Exploring Breast Cancer Drug Targets in the Third Dimension with Imaging

Carrie Jade Lovitt
Bachelor of Science (Honours)

School of Biomolecular and Physical Sciences
Science, Environment, Engineering and Technology
Griffith University

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Abstract

This project utilises innovative methodology to evaluate the suitability of novel three-dimensional (3D) cell culture models for investigating anti-cancer drug activity. 3D cell culture methodology was utilised as this in vitro approach is considered to recapitulate the in vivo conditions more accurately than two-dimensional (2D) monolayer cell culture. Two separate 3D cell culture model formats were developed which are amenable to automated liquid handling systems, and a variety of instruments for total well fluorescence and confocal imaging. The first 3D cell culture assay developed was in a 384-well format, and was validated as suitable for use for drug discovery. The second 3D cell culture assay was optimised for 1536-well format, specifically created for extensive drug combination studies.

The 3D breast cancer cell culture assay developed for drug discovery utilised the breast cancer cell lines of MDA-MB-231 (endocrine receptor- and ErbB2 receptor-negative), MCF-7 (endocrine receptor-positive) and BT-474 (ErbB2 receptor over-expression). This 3D cell culture assay was miniaturised to a 384-well format, developed to be semi-automated and was thoroughly characterised for sensitivity and reproducibility. In addition, measurements of metabolic activity or spheroid morphology can be utilised for determination of drug activity. To validate the 3D breast cancer cell culture assay for use in high-throughput applications, a pilot screen comprising of 741 clinically relevant drugs was completed. Results from the pilot screen identified a number of drugs with anti-breast cancer activity that warranted further investigation. The drugs of interest were a mixture of drugs with both novel and demonstrated anti-cancer activity.

One drug of interest from the pilot screen was ciclopirox olamine, a drug currently used to treat fungal infections, which was recently identified as having anti-breast cancer activity. To further investigate the potential use of ciclopirox olamine in breast cancer therapy regimes in a pre-clinical setting, a novel 3D breast cancer cell culture assay was specifically developed for drug/compound combination studies in a high-throughput manner. This 3D breast cancer cell culture model was miniaturised to a 1536-well format and characterised for assay quality and reproducibility. Ciclopirox
Abstract

Olamine was further researched for use in breast cancer therapy in conjunction with doxorubicin, as ciclopirox olamine has been identified as an iron chelator. Iron chelators can be administered in combination with doxorubicin in breast cancer therapy regimes. However, more potent iron chelating drugs may also enhance anti-breast cancer activity. In the combination studies undertaken, results show that ciclopirox olamine is a potential candidate for anti-breast cancer therapy.

Culturing breast cancer cell lines in 3D cell culture has been shown to result in a range of different characteristics when compared to culturing cells in a traditional 2D monolayer. To determine if the 3D cell culture models demonstrated altered drug sensitivity due to the more biologically relevant culturing conditions, the activity of various standard of care breast cancer chemotherapy drugs was evaluated in both 2D and 3D cell cultures. Results showed significant drug resistance in 3D cell culture for anthracyclines and taxanes against selected breast cancer cell lines when compared to 2D cell culture. To investigate the mechanisms of anthracycline and taxane drug resistance observed in 3D cell culture models, a number of parameters which may contribute to altered drug sensitivity were examined. These parameters included: drug penetration, proliferation of cells in 3D culture, analysis of drug activity in 3D cell culture in the absence of extracellular matrix (ECM) proteins and altered expression of various key protein levels resulting from drug exposure.

Research revealed that doxorubicin penetrated breast cancer cells in 3D cell culture within hours of exposure at various drug concentrations. Therefore, the inability of doxorubicin to penetrate 3D cell culture was subsequently ruled out. However, cell lines were found to proliferate at a significantly lower rate in 3D cell culture compared to 2D cell culture, which may have impacted on drug sensitivity. In addition, there was cell line-dependent drug sensitivity differences detected in 3D cell cultures in the absence of ECM. The sensitivity changes observed in 3D cell cultures to doxorubicin in the absence of ECM were investigated further. Due to β1-integrin being intimately associated with the ECM, β1-integrin signalling was investigated for its potential role in doxorubicin resistance. Studies revealed that preventing β1-integrin signalling in 3D cell culture during drug exposure reduced the levels of Bcl-2 and Bcl-XL, suggesting that β1-integrin partially mediates drug resistance.
Collectively, the results obtained here show the reduced proliferation rate and expression of β1-integrin may contribute to drug resistance in breast cancer tumours.

These studies have resulted in the development of two miniaturised, well-characterised and reproducible assays using 3D breast cancer culture, which are suitable for high-throughput pre-clinical drug/compound evaluation and combination studies. Application of 3D cell culture increased our understanding of anti-breast cancer drug activity and enabled research into approaches for overcoming resistance mechanisms in breast cancer therapy.
Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Signature:                  Date:
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List of Abbreviations

% = Percentage
%CV = Percentage coefficient of variation
°C = Degrees Celsius
µl = Microliter/s
µM = Micromolar
2D = Two-dimensional
3D = Three-dimensional
ANOVA = Analysis of variance
AUC = Area under the curve
BF = Bright field
BRCA1 = Breast cancer 1, early onset
BSA = Bovine serum albumin
CMF = Cyclophosphamide/methotrexate/fluorouracil
Da = Daltons
DCIS = Ductal carcinoma in situ
DIC = Differential interference contrast microscopy
DMSO = Dimethyl sulfoxide
DNA = Deoxyribonucleic acid
ECM = Extracellular matrix
EDTA = Erythrylenediaminetetraacetic acid
EGF = Epidermal growth factor
EGFR = Epidermal growth factor receptor
ER = Oestrogen Receptor
ErbB2/HER2 receptor = Human Epidermal Growth Factor Receptor 2
FAK = Focal adhesion kinase
FGF = Fibroblast growth factor
FIC = Fractional inhibitory complex
FIX = Fractional inhibitory index
GEM = Genetically engineered mouse
GFR Matrigel = Growth factor reduced Matrigel
GRB2 = Growth-factor-receptor-bound-2
HCA = High-content analysis
HIF-1 = Hypoxia-inducible factor 1
HMG-CoA 3-Hydroxy 3-methylglutaryl coenzyme A
HTS = High-throughput screening
IC$_{50}$ = Half maximal inhibitory concentration
IDC = Invasive ductal carcinoma
IDC = Invasive ductal carcinoma
IF = Immunofluorescence
IGF = Insulin-like growth factor
IGF-1R = Insulin-like growth factor 1 receptor
ILC = Invasive lobular carcinoma
JNK = Jun-amino terminal kinase
LCIS = Lobular carcinoma in situ
MAPK = Mitogen-activated protein kinase
ml = Millilitre/s
mM = Millimolar
mTOR = Mammalian target of rapamycin
List of Abbreviations

NaCl = Sodium chloride
NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells
nM = Nanomolar
PAGE = Polyacrylamide gel electrophoresis
PAK = p21-activating kinase
PARP = Poly (ADP-ribose) polymerase
PBS = Phosphate-buffered saline
PDGF = Platelet-derived growth factor
PFA = Paraformaldehyde
PI3K = Phosphatidylinositol 3-kinase
PR = Progesterone receptor
PRF Matrigel = Phenol red-free Matrigel
PtdIns(3,4,5)P3 = phosphatidylinositol-3,4,5-trisphosphate
PTEN = Phosphatase and tensin homolog
rBM = Reconstituted basement membrane
RGD = Arg-Gly-Asp
RTK = Receptor tyrosine kinase
SD = Standard deviation
SDS = Sodium dodecyl sulphate
SEM = Standard error of the mean
SFKs = Src family kinases
SOS = Son-of-sevenless
TBS = Tris-buffered saline
TBS/T = Tris-buffered saline with Tween-20
TGF-β = Transforming growth factor beta
TLDU = Terminal duct lobular unit
v/v = Volume per volume
VEGF = Vascular endothelial growth factor
w/v = Weight per volume
Statement Acknowledging the Extent and Nature of Any Assistance Received in the Pursuit of the Research

Three publications have resulted from research undertaken as part of this PhD project to date. The papers are listed as follows:


I acknowledge the contribution of the co-authors within these publications. Only the research completed and the methodology developed as part of this PhD project is presented in this thesis and the research completed by others has been excluded or duly acknowledged and referenced. The design and development of this PhD project was completed in consultation with my supervisor, Professor Vicky Avery. The contribution of others to this work is detailed below.

Collaborative research with Todd Shelper was undertaken for the development of the miniaturised three-dimensional (3D) cell culture model for breast cancer. The results from the development and validation of this assay are described in research completed as part of Chapter Three, Chapter Four and Chapter Five of this thesis and a selection of these results is published in the manuscript entitled: “**Miniaturized Three-**
"Dimensional Cancer Model for Drug Evaluation." I acknowledge the contributions of my co-authors (Todd Shelper and Vicky Avery) for their contributions in this publication.

Application of methodology developed as part of this PhD was published within the manuscript entitled: “Nickel and Zinc Cyclam-amino Acid and Cyclam-peptide Complexes may be Synthesized with “Click” Chemistry and are Non-toxic.” The two-dimensional cell culture assay methodology utilised in the initial monolayer screening of the library of clinically relevant drugs in Chapter Five against MDA-MB-231 was utilised to obtain the breast cancer results published within this manuscript. The results obtained from the screening of the clinically relevant drug library were not included in this manuscript. I acknowledge the contributions of my co-authors (Mingfeng Yu, Jason Price, Paul Jensen, Todd Shelper, Sandra Duffy, Louisa Windus, Vicky Avery, Peter Rutledge and Matthew Todd) for their independent research presented in this publication.

Methodology developed in the course of this PhD project, and utilisation of images resulting from the 3D assay, were included within the manuscript recently accepted and in press entitled: “PCaAnalyser: A 2D-Image Analysis Based Module for Effective Determination of Prostate Cancer Progression in 3D Culture.” The methodology for determination of doxorubicin penetration into 3D breast cancer spheroids and a small portion of the results obtained from the research into drug penetration in 3D breast cancer cell culture conducted as a component of this PhD project (Chapter Six) was incorporated into the manuscript. I acknowledge the contributions of my co-authors (Md Tamjidul Hoque, Louisa Windus and Vicky Avery) for their independent research outcomes which have been presented in this publication.
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