable after treatment with miR-7 (up to 60 nM) but die as a result of cantharidin treatment. Furthermore, in HCT116 cells, while no miR-7 upregulation was detected as a result of cantharidin treatment, their viability is affected similarly to that of HT29.

6.3.5.5. Cantharidin effect is fast acting and potent

The effect of cantharidin is also relatively fast. miRNA upregulation and protein downregulation are both observable within a 24 h timeframe. Time course data indicated pAkt levels decreased within 4 h in HT29 cells (Figure 71).

EC₅₀ data determined after 72 h indicated that Huh7 cells were the most sensitive to cantharidin (EC₅₀ = 1.42 µM, Figure 66), and EC₅₀’s for both HT29 (EC₅₀ = 3.47 µM, Figure 62) and HCT116 (EC₅₀ = 2.89 µM, Figure 64) were of a comparable order of magnitude. In later Western blot experiments the concentration of cantharidin in Huh7 was reduced to 1 µM similar to its 72 h EC₅₀ in order to recover enough cells for protein collection after just 24 h. On the other hand, HT29 cells were treated with 10 µM cantharidin for Western blotting, three times its EC₅₀ value and remained viable at this concentration and time (24 h). This may suggest that cantharidin has a much more potent effect on Huh7 cell viability compared to HT29 cells in a 24 h period. Huan et al. similarly noted
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differences in a time- and dose-dependent manner upon cantharidin treatment in T24 bladder cancer cells compared with HT29 CRC cells. In particular, they noted greater toxicity in T24 cells within the first 6 h of treatment compared to HT29 cells, but subsequently greater toxicity in HT29 cells after the full 24 h period (Huan, Lee et al. 2006).

While nanomolar potency is generally desired in candidates for drug development, it is not necessarily a predictor of clinical efficacy (Wong, Cheng et al. 2012). Also, the toxicity of a potential drug candidate does not necessarily limit its potential for further development if the intended patient survival is not predicted beyond a few years (Strovel, Sittampalam et al. 2012). This is important as cantharidin displays micromolar potency shown here. While cantharidin has been involved in clinical trials for topical application of the treatment of the skin condition Molluscum Contagiosum (clinicaltrials.gov identifiers: NCT03017846, NCT00667225) and Verruca Vulgaris (common warts) (clinicaltrials.gov identifier: NCT01084824), it has not been previously trialled for the treatment of cancer.

6.3.6. 5-Fluorouridine (5-FUrd)

5-FUrd was selected as a potential hit compound following close analysis of raw luciferase counts. Apparent cytotoxic activity of the compound meant that it was analysed separately, yet still appeared to reduce the reporter luciferase counts relative to Firefly (Table 13). Here, 5-FUrd was confirmed to upregulate miR-7 in HT29 cells (Figure 55) and was then selected to be further tested in Huh7 cells given its relatively large and consistent upregulation of miR-7 in HT29 cells (section 6.2.2) when compared to the other hits. However, there was no apparent upregulation of miR-7 by 5-FUrd (Figure 61).

HT29 and HCT116 cells were highly sensitive to 5-FUrd and EC$_{50}$ concentrations were in the nanomolar range (Figure 63 and Figure 65). Huh7, on the other hand, did not appear to be as affected and 35% of cells still remained viable at the highest concentration tested (10 µM) after 72 h (Figure 67). Greater concentrations of 5-FUrd should be tested in Huh7 cells to confirm its EC$_{50}$. Like cantharidin, 5-FUrd appeared to take time to affect cell viability in HT29 cells. HT29 cells were treated at a concentration of 10 µM for 24 h
Results Chapter: Hit compound validation for the purposes of screening and Western blotting with enough cells still viable at the time of harvesting protein (24 h), however, after 72 h the EC$_{50}$ for HT29 was 0.0095 µM. My observations regarding cell viability indicated that HT29 and Huh7 cells appeared unaffected after 24 h of 10 µM 5-FUrd treatment, yet just 1 µM over the course of 24 h in HCT116 cells appeared to leave only ~50% viable. 5-FUrd therefore affected HCT116 cells much more rapidly than HT29 cells despite both having comparably low EC$_{50}$ values at 72 h.

Treatment with 5-FUrd (10 µM) resulted in a decrease in EGFR expression in HT29 cells (Figure 68) but not in Huh7 cells (Figure 70), consistent with miR-7 upregulation in HT29 but not Huh7. Further experimentation, similar to that done in anti-miR-7 rescue experiments with cantharidin (Figure 72) should be carried out to confirm if a link exists between the level of EGFR expression and miR-7 upregulation by 5-FUrd.

Unlike cantharidin, there is very little reported on 5-FUrd as a therapy, but work has been conducted with the related compound, 5-Fluorouracil (5-FU), including an assessment of miRNA modulation as a result of 5-FU treatment in CRC. The authors show up and downregulation of 22 miRNAs (out of 153 miRNAs as measured by RT qPCR) in response to 5-FU treatment. It is worth mentioning that the authors use clones derived from HT29 and HCT116 cells and miR-7 expression was not examined in their panel of miRNAs (Rossi, Bonmassar et al. 2007).

6.3.7. Summary

In summary, all hit compounds except one were confirmed as miR-7 regulators in HT29 cells (parental line, rather than the stable line generated for screening). Two of these confirmed hits, cantharidin and 5-FUrd were taken forward and examined in a further CRC cell line, HCT116, as well as in the HCC cell line, Huh7. Only cantharidin was confirmed as a miR-7 regulator in Huh7 cells. Cantharidin is supported by much research as a potential cancer therapy in various cancers and there have been several mechanisms of action described. Further characterisation of protein regulation by both cantharidin and 5-FUrd was carried out in HT29 and Huh7. While this was somewhat hampered by the discontinuation of a particular survivin antibody, 5-FUrd showed downregulation of EGFR and Akt in HT29 cells, while cantharidin showed
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downregulation of total Akt, pAkt and survivin in HT29 cells and pAkt in Huh7 cells. Downregulation of Akt and pAkt may in part be attributed to miR-7 upregulation by cantharidin. In the future, it is suggested that the HCT116 cell line (in which cantharidin did not upregulate miR-7) could be used as a control to identify the effects of cantharidin independent of miR-7 regulation. The aim of the project to develop a screening platform and identify compounds which regulate miR-7 activity was successful.
7. Thesis summary and future directions

This chapter summarises the key findings of this thesis and discusses them in context with current clinical therapies. Successes and limitations of both the nanoparticle-mediated miRNA delivery and compound screening aspects of this thesis are discussed. Finally, it outlines future directions for this project.

The focus of this thesis was to develop ways to increase miR-7 expression in cancer and in particular to either deliver miR-7 more efficiently to cancer cells or identify ways to upregulate miR-7 expression using small molecule modifiers. This aim was chosen given the emergence of miRNAs as potential therapeutics for cancer. During the course of this PhD, the field has significantly developed, as has the data for miR-7 as a clear therapeutic target given its actions as a tumour suppressor in many cancers.

Studies in this thesis were commenced with two parallel programs of work. In the first, the potential to increase miR-7 expression in CRC cells using a novel nanoparticle was investigated. Although nanoparticle-delivered miR-7 was successfully detected in cancer cells, subsequent demonstration that the intracellular miR-7 was functional was unsuccessful. This prompted a greater focus on the other program of work, which involved the identification of a novel small molecule that could increase miR-7 expression via screening of a custom small molecule library.

7.1. PGMA-PEI nanoparticles deliver microRNA-7 \textit{in vitro}

A key finding of this thesis was that a novel nanoparticle-miR-7 conjugate could deliver miR-7 to CRC cells but not with the functional activity desired. However, further development of this approach merits consideration. A study has since been published using similarly structured nanoparticles provided by the same laboratory as here (Iyer Lab, UWA) which showed success in siRNA/miRNA delivery and action (Tangudu, Verma et al. 2015). The authors demonstrated protein target knockdown with miRNA mimic and vector-based shRNA \textit{in vitro}, and reduced breast cancer tumour growth rate \textit{in vivo} following delivery with vector-based shRNA against c-Myc (pGIPZ). The authors conducted several aspects of their work differently to the studies carried out here. They
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used different miRNA mimics, siRNA and DNA vectors, with different targets. They also worked in different cells, which can affect transfectability, and importantly used a different binding ratio of nanoparticle to miRNA. As shRNA’s target a single protein, a modest amount may be sufficient to cause protein target knockdown in comparison to a miRNA which targets multiple mRNA transcripts. In addition, the binding ratio used here was informed by the Iyer Lab who supplied the nanoparticles and was 14:1 (nanoparticles:miRNA) incubated for 1 h whereas Tangudu et al. used a 4:1 nanoparticle to miRNA ratio for 4 h. This ratio is critical to optimise efficient miRNA binding and to retain the correct surface charge required for cellular uptake of nanoparticles by cells. The authors similarly outline that efficient induction of RNAi is dependent on high loading capacity. While similar in description, the exact composition of the nanoparticles may have been altered or improved upon by the time of this study, especially given that a different optimal binding ratio was determined. Therefore, the potential for miR-7 delivery to CRC cells and in vivo models is a real possibility and warrants further investigation especially with consideration to altering the nanoparticle-miRNA binding conditions.

Several different types of nanoparticles exist that have successfully delivered RNAi molecules to cells and these too may be considered in experimental application in the context of miR-7 (Wu, Wang et al. 2010, Crew, Tessel et al. 2012, Huschka, Barhoumi et al. 2012, Mohammadi, Salmasi et al. 2015, Ebrahimian, Taghavi et al. 2017, Xie, Murray-Stewart et al. 2017). The studies carried out here demonstrate in principle miRNA delivery by PGMA-PEI nanoparticles.

7.2. The design of the screening platform was effective

Meanwhile, the screening aspect of this thesis proved successful in the identification of a miR-7 regulator, namely cantharidin and several others in HT29 cells. There have been very few studies that have identified small molecule-mediated regulation of specific miRNAs, with most of these screens seeking miRNA inhibition (Gumireddy, Young et al. 2008, Young, Connelly et al. 2010, Xiao, Li et al. 2014, Lee, Lee et al. 2016). The screen conducted here, a cell-based luciferase screening platform, was similar in design to others aimed at identifying miRNA regulators.
During the writing phase of this thesis, a group published a paper in which they developed a very closely designed screening platform (Cinkornpumin, Roos et al. 2017). Their screen employed the psiCHECK-2 vector with eight let-7 target sites downstream of *Renilla* luciferase. They similarly cloned a neomycin resistance cassette into the psiCHECK-2 and generated stable Huh7 cells. In addition, they also sought a decrease in luciferase signal to identify let-7 activators. However, in contrast to the screen performed here, the screen by Cinkornpumin *et al.* was conducted in 384-well format. This validates the potential of the screen developed here to be miniaturised further for higher throughput. The $Z'\text{factor}$ achieved was 0.65 which is comparable to the $Z'\text{factors}$ of 0.5 (1 nM miR-7) and 0.8 (10 nM miR-7) achieved here.

In their screen, Cinkornpumin *et al.* reported a high rate of false positives due to off-target luciferase regulation (Cinkornpumin, Roos et al. 2017). To counteract this, they put in place parallel plates transiently transfected with control psiCHECK-2 vector or the let-7 reporter psiCHECK-2 vector. The inclusion of parallel transient plates was not necessary with the smaller library, and thereby a lesser number of false positives encountered herein. The inclusion of parallel plates would have been a costly and labour intensive modification to the screen. It would also not rule out those compounds that exhibited a reduced *Renilla*/Firefly ratio due simply to an increase in Firefly luciferase expression which was the reason many compounds were eliminated here. In the future, transient screening need not be conducted for the whole library of compounds in parallel to the screen, but rather, could be conducted as a secondary measure with hits identified to rule out false positives at this stage. However, with sterile tissue culture automation (which was not available to me) parallel transient screening could be more easily achieved. Nonetheless, the justification to carry this out would depend upon future throughput and the rate of false positives.

In addition, Cinkornpumin *et al.* ruled out toxic compounds through the incorporation of a luminescent cell viability assay (CellTiter-Glo (Promega)) as an additional measure in their screen (Cinkornpumin, Roos et al. 2017). They were able to conduct this using the live-cell luciferase reagent ViviRen (Promega) when measuring *Renilla* luciferase expression. In contrast, the reagent used for luciferase detection here (Dual-Glo) lyse the cells making a subsequent measure of cell viability impossible. On the other hand, as miR-7 expression results in reduced cell viability, it was decided not to eliminate toxic
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compounds immediately but instead to examine raw data of ‘dark’ wells before removing compounds. Keeping in mind the miR-7 regulator 5-FUrd was chosen from a selection of apparently cytotoxic compounds. Despite their toxicity, these compounds could still hold key information on compound structure required for miR-7 regulation and further optimisation could reduce their toxicity for clinical use.

Cinkornpumin et al. were similarly successful in identifying compounds that regulated their miRNA of interest and its targets which further demonstrates the success of this screen design elsewhere.

7.3. Further optimisation of the platform

There are many ways in which this screen could be modified and optimised for future use. A small molecule would have been preferable to a miRNA mimic as a positive control. However, the controls used in the screen for let-7 activators were similarly let-7 mimics. Longer incubation times in screening and validation stages could be considered, for example, 48 h as opposed to 24 h as some compounds may be slower acting, although this may exclude some hit compounds that act and reduce cell viability quickly. Further, miniaturisation of the platform would allow for greater throughput of compounds and would also reduce assay costs.

7.4. Compound library selection was appropriate for the screen

Advantages of the library utilised here is, firstly, that it is relatively small which suited the assay design in terms of throughput and cost. Secondly, it is composed of a diverse range of natural products which have been proven to produce many lead compounds. Finally, it has previously been screened for and resulted in, successful identification of a miRNA modulator, Rubone (Xiao, Li et al. 2014). All in all, the greater the number and variety of compounds screened provides a greater opportunity for identifying hits and therefore screening larger similarly diverse libraries in the future would be advantageous in this regard.
7.5. Cantharidin was identified as a hit compound with downstream effects on microRNA-7 targets

There were several hits identified and confirmed as regulators of miR-7 in the HT29 parental cell line; rifampicin, 5-FUrd, harmine, 6-benzylaminopurine riboside and cantharidin. Cantharidin and 5-FUrd were followed up for further testing as they appeared to induce the greatest fold upregulation of miR-7. Cantharidin which was confirmed to upregulate miR-7 in HT29 and Huh7 cells showed downstream pathway effects on miR-7 protein target expression, namely, pAkt in both lines. Also, 5-FUrd showed EGFR downregulation in HT29 cells. However, it must be noted that while EGFR is a direct miR-7 target, Akt and pAkt are indirect miR-7 targets and are therefore prone to effects independent of miR-7. In future studies, several other direct miR-7 targets such YY1 (Zhang, Li et al. 2013) and XRCC2 (Xu, Chen et al. 2014) which have been examined in the context of CRC would be beneficial in evaluating the downstream effects of cantharidin-mediated upregulation of miR-7.

There was also some indication that cantharidin induces apoptosis in HT29 and Huh7 cell lines in line with many reports that have also attributed cantharidin to apoptosis in other cancer cell lines (Huh, Kang et al. 2004, Huan, Lee et al. 2006, Sagawa, Nakazato et al. 2008, Kim, Kim et al. 2013).

7.6. Further work in compound validation

To improve upon the validation of hit compounds several different measures could be included. A time course of cantharidin induced miR-7 upregulation would be insightful to determine the speed of regulation. Especially given, pAkt downregulation was observed in just 4 h. A study reported miR-7 upregulation by Trichostatin A in just 8 h and dramatic EGFR inhibition in 24 h in MDA-MB-231 (Tu, Chen et al. 2014). An examination of mature, pre- and pri- miRNA transcripts via RT qPCR would also indicate whether the compound was acting transcriptionally or post-transcriptionally. It is suggested in the literature that cantharidin may generally affect miRNA expression and utilising RT qPCR for several other miRNAs may confirm this, such as miR-122 and miR-
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200c which were also identified to be highly upregulated as a result of cantharidin treatment in MCF-7 cells (Zhang and Yan 2015). Western blot analysis performed here is well suited and a direct measure for confirming compound action on target proteins. During the validation phase of the let-7 screen, Cinkornpumin et al. use RT qPCR to measure let-7 target protein mRNA (Cinkornpumin, Roos et al. 2017), however, mRNA levels do not always correlate with protein expression (Schwanhausser, Busse et al. 2011) making Western blot the preferred method here. Further assays that characterise the consequential effect of the drug such as proliferation, invasion and migration assays should be carried out (apoptosis was considered here). In addition, determining the mechanism of miR-7 upregulation and examining any effect cantharidin may have on epigenetic markers should also be considered.

Furthermore, as several other hits were confirmed in HT29 cells validation of all others could be carried out in different cancer cell lines.

7.7. Lead optimisation and testing of cantharidin analogues

Following the identification of lead compounds, comes the generation and testing of compound analogues to optimise activity as hit compounds will likely have rudimentary activity (Guo 2017). Natural compounds are unique in that they can be quite large and complex, with many structures that may not play a functional role and these can be removed at this stage to simplify the compound structure. This limits the potential for off-target interactions as well as simplifying chemical synthesis (Guo 2017). Building in lead-like properties guides the design of potential drug candidates. The aim is to improve activity, potency, selectivity, stability, solubility, minimise adverse effects and simplify structural complexity (Guo 2017).

Cantharidin is a relatively small and simple compound and the modification of its moieties will assist in identifying its structure-activity relationship with miR-7. For example, the demethylated version, norcantharidin, was not identified here as a regulator of miR-7 expression suggesting that the methyl group may be central to this activity. There have been several studies that have produced and tested cantharidin analogues in the context of cancer (McCluskey, Ackland et al. 2003, Yeh, Su et al. 2010).
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For example, an analogue of cantharidin which retained activity without any apparent toxicity reduced xenograft growth and lung metastasis via enhancing Doxorubicin activity in aggressive sarcoma (Zhang, Peng et al. 2010). In summary, there is potential for the improvement of cantharidin as a lead compound.

7.8. Clinical potential for small molecule regulators combined with nanoparticle delivery systems

While the delivery of oligonucleotide-based drugs by nanoparticles has been described thoroughly in previous chapters, the delivery of small molecule miRNA regulators by nanoparticles is concurrently being investigated. Rubone, identified as a miR-34a regulator has since been delivered in combination with paclitaxel in polymer-based PEG micelles and inhibited orthotopic prostate tumour growth in vivo (Wen, Peng et al. 2017).

In addition, another study has examined PEGylated liposomal delivery of cantharidin to HCC in vitro and in vivo. They showed improved therapeutic benefit of liposomally delivered cantharidin when compared to free cantharidin, greater anti-proliferative effect and, like the many studies mentioned in the previous chapter, G2/M phase arrest and apoptosis in HepG2 cells in vitro (Zhang, Lin et al. 2017). Therefore, there is much future scope for both aspects of this research.

7.9. Summary

In summary, aberrant miRNA expression is well recognised in cancer development, progression and response to treatment. However, despite recent advancements, their therapeutic delivery remains a considerable challenge. Whilst there are several clinical trials underway, severe side effects and related deaths associated with the MRX34 miR-34a mimic clinical trial that ceased in late 2016 has raised considerable concern regarding the safety of miRNA-based therapeutics and their delivery systems. Meanwhile, miRNA regulation by small molecules has shown great potential for cancer treatment in vitro and in vivo (Xia, Li et al. 2015). A large base of clinical experience with small molecule-based drugs and the safety concerns associated with miRNA delivery
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highlight the importance of studies into small molecule-mediated miRNA regulation. On the other hand, whilst the adverse events of the MRX34 trial could not be attributed to the carrier or to the mimic, there still remains much scope for the improvement of delivery systems in this regard.

This thesis demonstrated successful novel PGMA-PEI nanoparticle uptake by CRC cell lines, showed their successful capacity for miR-7 delivery, and assessed subsequent protein expression. In addition, an assay was developed that could identify modulators of miR-7 expression suitable for the screening of compound libraries. Potential host cell lines were assessed for suitability, including consideration for basal miR-7 expression, capacity for miR-7 upregulation and miR-7 dependent modulation of target protein expression. Dual-luciferase vectors with consensus and mutant miR-7 targets were generated and subsequently further modified for the generation of a stable cell line. Pilot studies were carried out and demonstrated that the system was sensitive to endogenous upregulation of miR-7 by HOXD10. Eight hundred naturally sourced compounds were screened. Hit compounds were determined after rigorous compound triaging and elimination. Identified hit compounds were confirmed to upregulate miR-7 by RT qPCR and assessed in further cell lines. Further characterisation of select compounds was carried out to determine dose-dependent luciferase inhibition, EC50 values, the effect on downstream target proteins, whether miR-7 could rescue protein inhibition and finally, to explore these compounds in the context of apoptosis. This study identified several miR-7 regulators. These compounds, in particular, cantharidin, offer new possibilities for the treatment of CRC and HCC and sets the scene for the development of novel cantharidin-based small molecule modifiers of miRNA expression. A screen for small molecule modifiers of miR-7 has not been developed or conducted before, and this platform could be used to continue screening further libraries for miR-7 regulators or, by changing the target sequence, could be applied to any other miRNA of interest.
References


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Appendices

Appendix I – Vector map of psiCHECK-2
Generated using SnapGene software (GSL biotech).

psiCHECK-2 vector sequence reference points:

- Synthetic Renilla luciferase gene (hRluc) 694 – 1629
- Multiple cloning region 1636 – 1680
- Synthetic firefly luciferase gene (hluc+) 2532 – 4184
- β-lactamase (Amp\(^{n}\)) coding region 4587 – 5447
Appendix II – Vector map of psiCHECK-2 (3mer)
Generated using SnapGene software (GSL biotech).

psiCHECK-2 (miR-7 target 3mer) vector sequence reference points:

- Synthetic Renilla luciferase gene (hRluc) 694 – 1629
- miR-7 target concatemer (three repeats) 1643 – 1741
- Synthetic firefly luciferase gene (hluc+) 2599 – 4251
- β-lactamase (AmpR) coding region 4655 – 5515
Appendices

Appendix III – Vector map of psiCHECK-2 (6mer)
Generated using SnapGene software (GSL biotech).

psiCHECK-2 (miR-7 target 6mer) vector sequence reference points:

- Synthetic Renilla luciferase gene (hRluc) 694 – 1629
- miR-7 target concatemer (six repeats) 1643 – 1824
- Synthetic firefly luciferase gene (hluc+) 2682 – 4334
- β-lactamase (Amp^n) coding region 4738 – 5598
Appendix III – Vector map of psiCHECK-2 (6mer + NeoR)
Generated using SnapGene software (GSL biotech).

psiCHECK-2 (6mer + NeoR) vector sequence reference points:

Synthetic Renilla luciferase gene (hRluc) 694 – 1629
miR-7 target concatemer (six repeats) 1643 – 1824
Synthetic firefly luciferase gene (hluc+) 2682 – 4334
Neomycin/ Kanamycin resistance gene 5069 – 5863
β-lactamase (AmpR) coding region 6352 – 7212