In Vitro and *In Vivo* Models to Assess the Mechanism of Lapatinib-Induced Diarrhoea

A Thesis Submitted for the Degree of Doctor of Philosophy by

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This thesis is dedicated to my loving husband, Yasser and my precious children, Ammar and Yasmin for all their loves, supports and sacrifices.

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List of Abbreviations

$2^{-\Delta Ct}$	Delta threshold cycle
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ASCO	American Society of Clinical Oncology
AST	Aspartate aminotransferase
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
b.i.d	Twice daily administration
B2M	Beta-2-microglobulin
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
$CaCl_2.2H_2O$	Calcium chloride dihydrate
cAMP	Cyclic adenosine 5'-monophosphate
CFTR	Cystic fibrosis transmembrane conductance regulator
Cl^-	Chloride ion
CO2	Carbon dioxide
COX-2	Cyclooxygenase 2
Ct	Threshold cycle
CYP	Cytochrome
DAB	3, 3'-diaminobenzidine
DAPI	4′,6-Diamidino-2-phenylindole dihydrochloride
DEGs	Delayed early genes
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMEA	European Medicines Agency
ErbB1	Epidermal growth factor receptor 1
ErbB2	Epidermal growth factor receptor 2
ERK	Extracellular regulated kinase
EtOH	Ethanol
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FDA	US Food and Drug Association
FITC	Fluorescein isothiocyanate
G	Conductance
GGT	Gamma-glutamyl transferase
GI	Gastrointestinal
GPCR	G-protein coupled receptor
Grb2	Growth factor receptor bound protein 2
GSK	Glaxo Smith Kline
H&E	Haematoxylin and eosin
H_2O	Water
HB-EGF	Heparin-binding epidermal growth factor
HER	Human epidermal growth factor receptor
IC_{50}	Half maximal inhibitory concentration

IEC-6	Rat jejunal cell line
IEGs	Immediate early genes
IgG	Immunoglobulin
I_{sc}	Short circuit current
K^+	Potassium ion
K_2HPO_4	Potassium hydrogen phosphate
kDa	kilodalton
L200 2x	Lapatinib 200 mg/kg twice daily
L240 1x	Lapatinib 240 mg/kg once daily
LAP	Lapatinib
LD	Lactate dehydrogenase
LPS	Lipopolysaccharide
M.C.H	Mean corpuscular haemoglobin
M.C.H.C	Mean corpuscular haemoglobin concentration
M.C.V	Mean corpuscular volume
mAbs	Monoclonal antibodies
MAPK	Mitogen activated protein kinase
MBC	Metastatic breast cancer
mCRC	Metastatic colorectal cancer
$MgCl_2.6H_2O$	Magnesium chloride hexahydrate
mRNA	Messenger ribonucleic acid
Na^+	Sodium ion
Na^+/K^+-ATPase	Sodium potassium adenosine triphosphatase
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate

NEC	Necrotising enterocolitis
NGS	Normal goat serum
NSCLC	Non-small cell lung cancer
P.C.V	Packed cell volume
PAC	Paclitaxel
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pErbB1	Phosphorylated ErbB1
pErbB2	Phosphorylated ErbB2
Pgp	P-glycoprotein
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-kinase
PS	Phospholipid phosphatidylserine
q.d.	Once daily administration
R.B.C	Red blood cell
R.D.W	Red blood cell distribution width
Raf	Rapidly accelerated fibrosarcoma
Ras	Reticular activating system
RM-SCCHN	Recurrent and/or metastatic squamous cell carcinoma of head and neck
RNA	Ribonucleic acid
S.E.M	Standard error mean
SCCHN	Squamous cell carcinoma of head and neck
Sh2	Src-homology 2
T84	Human colon carcinoma
TEER	Transepithelial electrical resistance

TGF-α	Transforming growth factor alpha
TKI	Tyrosine kinase inhibitor
TNF-α	Tumour necrosis factor alpha
TX-100	Triton X-100
UBC	Ubiquitin C
Walker 256	Rat breast carcinoma cell line
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

Abstract

Lapatinib, an ErbB1/ErbB2 tyrosine kinase inhibitor is effective in breast cancer treatment but is associated with diarrhoea. ErbB1 inhibition by lapatinib may interfere with the normal functioning in the intestines. However, the underlying mechanisms remain unclear. This PhD project was conducted to investigate the mechanism of lapatinib-induced diarrhoea.

First study was conducted to determine the cytotoxic properties of lapatinib on rat breast carcinoma (Walker 256) and jejunal (IEC-6) cells and to evaluate the relationship between ErbB1 expression and sensitivity to growth inhibition by lapatinib. The cytotoxic effect of lapatinib on Walker 256 and IEC-6 was evaluated via XTT assay and FACS analysis. Cell lines were incubated with lapatinib for 6, 24 or 48 hours before evaluation. ErbB1 and ErbB2 mRNA and protein expression were determined via RT-PCR and immunofluorescence staining, respectively. Lapatinib inhibited 50% Walker 256 and IEC-6 cell growth at 8.2 ± 0.18 and 3.03 ± 0.26 μM respectively. Higher percentage of necrotic cells (37.91 ± 7.08 %) were observed in lapatinib-treated Walker 256 compared to control untreated cells (11.86 ± 5.62 %) ($p<0.01$), at 48 hours. Lapatinib-treated IEC-6 at 24 hours showed higher percentage of late apoptotic cells (53.56 ± 15.37 %) compared to controls (12.91 ± 4.70 %) ($p<0.01$). Similarly, at 48 hours incubation lapatinib-treated IEC-6 showed a higher percentage of late apoptotic cells (56.82 ± 11.53 %) compared to the control untreated samples that exhibited 22.70 ± 12.81 % late apoptotic cells ($p<0.05$). *ErbB1* mRNA was unable to be detected in Walker 256 due to low expression. Both mRNA expression of *ErbB1* and *ErbB2*, as well as immunofluorescence staining of ErbB1, ErbB2, pErbB1 and pErbB2 showed no differences between control untreated and lapatinib-treated cells. Lapatinib induced necrosis in tumour cells, while inducing late apoptosis in jejunal cells. This may explain lapatinib-induced diarrhoea in patients administered with the drug which could be due to apoptosis of small intestinal epithelial cells leading to barrier disruption and

consequently diarrhoea. However, much remains to be learned on the molecular mechanisms related to lapatinib's cytotoxic effect.

The second study was performed to develop Walker 256 tumour-bearing rat model to study lapatinib-induced diarrhoea. Walker 256 cells were then injected into the right flanks of female albino Wistar rats and grown over 3 weeks. This correlates with the peak of lapatinib-induced diarrhoea in Wistar rats. A concentration of $2x10^6$ cells/0.2 ml resulted in consistent tumour growth. Tumours were measurable by day 4 and reached 10% of body weight 25 days post-inoculation, without metastasis to distant sites. *ErbB1* and *ErbB2* mRNA were expressed in the tumour tissue. Walker 256 tumour did not cause any changes in jejunum and colon, thus there will be no interference of tumour on the intestines in the study of lapatinib-induced diarrhoea. As tumour regression was seen, this matter was taken into consideration in the following study in which the tumour growth was observed intently.

The animal model now provides a framework which enables the study of lapatinib-induced diarrhoea in tumour-bearing animals. Thus, the third study was carried out which aimed to identify histological changes in intestines following lapatinib treatment and to determine the mechanism of diarrhoea related to the treatment. Female albino Wistar rats were injected subcutaneously with Walker 256 breast tumour cells. When the tumour reached 0.01% of body weight, rats were divided into three groups: control, lapatinib 240mg/kg once daily gavaged (L240 1x) and lapatinib 200mg/kg twice daily gavaged (L200 2x). Rats were assessed for indicators of intestinal injury. Upon necropsy, jejunum and colon were collected for histological assessment via H&E staining. Expression of ErbB1, ErbB2 and markers for apoptosis (caspase-3) and proliferation (ki-67) were detected via immunohistochemistry. From the results, diarrhoea was seen in L200 2x group but not in other groups. However, both L240 1x and L200 2x showed significant changes in the intestines compared to controls such as villus blunting in jejunum (L240 1x $p<0.01$, L200 2x $p<0.0001$) and increased apoptosis in colon (L240 1x $p<0.01$, L200 2x $p<0.001$). ErbB2

expression in jejunal crypt was significantly lower than controls in both L240 1x ($p<0.05$) and L200 2x ($p<0.05$). Lapatinib twice daily administration caused diarrhoea. However, it was not related to ErbB1 expression as was expected. The current study was unable to find a dose and a schedule of lapatinib that was able to induce diarrhoea with tolerable levels of systemic toxicity. As such, further research is required to test a number of different schedules to find an appropriate way to model lapatinib-induced diarrhoea in breast cancer-bearing rats. This will enable future work to focus on uncovering mechanisms of lapatinib-induced diarrhoea and to test interventions for diarrhoea management.

The fourth and final study was carried out to investigate the effect of lapatinib and paclitaxel treatment on chloride secretion in the T84 model of colonic epithelium. Lapatinib and paclitaxel were first tested for their cytotoxic effect on proliferating cells that revealed T84 cells were relatively resistant to lapatinib with an IC_{50} of 26.48 ± 1.64 µM in serum-free-medium, 43.24 ± 2.73 µM in 5% serum-medium and 58.59 ± 1.37 µM in 10% serum-medium. In comparison, the effect of paclitaxel was more potent with an IC_{50} reached at 7.52 ± 0.25 µM in serum-free-medium, 12.58 ± 1.13 µM in 5% serum-medium and 18.48 ± 0.77 µM in 10% serum-medium. The lack of response of T84 cells to lapatinib is likely due to low target expression. As it has been shown in the current study that lapatinib and paclitaxel have cytotoxic effects on the T84 human colonic epithelial cell line, TEER experiments were conducted to determine the effect of both drugs on cell permeability. It was observed that lapatinib at both higher and lower concentrations, treated either from apical, basal or both apical and basal sides, as well as at different incubation hours does not affect colonic epithelial cell permeability, suggesting that lapatinib does not damage cell-cell adhesion properties, and likely spares epithelial tight junctions. To examine the effect of lapatinib as well as paclitaxel on intestinal chloride transport, T84 human colonic epithelial cell monolayers were mounted in Ussing chambers and the changes in short circuit current (I_{sc}) were measured. Experiments were conducted at established monolayer resistance and it was

observed that pre-treatment of T84 cells with lapatinib or paclitaxel did not affect baseline $I_{sc.}$ Chloride secretion was measured as I_{sc} across the T84 cell monolayers that were mounted in Ussing chambers, thus the findings reflected that pre-treatment with both drugs does not alter baseline chloride secretion. The present study exhibited higher baseline conductance of cell monolayer pre-treated with a high concentration of paclitaxel which reflected higher permeability, consequently lower barrier function/resistance, compared to control untreated monolayers. The finding is supported by the TEER results that showed that higher concentration of paclitaxel increased cell monolayer permeability. Lapatinib and paclitaxel also do not affect chloride secretion, however, due to lapatinib being an ErbB1 inhibitor which could interfere with chloride secretion, further investigations are required. Effect of lapatinib on chloride secretion might occur via other mechanisms unrelated to calcium or cAMP regulated chloride secretion. The mechanism of lapatinib-induced diarrhoea may be mediated by other unknown mechanisms. The cytotoxic effect of lapatinib as well as paclitaxel on T84 colonic cell line was also shown not to contribute to its effect on T84 monolayer permeability and chloride secretion. Overall, further investigations are needed to clarify the possible mechanisms of lapatinib-induced diarrhoea.

Finally, investigations in this PhD study managed to describe the minor impact of lapatinib on the gastrointestinal mucosa. Thus, much remains to be learned on the mechanisms related to lapatinib or ErbB1 targeted therapy-induced diarrhoea. Understanding the mechanism of damage will lead to an ability to target prevention or treatment as well as managing targeted therapy-induced diarrhoea appropriately.

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Presentations at scientific meeting

1. *Investigating Walker 256 Tumour-Bearing Rat Model to Study Lapatinib-Induced Mucosal Injury,* Australian Society for Medical Research (ASMR) South Australia (SA) Annual Scientific Meeting, 8 June 2013, Adelaide Convention Centre, Adelaide, South Australia.
2. *Development of a Walker 256-TC Tumour-Bearing Rat Model to Study Lapatinib-Induced Mucosal Injury*, Multinational Association of Supportive Care in Cancer/International Society of Oral Oncology (MASCC/ISOO) International Cancer Care Symposium, 27-29 June 2013, Berlin, Germany.
3. *Effects of the Dual ErbB Tyrosine Kinase Inhibitor, Lapatinib, on Walker 256 and IEC-6 cell lines.* Australian Society for Medical Research (ASMR) South Australia (SA) Annual Scientific Meeting, 4 June 2014, Adelaide Convention Centre, Adelaide, South Australia.
4. *Comparative Effects of the Dual ErbB Tyrosine Kinase Inhibitor, Lapatinib, on Tumour and Intestinal Cell Lines*, Florey International Postgraduate Research Conference, 25 September 2014, National Wine Centre of Australia, Adelaide, South Australia.
5. *Investigating Lapatinib-Induced Diarrhoea in a Tumour-Bearing Rat Model*, Malaysian Society of Pharmacology and Physiology 29th Scientific Conference, 24-25 August 2015, Setia City Convention Centre, Shah Alam, Selangor, Malaysia.
6. *Cytotoxic Effects of the Dual ErbB Tyrosine Kinase Inhibitor, Lapatinib, on Tumour and Intestinal Cell Lines,* Malaysian Society of Pharmacology and Physiology 30th Scientific Conference, 15-16 August 2016, Shangri-La Putrajaya, Putrajaya, Selangor, Malaysia.

Thesis explanation

This thesis is composed of eight chapters as follows: literature review, general methods, four distinct research chapters, general discussion, references and appendix. Each research chapter was written with introduction, material and methods, results, discussion and conclusion. All references are included in the final chapter.