DESIGN AND DEVELOPMENT OF NOVEL TOOLS FOR THE SCREENING AND IDENTIFICATION OF INHIBITORS OF HER RECEPTOR FAMILY AND NRF2 FOR OVARIAN CANCER THERAPY

A thesis submitted for the degree of Doctor of Philosophy (PhD)

By

Ibrahim Hamza Kankia,
BSc, MSc, MRSB

School of Science, Engineering and Technology, Abertay University
Dundee, United Kingdom

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Declaration

Candidate's declaration

I, **IBRAHIM HAMZA KANKIA** hereby certify that this thesis submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy (PhD), Abertay University, is wholly my own work unless otherwise referenced or acknowledged. This work has not been submitted for any other qualification at any other academic institution.

Signed……
Date……

Supervisor's Declaration

I, **DR. YUSUF DEENI** hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Doctor of Philosophy (PhD) in Abertay University and that the candidate is qualified to submit this thesis in application for that degree.

Signed……
Date……

Certificate of Approval

I certify that this a true and accurate version of the thesis approved by the examiners, and that all relevant ordinance regulations have been fulfilled.

Supervisor……
Date……..……

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Dedication

This thesis is dedicated

To:

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My Parents, Late Alhaji Hamza Kankia and

Late Hajiya Ramatu Hamza Kankia

And

The over 14.1 million people with cancer cases in the world.

Publications Relating to Thesis

Publications

- ✓ Hilal S. Khalil, Simon P. Langdon, **Ibrahim H. Kankia**, James Bown, and Yusuf Y. Deeni 'NRF2 Regulates HER2 and HER3 Signalling Pathway to Modulate Sensitivity to Targeted Immunotherapies', Oxidative Medicine and Cellular Longevity, pp. 22. http://dx.doi.org/10.1155/2016/4148791

- ✓ **Ibrahim H. Kankia**, Hilal S. Khalil, Simon P. Langdon, Peter R. Moult, James L. Bown, and Yusuf Y. Deeni 'NRF2 Regulates HER1 Signalling Pathway to Modulate the Sensitivity of Ovarian Cancer Cells to Lapatinib and Erlotinib', Oxidative Medicine and Cellular Longevity, Oxidative Medicine and Cellular Longevity (Submitted and is under review).

- ✓ **Ibrahim H. Kankia**, Hilal S. Khalil, Simon P. Langdon, Peter R. Moult, James L. Bown, and Yusuf Y. Deeni 'NRF2 Regulates HER4 Signalling Pathway in Ovarian Cancer Cells (In preparation for submission).

- ✓ **Ibrahim H. Kankia**, Hilal S. Khalil, Simon P. Langdon, Peter R. Moult, James L. Bown, and Yusuf Y. Deeni 'Targetting HER4 Signalling Pathway to Modulate the Sensitivity of Ovarian Cancer Cells to RTK Chemotherapy (In preparation for submission).

Oral Presentations

- ✓ **Ibrahim H. Kankia**, Hilal S. Khalil, James L. Bown, and Yusuf Y. Deeni 'Processes of Cloning' Presented during the YLS Symposium 2015: Modelling approaches in molecular signalling 13th November 2015 at University College London

- ✓ **Ibrahim H. Kankia**, Hilal S. Khalil, James L. Bown, and Yusuf Y. Deeni '' Design and Development of Novel tools for the Screening and Identification of HER receptor Family and NRF2 inhibitors for Ovarian Cancer Therapy' Presented during Abertay Postgraduate Research Student Conference, 22- 24, June 2016 at Graduate School Seminar Room, Abertay University.

Poster Presentations

- ✓ **Ibrahim H. Kankia**, Hilal S. Khalil, James L. Bown, and Yusuf Y. Deeni '' Polymerase Chain Reaction (PCR) as one of the steps to start Cloning' Presented during Abertay Postgraduate Research Student Conference, 11- 12, November 2015 at Graduate School Area, Abertay University.

- ✓ **Ibrahim H. Kankia**, Hilal S. Khalil, James L. Bown, and Yusuf Y. Deeni '' Cloning of proximal promoter regions of human *HER1* and *HER4* genes' Presented at 2nd International conference on CLINICAL SCIENCES AND DRUG DISCOVERY held at University of Dundee, on 27-29 July 2016.

- ✓ **Ibrahim H. Kankia**, Hilal S. Khalil, Simon P. Langdon, Peter R. Moult, James L. Bown, and Yusuf Y. Deeni" NF-E2-related factor (NRF2) cross-talks with HER receptor signalling pathway to modulate the anti-cancer effectiveness of RTK inhibitors in ovarian cancer cells' Presented at Cell Symposia: Cancer, Inflammation, and Immunity, June 11-13, 2017-San Diego, CA, USA

Abstract

Cancer, which is characterised by aggressiveness and increased capacity for metastatic spread still requires basic researchers and clinicians to direct enormous efforts toward the development of novel therapeutic targets. Potential novel targets can be identified and exploited in combination with currently existing therapeutic approaches to improve their efficacy and overcome treatment resistance of tumour cells, protecting the patient from recurrence. To achieve this, different strategies and techniques can be proposed to identify the most promising candidate molecules for further exploitation as therapeutic targets. Human epidermal growth factor receptors (HERs) and NF-E2-related factor 2 (NRF2) are regulators of cellular proliferation and determinants of cancer initiation and progression. NRF2 and HERs confer cancers with resistance to several therapeutic agents. Nevertheless, there is limited understanding of the regulation of HER expression and activation, and the link between NRF2 and HER signalling pathways. This research has demonstrated that pharmacological activation of NRF2 by tert-butyl hydroquinone (tBHQ) upregulates the expression of HER family receptors, HER1 and HER4, elevates phospho protein kinase B (pAKT) levels, and enhances the proliferation of ovarian cancer cells. Pharmacological inhibition using retinoic acid (RA) and bexarotene and genetic inhibition using small interfering RNA (siRNA), did the opposite. Further, tBHQ caused transcriptional induction of HER1 and HER4 with different levels of expression, while siRNA-mediated knockdown of NRF2 prevented this and further caused transcriptional repression. A panel of potent NRF2 inhibitors were screened with the hope of finding the most potent for further investigation. Bexarotene was found to be the most potent and was used either alone, or in combination with lapatinib or erlotinib. The use of these drugs in combination with bexarotene resulted in the repression of HER1, HER2, HER3 and HER4 expression, inhibition of NRF2, elevation of ROS, depletion of glutathione and enhanced cytotoxicity in PEO1, OVCAR3, SKOV3 and MCF7-AREc32 cell lines. This explained the crosstalk mechanism between HER receptor family and NRF2 and the role of NRF2 in drug resistance and as a relevant anti-cancer target which opens up novel avenues of targeting HER receptor kinase family and NRF2 pathways for improving cancer therapy.

Abbreviations, Symbols, Units and Notations

AKT	Protein kinase B
ALDH1A1	Aldehyde dehydrogenase 1 family, member A1
ALDH3A1	Aldehyde dehydrogenase 3 family, member A1
ANOVA	Analysis of variance
AR	Amphiregulin
ARE	Antioxidant responsive element
ATP	Adenosine Triphosphate
Bach1	BTB and CNC homology 1
Bach2	BTB and CNC homology 2
bHLH	Basic helix-loop-helix
BTB	Broad complex Tramtrack and Bric-à-Brac
BTC	Betacellulin
BLAST	Basic local alignment search tool
BSA	Bovine Serum Albumin
BRCA1	Breast cancer typ1 susceptibility protein
BRCA2	Breast cancer typ2 susceptibility protein
Bp	Base pair
bZIP	Basic region-leucine zipper
CI	Combination Index
CREB	cAMP response element-binding protein
CBP	CREB-binding protein
CDK	Cyclin-dependent kinase

c-Fos	Fos family transcription factors
c-Jun	Jun family transcription factors
c-Myc	Myc family transcription factors
CHD	Chromodomain helicase DNA-binding
CHD6	Chromodomain helicase DNA binding protein 6
CHX	Cycloheximide
CNC	Cap'n'collar
Cul3	Cullin3
dH2O	Double distilled water
DMEM	Dulbeccos‟s modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EPR	Epiregulin
ERK	Extracellular signal-related kinase
et al.	et alia (and others)
ETF	EGFR-specific transcription factor
FBS	Foetal bovine serum
5-FU	5-fluorouracil
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCLC	Glutamate cysteine ligase catalytic subunit

GCLM	Glutamate cysteine ligase modulatory subunit
Grb2	Growth factor receptor-bound protein 2
GSK3β	Glycogen synthase kinase-3β
h	hour
H_2O_2	Hydrogen peroxide
HB-EGF	Heparin-binding EGF-like growth factor
HER1	Human epidermal growth factor receptor 1
HER2	Human epidermal growth factor receptor 2
HER3	Human epidermal growth factor receptor 3
HER4	Human epidermal growth factor receptor 4
HO-1	Heme oxygenase-1
HOCl	Hypochloride
HRG- β1	Heregulin-β1
HRP	Horse radish peroxide
HSP	Heat-shock protein
Ig	Immunoglobulin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
INRF2	Inhibitor of NRF2
IVR	Intervening region
JNK	c-Jun N-terminal kinase
KEAP1	Kelch-like ECH-associated protein 1
LAR	Luciferase assay reagent
LDS	Lithium dodecyl sulfate

MAPK	Mitogen-activated protein kinase
mg	Milligram
mins	Minutes
mL	Milliliter
mM	Millimolar
mm	Millimetre
mRNA	Messenger ribonucleic acid
MRP	Multidrug resistance associated protein
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NCBI	National Centre for Biotechnology Information
NF-Kb	Nuclear factor kappa-light-chain-enhancer of activated B cells
nm	Nanomolar
NO•	Nitric oxide
NO_2^+	Nitronium
N_2O_2	Dinitrogen dioxide
NQO1	NAD(P)H dehydrogenase, quinone 1
NRF1	Nuclear factor (erythroid 2) – Like factor 1
NRF2	Nuclear factor (erythroid 2) – Like factor 2
NRF3	Nuclear factor (erythroid 2) – Like factor 3
^oC	Degrees Celsius
$^1O_2$	Singlet oxygen
O2•−	Superoxide
O3	Ozone

OH	Hydroxyl radical
OD	Optical density
ONO−	Peroxynitrite
O2NOCO2−	Nitrocarbonate anion
O=NOOCO2−	Nitrosoperoxycarbonate anion
Opti-MEM	Reduced-Serum Minimal Essential Medium
pAKT	Phospho protein kinase B
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pGL3-Basic	Firefly luciferase reporter basic vector
PI3K	Phosphatidylinositol 3-kinase
pmol	Picomolar
PPAR	Peroxisome proliferation receptor
pRL-CMV	Renilla luciferase internal control vector
PVDF	Polyvinylidene difluoride
R•	Organic radicals
RA	Retinoic acid
RAC3/AIB1/SRC-3	Receptor-associated coactivator 3/ Amplified in breast 1/ Steroid receptor coactivator-3
RAR	Retinoid acid receptor
RARE	Retinoic acid response elements
RAS	Rat sarcoma viral oncogene homolog
Rb	Retinoid binding

RFP	Red fluorescence protein
RIPA	Radioimmunoprecipitation assay
RO•	Alkoxyl radicals
ROO•	Peroxyl radicals
ROOH	Organic hydroperoxides
ROS	Reactive oxygen specie
ROS•	Sulfonyl radicals
RPM	Revolution per minute
RPMI Medium	Roswell Park Memorial Institute medium
RS•	Thiyl radicals
RSOO•	Thiyl peroxyl radicals
RSSR	Disulfides
RTK	Receptor Tyrosine Kinase
RTKi	Receptor Tyrosine Kinase inhibitors
RXR	Retinoid X receptor
s	second
Shc	SHC-transforming protein 1 adaptors
SDS	Sodium dodecyl sulfate
SDSPAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sMAF	small musculoaponeurotic fibrosarcoma
SOB	Super optimal broth
Sp1	Specificity protein 1
tBHQ	tert-butylhydroquinone

TAD	Transactivation domain
TAE	Tris Acetate EDTA buffer
TB	Terrific Broth
TBS	Tris-buffered saline
TBST	Mixture of tris-buffered saline and Tween 20
TGFα	Transforming growth factor-α
TNBC	Triple-negative breast cancer
U	Units
UK	United Kingdom
USA	United States of America
UV	Ultra violet
V	Volts
WHO	World Health Organisation
x	how many times (concentration)
µ	micro
µg	microgram
µL	microliter
µM	micromolar
%	percent
>	Greater than
<	Less than
=	Equals to

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Structure of the Thesis

This thesis is designed into seven chapters and sections of references and appendices, chapter one is a general introduction and literature review and chapter two is the materials and methods. Chapters three, four, five and six are in line with the research aims and objectives of the thesis. Chapter seven is the discussion and conclusion while the other sections are references and appendices respectively. The following are the brief description of each chapter:

Chapter One: Provides the general introduction of cancer, then ovarian cancer specifically, followed by the deep review of what has been done, what needs to be done by the research in the area of this study. This chapter provides a background of this research which gives an insight that paved a way of developing hypothesis and aims and objectives of this study.

Chapter Two: Provides material and methods followed for the successful completion of this research which involves small portion of bioinformatics and all the rest are laboratory based. It gives a complete description of the materials used and methods followed during the data collection and analysis.

Chapter Three: Is the first research chapter and it explains how the first objective is achieved. This section of the study reported on cloning of promoter regions of human HER1 and HER4 genes which subsequently provides an insight into the study in the next research chapter.

Chapter Four: Is the second research chapter and it explains how the second objective is achieved. It provides the molecular basis for regulation of the expression of these genes by NRF2. It explains the regulation of HER1 and HER4 upon NRF2 activation with tBHQ and inhibition with RA and knocking down with siRNA, there by highlighting the possibility of direct linkage between ROS, HER family and NRF2 signalling pathways.

Chapter Five: Is the third research chapter and it explains how panels of compounds expected to be the potent NRF2-ARE inhibitors were screened to uncover the most potent compound for the next research chapter in this study.

Chapter Six: Is the fourth and the last research chapter which explains how the last objective of this research is achieved. In this section, all the HER receptor family were investigated following treatments with either bexarotene or in combination with lapatinib or erlotinib. A series of experiments such as cytotoxicity assays, immunoblotting, ROS level detection as well as total glutathione were performed.

Chapter Seven: Discusses and provides key findings and concludes this research. It also gives recommendations for future studies.

References section: Contains all the references cited in this research in alphabetical order and in Harvard referencing style recommended by Abertay University.

Appendices section: Provides the information of the materials, equipment, reagents, chemicals used in this study as well as their nature, source and the company they are purchased or name of the donor.

1. CHAPTER ONE

General Introduction and Literature Review

1.1. Cancer

Cancer is a complex disorder characterised by abnormal growth and alterations in cells, reduced cell death and increased cellular energy metabolism (Ivey et al., 2016, Hojjat-Farsangi et al., 2014, Hojjat-Farsangi et al., 2017, Hanahan and Weinberg, 2011). Cell division and apoptosis are considered the two most common physiological processes that regulate normal tissue homoeostasis, and their disruption could lead to the pathogenesis of cancer, a fatal disease that consists of immortal cells (Hojjat-Farsangi et al., 2014, Nwabo Kamdje et al., 2014, Hojjat-Farsangi et al., 2017). Cancer is initiated by repeated cell attrition and repopulation involving two concepts of cancer initiation. Firstly, cancer is the result of a series of genetic changes that subsequently transform normal cells into malignant ones. Secondly, deregulation of specific biological processes during tumour development helps and sustains cancer initiation and progression (Labi and Erlacher, 2015). Cancer is considered one of the leading causes of death worldwide, with reported cases in 2012 of approximately 14 million (Torre et al., 2015, Ferlay et al., 2013, WHO, 2017, Ferlay et al., 2015). In the next 20 years, the number of cases of cancer is expected to rise by 70% to 25 million (WHO, 2017). Possible causes of cancer include tobacco smoking, obesity, poor diet, infections such as hepatitis B, hepatitis C and human papillomavirus (HPV), exposure to ionising radiation and environmental pollutants (WHO, 2017). Different cancers may have dissimilar signs and symptoms depending on the organ affected and gender of the sufferer. For example, bladder and kidney cancer is associated with blood in urine, have pain increased urination and urinary tract infection and interstitial cystitis. Breast cancer has symptoms such as lump or thickening of lumps (not all lumps are cancerous), itching, redness or soreness of the nipples. Ovarian cancer often has no symptoms until in the later stages of development, symptoms include weight loss, fatigue, bloating, and abdominal pain (WHO, 2017). Cancer can be detected by physical examinations, screenings such as blood tests, scanning or medical biopsy (WHO, 2017).

1.2. Ovarian Cancer

Ovarian cancer is a fatal type of cancer affecting the female reproductive tract and is the leading cause of death from gynaecological malignancies (Permuth-Wey and Sellers, 2009, Merino and Jaffe, 1993, Barber, 2012, Barber, 1986, Colombo et al., 2006, Jacobs et al., 2016). It is estimated that every year up to eight out of 100,000 women die of ovarian cancer (Permuth-Wey and Sellers, 2009, Merino and Jaffe, 1993, Barber, 2012, Barber, 1986, Colombo et al., 2006, Jacobs et al., 2016). About 50% of ovarian cancers are diagnosed in women over the age of 65, and this proportion is expected to increase in coming years as populations and life expectancies increase. This is why ovarian cancer cases tend to worsen as the age of a patient rises (Edwards et al., 2002, Oberaigner et al., 2012, Tew, 2016). Decreased survival in older women with ovarian cancer is as a result of cancer being more aggressive with advanced age, an inherent resistance to chemotherapy, and individual patient factors such as multiple concurrent medical problems, cognitive impairment, depression, frailty, poor nutrition and limited social support leading to greater toxicity of the therapy. Others factors can also include physician and health care bias towards the elderly which can result in inadequate surgery, suboptimal chemotherapy and poor enrolment in clinical trials (Tew, 2016, Tew et al., 2014).

1.2.1 Types of Ovarian Cancer

Ovarian cancer is classified according to the type of cell from which it starts. Below are descriptions of different types of ovarian cancer.

1.2.1.1 Epithelial tumours

Epithelial tumours of the ovary constitute almost 90% of all malignant ovarian tumours. This form of ovarian cancer generally occurs in postmenopausal women. Epithelial tumours are thought to derive from the surface epithelium (coelomic epithelium or mesothelium) covering the ovary and the underlying stroma. Although the ovary is not of Müllerian origin, the source of these neoplasms, namely the surface epithelium, is derived from the coelomic epithelium, which in the embryo gives rise to the Müllerian ducts. The latter form the fallopian tubes, uterine body, cervix and possibly the upper part of the vagina

with their large variety of epithelia (Seidman et al., 2011). It is generally agreed that a large subpopulation of epithelia ovarian cancers arise from fallopian tube mucosal epithelium. However, there is still some debate as to whether the cancers originate from secretory or ciliate cell lineage (Rohozinski et al., 2017).

The fallopian tube and associated infundibulum epithelium primarily contain two different cell types, ciliated and secretory, that can give rise to epithelial ovarian cancers. Whilst the ciliated cells have motile cilia that help in the movement of the egg released from the ovary to the uterus, the secretory cells have non-motile primary cilia that are thought to act as sensory structures (Rohozinski et al., 2017, Hagiwara et al., 2008). Therefore, tracing the patterns of gene and protein expression in ovarian tumours may help to determine the cell type that contributes to the cancer's origin. This type of approach was reported by Cheng et al., (2005) when they noted that HOX gene expression can be used to clearly and successfully identify the region of origin of tissue within the female reproductive tract (Cheng et al., 2005).

Surgery is often the initial treatment of choice for ovarian cancer including epithelial types, provided that patients are medically fit. Patients who are not candidates for optimal debulking should be considered for neo-adjuvant chemotherapy followed by interval debulking surgery and then further chemotherapy. Patients who are not fit for surgery may be given chemotherapy and considered for surgery later, or treated solely with chemotherapy (Holschneider and Berek, 2017).

1.2.1.2 Germ cell carcinoma tumours

Germ cell carcinoma tumours make up approximately 5% of ovarian cancer cases. In disparity with epithelial ovarian cancers that arise from the surface coelomic epithelium, ovarian germ cell tumours are believed to originate from primordial germ cells that migrate into the gonadal ridge at six weeks of embryonic life. This type of tumour might exhibit a spectrum of histologic differentiation that impersonates a primitive developing embryo. The concept of germ cell tumours as a specific group of gonadal neoplasms has emerged over recent decades. The concept is based on the common histogenesis of these neoplasms, the relatively frequent presence of histologically different neoplastic

elements within the same tumour, the presence of histologically similar neoplasms in extragonadal locations along the line of migration of the primitive germ cells from the wall of the yolk sac to the gonadal ridge and the remarkable homology between the various tumours in the male and female (Talerman and Vang, 2011a, Talerman and Vang, 2011b). For instance, dysgerminoma appears to be descended from relatively undifferentiated cells, whereas yolk sac tumours show malignant change in a cell line committed to extraembryonic differentiation. The immature teratomas are derived from cells predisposed to somatic differentiation and recapitulate tissue from all three primitive germ cell layers, namely ectoderm, endoderm and mesoderm. Taken together, the dysgerminoma, yolk sac tumour, immature teratoma and their hybrid mixed germ cell tumour comprise more than 90% of malignant germ cell tumours. Although germ cell carcinoma tumours account for less than 5% of ovarian cancers, their importance is greater than their numerical incidence implies because they occur in children and women in their early 20s (Talerman and Vang, 2011a, Gershenson, 1994, Lee-Jones, 2003).

The preferred initial approach for patients with suspected germ cell tumours for both diagnostic and therapeutic intent is surgery, and the safety of such an approach has been well established. In the past, radiation therapy was the traditional approach for patients with germ cell malignancies and dysgerminomas in particular, because dysgerminomas are sensitive to radiation therapy with overall survival rates of between 70% and 100%. However, recent advances have paved the way for success in combination chemotherapy. The excellent response of germ cell malignancies to adjuvant chemotherapy has allowed a tailored approach to the surgical management of this disease in women who desire fertility preservation (Emily R. Penick and Charlotte S. Marcus, 2018).

1.2.1.3 Stromal carcinoma tumours

Stromal carcinoma tumours are the category of ovarian tumours that includes all those containing granulosa cells, theca cells and luteinized derivatives, Sertoli cells, Leydig cells and fibroblasts of gonadal stromal origin. These types of tumours originate from the ovarian matrix, and consist of cells from the embryonic sex cord and mesenchyme. About 90% of hormonally active ovarian tumours

belong to this category, and are associated with physiologic and pathologic signs of estrogen or androgen excess (or both) including isosexual precocity, hirsutism, abnormal bleeding, endometrial hyperplasia or carcinoma and increased breast cancer risk. Amongst all types of ovarian cancers, 5% to 10% belong in the stromal carcinoma tumour group. Most of these (70%) are granulosa cell tumours, which are low-grade malignancies with a relapse rate of 10% to 33% (Emily R. Penick and Charlotte S. Marcus, 2018).

Because the majority of stromal carcinoma tumours are benign or low malignant potential tumours, surgical therapy is the preferred approach for treating this type of cancer. Furthermore, for patients who wish to retain fertility, unilateral salpingo-oophorectomy with preservation of the uterus, contralateral ovary and full surgical staging, is an appropriate therapy for patients with stage IA disease. Advanced-stage disease and disease in older women should be managed with complete staging and hysterectomy with bilateral salpingo-oophorectomy. Patients with early-stage disease can be treated with surgical therapy alone and can expect an excellent prognosis. However, those with stage IC or greater should be counselled about adjuvant therapy. The most common chemotherapy regimen is bleomycin, etoposide and cisplatin (Emily R. Penick and Charlotte S. Marcus, 2018).

1.2.1.4 Small cell carcinoma of the ovary

Small cell carcinoma of the ovary is a very rare tumour that affects mainly women in their early 20s. Approximately two-thirds of patients with ovarian small cell carcinoma have hypercalcemia. Small cell ovarian cancer is very aggressive, grows very quickly and is a highly malignant tumour with a poor prognosis. Although small cell carcinoma was briefly mentioned in a 1979 monograph by Scully (Scully, 1979), its entity was first fully described by (Richard Dickersin et al., 1982). Presently, there are around 300 cases of this type of tumour reported in the literature (Kapoun et al., 2015). Tumour markers, which are useful in epithelial ovarian cancers, are non-informative for this type of tumour. The histological diagnosis of small cell carcinoma of the ovary is based on the finding of a small cell population with high nuclear atypia, numerous mitoses and frequent necrosis. The growth pattern is usually solid and trabecular, with typical

and almost pathognomic follicle-like spaces. In addition, some unusual findings have been described such as mucinous glands, mucinous signet ring cells, spindle cell sarcomatoid change, large cells and rhabdoid cytomorphology (Witkowski et al., 2016, Kascak et al., 2016).

Treatment of this type of ovarian tumour is stage dependent. For example, in early stages, surgery (as with ovarian cancers) is preferred, followed by adjuvant chemotherapy with cisplatin and etoposide. Most patients with this type of tumour respond to chemotherapy treatment employing a combination of agents such as cisplatin, etoposide, doxorubicin and bleomycin, or cyclophosphamide and vinblastine (Witkowski et al., 2016, Kascak et al., 2016). There are different opinions on approaches in the early stages of this type of ovarian tumour. Some reports recommend fertility-sparing surgery followed by adjuvant chemotherapy, others suggest radical staging surgery with pelvic and paraaortic lymphadenectomy (Powell et al., 1998, Pautier et al., 2007). For late stages, it is recommended that neoadjuvant chemotherapy followed by radical staging surgery should be considered (Harrison et al., 2006)**.** Due to the highly aggressive nature of this tumour, prognosis is very poor and despite various approaches, one-year survival of patients with this type of tumour is only around 50% (Witkowski et al., 2016, Richard Dickersin et al., 1982).

Despite approaches such as surgical and chemotherapeutic treatment approaches, metastatic ovarian cancer is still incurable mainly as a result of a lack of effective therapeutic strategies. Because of this, intense efforts are being made to define potential molecular targets that may augment the survival of patients with ovarian cancer (Williams et al., 2007). In this respect, several reports have identified potential therapeutic targets, with the efficacy of treatments against such targets being analysed in clinical trials in various phases of development. Amongst such experimental treatments, agents acting on inhibiting tyrosine kinases have received particular attention (Alečković and Kang, 2015, Goltsov et al., 2014a, Jeong et al., 2013, Lee et al., 2016b, Præstegaard et al., 2016).

Many reports have implicated the HER receptor tyrosine kinase (RTK) family as an alternative target in cancer treatment, including ovarian cancer (Gschwind et

al., 2004, Mendelsohn and Baselga, 2000, de Bono and Rowinsky, 2002, Normanno et al., 2003, Reese and Slamon, 1997). Up to 70% of ovarian cancers have HER1 overexpression and most of the drugs targeting this receptor are already in clinical use. HER2 overexpression has also been reported in ovarian cancer and most of the drugs used in targeting the receptor, especially in breast cancer, are in clinical use and being given to patients. A study by Sheng and colleagues also reported the involvement of HER3 as a potential target in ovarian cancer (Montero et al., 2015). Similarly, high incidences of HER4 expression in ovarian cancer have also been reported (Davies et al., 2014). Because of the relevance of tyrosine kinases in ovarian cancer, the use of HER family-targeting monoclonal antibodies and drugs such as trastuzumab, pertuzumab lapatinib and erlotinib are increasingly attractive (White et al., 2014, Khalil et al., 2016b, Montero et al., 2015, Mullen et al., 2007, Langdon et al., 2010).

In contrast, NRF2 has been implicated in cancer development and progression as causing resistance to chemotherapy. NRF2 has now become a molecular target to overcome chemoresistance in cancers including ovarian cancer (Khalil et al., 2016a, Khalil and Deeni, 2015, Wang et al., 2008, Ren, 2011, Olayanju et al., 2015, Chian et al., 2014b, Tang et al., 2011, Wu et al., 2014). NRF2 inhibitors such as bexarotene and RA, whose mechanism of action involves blocking NRF2 nuclear import and its subsequent binding to deoxyribonucleic acid (DNA) (Wang et al., 2007, Wu et al., 2014, Wang et al., 2013), trigonelline and ascorbic acid that block NRF2 from translocating to the nucleus (Arlt et al., 2013, Sirota et al., 2015, Tarumoto et al., 2004) have been identified. Other inhibitors identified include brusatol that down-regulates NRF2 levels by affecting NRF2 translation and post-translational regulation (Ren, 2011, Olayanju et al., 2015) and luteolin, which degrades NRF2 at the messenger ribonucleic acid (mRNA) level (Chian et al., 2014b, Tang et al., 2011). In addition, Chrysin, which down-regulates phosphoinositide 3-kinase /protein inase B (PI3K/Akt) and extracellular signal-related kinase (ERK) pathway leading to NRF2 repression (Gao et al., 2013a), and apigenin, which inhibits NRF2 via suppressing PI3K/Akt pathways (Gao et al., 2013b), are amongst the inhibitors reported. The discovery of additional inhibitors that would inhibit the over-activation of NRF2 in such a way as to sensitise cancer cells to chemotherapy is in progress (Arlt et al., 2013, No et al.,

2014). Other methods to improve cancer therapy involve gene therapy using small siRNA. Gene therapy involves the insertion of genetic material into the cells of a patient, resulting in a therapeutic benefit (Kanninen et al., 2015). siRNA-based therapy has shown great promise for many diseases and many targets for siRNA therapy including oncogenes such as NRF2, which is involved in cancer survival and resistance to chemotherapy (Huang et al., 2008).

Below is a detailed description of the HER receptor family and NRF2, their expression and how they are being targeted in cancers, including ovarian cancer.

1.3. HER Family

HER family receptors are one of the families belonging to RTKs that regulate key signal transduction pathways. HER family receptors become active when their ligands, such as epidermal growth factor (EGF)-like proteins and neuregulins, bind to them. It is also reported that there are about 30 ligands and four receptors in the HER receptor family in humans that make up the complex signal-transduction network, and where activated, receptor-ligand complexes vary in both the strength and type of cellular responses that they induce (Cook et al., 2014, Yarden, 2001). The process of transducing extracellular signals occurs when growth factor receptors such as HER family receptors, activate intracellular messengers or directly use receptor translocation to the nucleus, leading to many conformational changes and receptor activations (Wieduwilt, 2008, Cook et al., 2014, Lee et al., 2016a, Zaczek et al., 2005). This activation subsequently leads to the induction of a number of signalling pathways including Ras/ Raf and PKI3/Akt MAPK, leading to DNA synthesis cell migration, survival, adhesion and proliferation (Cook et al., 2014, Wieduwilt, 2008). The different arrangements that the HER family possess to form dimers, either homodimers or heterodimers (Figure 1.1), lead to a diversity of distinctive purpose (McCubrey et al., 2015, Cook et al., 2014).

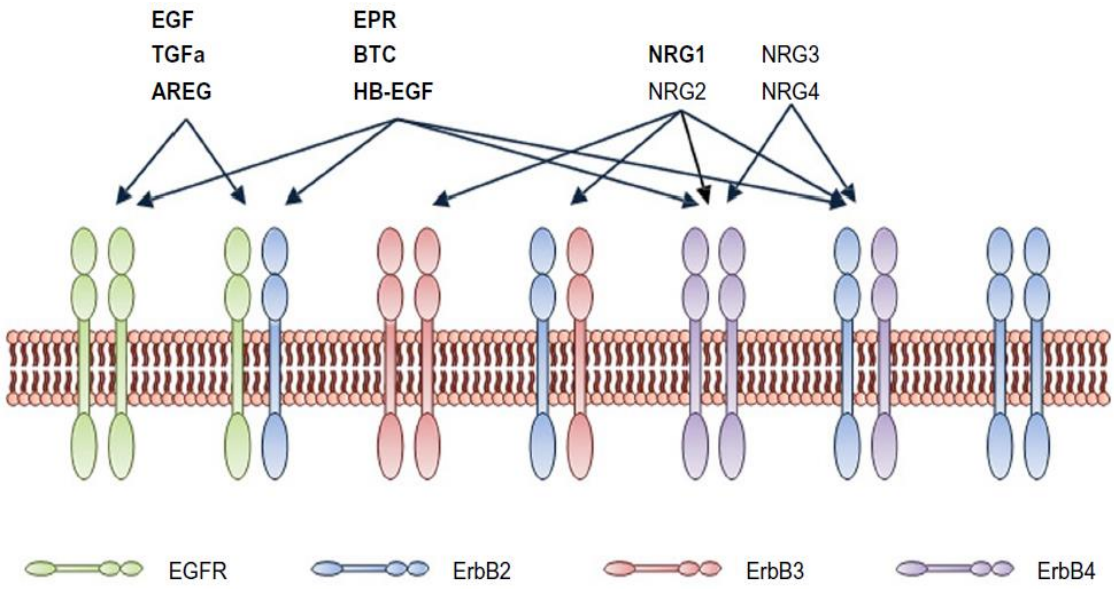


Figure 1.1: HER family receptors and their respective ligands. The receptors are shown based on their ability for homodimerization or heterodimerisation alongside the specific ligands. The figure is retrieved from (Cook et al., 2014).

RTK pathways, including the HER receptor family, are frequently impaired in cancer and are commonly targeted in cancer treatment, with the treatment targeting them being promising (Wieduwilt, 2008, Cook et al., 2014, Lee et al., 2016a, Zaczek et al., 2005).

1.3.1 HER1

HER1 is one of the four members of the HER family receptors. It is a glycoprotein with a molecular weight of 175 kDa. This cellular transmembrane protein is activated when its ligands such as EGF, transforming growth factor-α (TGFα), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), betacellulin (BTC), epi-regulin (EPR) and epigen bind to it, an event that leads to homodimerization of this receptor or heterodimerization with other receptors from the family. Following this, activation and autophosphorylation of HER1 initiate a series of intracellular signalling pathways that steer important cellular processes such as proliferation, adhesion, migration and apoptosis. However, studies have reported the over-expression of HER1 protein in a variety of cancers such as breast, ovarian head/neck and colorectal cancers (Moulder et al., 2001, Witton et al., 2003, Lee et al., 2005, Gordon et al., 2005, Reid et al., 2007, Mullen et al.,

2007). This increase in HER1 expression has been associated with poor treatment outcomes (Cook et al., 2014, Ilkhani et al., 2015, Schneider, 2009, Witton et al., 2003).

1.3.2. HER2

HER2 is a monomeric protein that belongs to the HER family of receptors, and is a membrane-bound tyrosine kinase and glycoprotein of 185 kDa. It has three different segments including the extracellular amino end segment, which contains four domains. These include the hydrophobic transmembrane segment and the carboxyl end segment, which also contains another three domains with autophosphorylation sites known as the juxtamembrane, the tyrosine kinase and the C-terminal tail parts. Amongst all the family members, HER2 is the only one that has no known ligand yet heterodimerizes with other members of the HER family following ligand binding, which leads to the activation of various intracellular signal transduction pathways involved in cellular processes and growth (Cook et al., 2014, Wieduwilt, 2008, Mass, 2004, Laughner et al., 2001, Rubin and Yarden, 2001, Klapper et al., 1999, Johnston and Leary, 2006).

HER2 is reported to be amplified and overexpressed in clear cell and mucinous tumours of breast and ovarian origin (McAlpine et al., 2009, Tapia et al., 2007, Schraml et al., 2003, Lin et al., 2011, Langdon et al., 2010, Mullen et al., 2007). Trastuzumab was the first monoclonal antibody used to try and target HER2, and a phase II trial indicated a response rate of 7.3%. Following that, another humanised antibody targeting HER2 emerged (pertuzumab), and it was given a clinical evaluation in ovarian cancer that indicated an improved response of 14.5%. Numerous approaches have uncovered more effective ways of targeting HER2 over-expressing cancers with the use of a combination of trastuzumab with pertuzumab having demonstrated much improvement over the use of single agents (Omar et al., 2015, Goltsov et al., 2014a, O'Sullivan and Connolly, 2014, Limaye et al., 2013, Scheuer et al., 2009, Baselga et al., 2012, Nahta et al., 2004, Langdon et al., 2010, Nagumo et al., 2009b, Sims et al., 2012b). In 2016, a study by Li et al. reported a safe and effective continuing treatment of trastuzumab beyond first line therapy for HER2 positive advanced gastric cancer (Li et al., 2016).

1.3.3. HER3

HER3 is a transmembrane glycoprotein with a molecular weight of 180 kDa. It is the third member of the HER family and interacts with various ligands such as neuregulin NRG-1 and NRG -2. However, HER3 is different from other HER family members for two important reasons: firstly, it lacks a functioning kinase domain and evidence suggests that even though HER3 can bind ATP, the phospho-transfer reaction is catalytically unstable; and secondly, HER3 has the ability to induce the PI3K-AKT pathway which in turn induces cancer cell proliferation (Cook et al., 2014, Wang et al., 2014).

Several studies have shown that HER3 is an indispensable chaperone to HER2. This is because as HER2 has no known ligand and HER3 has no functional kinase activity, they become suitable in making complexes with each other (Calderwood et al., 2006, Gajria and Chandarlapaty, 2011, Khalil et al., 2016a, Khalil et al., 2016b). The dimerization of HER2 and HER3 has been implicated in cancer initiation and progression (Wang et al., 2014).

1.3.4. HER4

HER4 is another member of the HER receptor family. It is a transmembrane glycoprotein with a molecular weight of 180 kDa, multiple furin-like cysteine rich domains, a tyrosine kinase domain, a phosphotidylinositol-3 kinase binding site and a PDZ domain-binding motif. HER4 is activated following its binding by neuregulins (NRG)-2 and -3, a heparin-binding EGF-like growth factor and betacellulin. This ligand binding leads to the induction of various cellular responses including mitogenesis and differentiation. Overexpression or mutation of HER4 has been associated with cancers, including ovarian (Okazaki et al., 2016, Qiu et al., 2008). Not much is known about the role of HER4 in cancer; however, a recent study by Okazaki et al (2016) reported developing a novel monoclonal antibody called P6-1 which significantly suppressed NRG-1, one of the ligands known to bind to human HER4 protein. The study confirmed that the antibody clearly suppresses NRG-1 ligand, which leads to reduced expression of HER4 in a number of cell lines used, including T47D and MCF7 cells. This,

therefore, suggests that targeting HER4 might be an important strategy for treating cancers, especially HER4 expressing ones (Okazaki et al., 2016).

1.4. Composition and signalling patterns of HER family receptors

The HER family of four are membrane-bound receptors that protrude into the cytoplasm. They have some common extracellular ligand-binding domains and a single transmembrane domain (Tanner et al., 2006, Linggi and Carpenter, 2006, Maihle et al., 2002). Their intracellular tyrosine kinase domain and C-terminal non-catalytic signalling tail are also similar across the HER family. All the HER family members have ligand binding domains except HER2, and once the ligand binds to the extracellular domain of any corresponding receptor, it induces a structural reconfiguration of the receptor leading to dimerization (typically homodimerization or heterodimerization) with other family members. This, in turn, leads to activation of the receptors resulting in autophosphorylation, transphosphorylation and subsequent induction of downstream signalling pathways. In another scenario, HER family receptors can be activated without any ligand binding, and this involves activation by non-physiological stimuli such as oxidative stress, ultraviolet light or γ-irradiation, amongst others (Sheng and Liu, 2011).

HER2 is considered the preferred dimerization partner of all the HER family. It has the strongest kinase activity amongst the family members, even though it has no known ligand. Hence HER2 can only be activated when it forms a dimer with any of the other members. In contrast, HER3 can have a ligand bind to it but has no functional kinase activity. It is therefore dependent on other members such as HER2 forming heterodimers and phosphorylating its signalling tail, which leads to downstream signalling effects. EGF, TGF-α and amphiregulin are HER1 ligands that specifically bind to it, whilst HER4 has NRG -3, NRG-4 and tomoregulin as ligands. Other ligands such as epiregulin, β-cellulin and heparin-binding EGF-like growth factors have dual specificity for both HER1 and HER4. In addition, NRG-1 and NRG-2 are ligands for both HER3 and HER4 (Sheng and Liu, 2011).

The activation of HER family members leads to the stimulation and recruitment of various adaptors and signalling molecules such as the extracellular signal-

regulated kinase (ERK) 1/2 via recruitment of growth factor receptor-bound protein 2 (Grb2), or SHC-transforming protein 1 (Shc) adaptors and the activation of ERK1/2 and phosphatidylinositol 3-kinase (PI3K)/AKT pathway, which have a role in cell proliferation and survival. Additionally, phosphorylation and activation of HER family and subsequent ERK1/2 and PI3K activation can also lead to the activation of a number of transcription factors. These include signal transducers and activators of transcription, as well as zinc-finger transcription factor and Fos family transcription factors such as (c-Fos), Jun family transcription factors such as (c-Jun), Myc family transcription factors (c-Myc) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-Kb) (Sheng and Liu, 2011).

1.4.1. HER1 expression in ovarian cancer

A comprehensive review by Siwak et al. (2010) reported on the role of HER1 in ovarian cancer and various ways of targeting it. The increased amplification and mutation of HER1 in ovarian cancer for up to 4 – 22% and <4% respectively, has also been reported (Siwak et al., 2010). Several studies have reported on the use of small molecule inhibitors such as gefitinib, lapatinib and erlotinib to block the kinase activity of HER1 (Siwak et al., 2010, Burris Iii et al., 2005, Montemurro et al., 2007, Kuang et al., 2010, Reid et al., 2007, Mester and Redeuilh, 2008, Murphy and Stordal, 2011, Matar et al., 2004, Johnston and Leary, 2006). Most of these drugs are already in clinical use and are being used as either single treatments or in combination, but most patients using them have reported resistance to the drugs (Siwak et al., 2010, Blackwell et al., 2010, Li et al., 2008b). Monoclonal antibodies such as cetuximab and futuximab targeting the HER1 receptor in ovarian cancer have also been reported, though with limited activity due to resistance (Sheng and Liu, 2011, Siwak et al., 2010, Yap et al., 2009, Matar et al., 2004).

1.4.2. HER2 expression in ovarian cancer

Reports have shown HER2 overexpression in ovarian cancer, for example SKOV3 cell line that was extracted from an ovarian cancer patient was known to have HER2 overexpression (Langdon et al., 1988, Mullen et al., 2007, Meden and Kuhn, 1997, Verri et al., 2005, Bookman et al., 2003, Serrano-Olvera et al.,

2006). Studies have connected a worse prognosis in ovarian cancer as a result of overexpression of HER2 (Bookman et al., 2003, Gordon et al., 2006, McAlpine et al., 2009, Serrano-Olvera et al., 2006, Berchuck et al., 1990). Although clinically HER2-targeted therapies have not shown significant promise as therapeutic agents in ovarian cancer, monoclonal antibodies such as trastuzumab and pertuzumab have been used to successfully treat HER2-overexpressing breast (BarthÉLÉMy et al., 2014, Langdon and Cameron, 2013), and ovarian (Langdon et al., 2010, Mullen et al., 2007, Sims et al., 2012b, Goltsov et al., 2014a) cancers, though with a low or partial response rate. Lapatinib, which is a dual inhibitor of HER1 and HER2 (Johnston and Leary, 2006, Kuang et al., 2010, Montemurro et al., 2007), is in clinical use and is used either alone or in combination with other anti-cancer drugs such as carboplatin, for treating patients with platinum-sensitive recurrent ovarian cancer (Sheng and Liu, 2011, Kimball et al., 2008, Lheureux et al., 2012).

1.4.3. HER3 expression in ovarian cancer

HER3 has been reported to be amplified and over-expressed in epithelial ovarian cancers (Sheng and Liu, 2011, Tanner et al., 2006, Nagumo et al., 2009b). A number of reports have connected the overexpression of HER3 with poor prognosis in cancers, including ovarian (Zhang et al., 2009, Reschke et al., 2008, Hayashi et al., 2008, Xu et al., 1999, Tanner et al., 2006, Nagumo et al., 2009b). Besides the full-length HER3, several segments of HER3 isoforms that consist of its extracellular domains have been mentioned and studied in ovarian cancer, though their functional significance remains unclear (Sheng and Liu, 2011, Lee and Maihle, 1998). HER3 has been reported to contribute towards resistance of drugs targeting HER1, HER2 and HER4 (Amin et al., 2010, Campbell et al., 2010, Hynes and Lane, 2005, Gala and Chandarlapaty, 2014, Ma et al., 2014). HER3 activation is increased, potentially through increased localisation of HER3, to the cytoplasmic membrane. Also, HER3 activation is reported to overcome resistance to gefitinib, especially in HER1-mutant non-small-cell lung cancer cell lines upon MET amplification (Engelman and Jänne, 2008, Turke et al., 2010). Not long ago, the inhibition of AKT was implicated in up-regulating HER receptors expression and phosphorylation, suggesting that HER3 may also have a role in mediating

resistance to PI3K/AKT pathway inhibitors (Sheng and Liu, 2011, Chandarlapaty et al., 2011, Sergina et al., 2007).

Several monoclonal antibodies such as pertuzumab and trastuzumab are already in clinical use for the treatment of breast and ovarian cancers (Langdon et al., 2010, Langdon and Cameron, 2013, Khalil et al., 2016b, Khalil et al., 2016a, Nahta et al., 2004, Molina et al., 2001, Hudis, 2007, Ajgal et al., 2017, Luen et al., 2017). These antibodies are used to block the binding sites of the ligands for HER2, thereby antagonising the possible homodimerization of HER2 or heterodimerization of HER2 with either HER3 or other HER family members. The use of the combination of pertuzumab with gemcitabine or of gemcitabine alone to treat ovarian cancer has also been reported, though with little improvement (Makhija et al., 2010). More recently, signalling through the HER3 receptor pathway has been reported to correlate with expression of its natural ligand, neuregulin-1 (NRG1), in both ovarian cancer cell lines and in primary human ovarian cancer cells (Srinivasan et al., 1999, Gilmour et al., 2002). The expression of NRG1 has been observed in 30 – 83% of ovarian carcinomas (Gilmour et al., 2002, Sheng et al., 2010). So far, the potential activity of these immunotherapeutics and other drugs as single agents or in combination with additional cytotoxic or biological therapies in ovarian and other cancers, is currently an area of interest and remains under focus (Sheng and Liu, 2011, Sheng et al., 2010, Ciardiello et al., 2000, Vanneman and Dranoff, 2012, Agarwal and Kaye, 2003, Gerber and Ferrara, 2005).

1.4.4. HER4 expression in ovarian cancer

HER4 has as yet not been well studied, therefore its full role in cancer is not fully understood (Madhusudan and Ganesan, 2004). HER4 is reported to be expressed in ovarian cancer (Gilmour et al., 2001), with many cancer cases such as melanoma having reported that an increase in the proliferation of cells was as a result of the mutation of HER4 (Madhusudan and Ganesan, 2004, Sheng and Liu, 2011, Gilmour et al., 2002). This suggests that HER4 has a role in cancer initiation and progression (Madhusudan and Ganesan, 2004, Sheng and Liu, 2011, Gilmour et al., 2002).

Despite the unclear role of HER4, most of the known ovarian cancer cell lines with high HER4 expression have been derived from platinum-refractory tumours (Gilmour et al., 2002, Gilmour et al., 2001), thereby suggesting the possibility that platinum resistance is as a result of HER4 overexpression and may be associated with the development of platinum resistance. In addition, the HER4 rate of expression has been implicated in improved ovarian cancer cell survival (Gilmour et al., 2002). Moreover, it has been reported that the clone P6-1 monoclonal antibody blocks HER4 binding to NRG1(Okazaki et al., 2016), and that the antibody appeared to show a stimulatory effect in a number ovarian cancer cell lines investigated, thereby raising alarm as to the possible role of HER4 in cancer cells (Okazaki et al., 2016). It, therefore, appears that HER4 is overexpressed in ovarian cancer (Sheng and Liu, 2011). This suggests that HER4 could provide alternative and salvage cell signalling and proliferation pathway to promote and sustain cancers. Thus, it would be of interest to target HER4 as a way to improve ovarian cancer therapy.

1.5. HER family regulation and expression

Regulation of HER receptor activation is a complex process (Krähn et al., 2001, Wood et al., 2004, Katz et al., 2007, Jorissen et al., 2003). The HER family are activated following ligand binding and receptor dimerization. As noted earlier, the ligands are specific to their respective receptors, with some having a dual specification of binding where they bind to one or more different receptors in the family to evoke signal transduction cascades and outcomes (Krähn et al., 2001). For example, HER receptors can mediate a signal that activates other downstream signalling pathways such as AKT or MAPK, which subsequently leads to cell growth, movement, survival, differentiation and death. Some of the ligands of these HER family receptors are reported to be overexpressed, with this being probably due to mutation of the respective receptors in many cancers (Roskoski Jr, 2014, Cell, 2016, Ritter et al., 2007, Rusch et al., 1997, Cuello et al., 2001). For example, HER1 has been found to be mutated in gliomas and NSCLC due to the gene rearrangements, amplification and subsequent overexpression of their aberrant protein products (Schlegel et al., 1994, Nicholas et al., 2006). HER2 overexpression is also reported in breast, ovarian, bladder

and NSCLC cancers, amongst others (Roskoski Jr, 2014, Cell, 2016, Khalil et al., 2016b, Shattuck et al., 2008, Mullen et al., 2007).

HER receptors' signalling cascade is regulated by both positive and negative feed-back and feed-forward loops, including transcription-independent early loops and late loops mediated by newly synthesised proteins and microRNAs (review reported by (Avraham and Yarden, 2011)). For example, a receptor that has been activated can be "switched off" through receptor dephosphorylation, receptor ubiquitination or the removal of active receptors from the cell surface through endosomal sorting and lysosomal degradation (Cell, 2016, Roskoski Jr, 2014).

1.6. Targeting the HER receptor family pathway for therapeutic intervention in cancer

Several studies on targeting HER signalling pathways have noted the use of small molecules and monoclonal antibodies in cancer chemoprevention and tumour suppression (Hynes and Lane, 2005, Fabian et al., 2005, Sequist et al., 2008, Langdon et al., 2010, Langdon and Cameron, 2013, Huang et al., 2004, Sartore-Bianchi et al., 2009, Scartozzi et al., 2004, Xiong et al., 2004, Gonzales et al., 2016, Cell, 2016). These drugs are used either in single or combination treatments to obtain a desired best result, with a number of suggestions having been made both pre-clinically and clinically that targeting more than one receptor could display better efficacy than just a single treatment approach (Cell, 2016, Gilmour et al., 2001, Zhou et al., 2004).

Combination therapies are being actively pursued to expand therapeutic options, as well as to deal with chemoresistance conferred by cancer. Approaches to uncovering effective combination treatments have focused on drugs targeting intracellular processes of the cancer cells, and in particular on small molecules that target aberrant kinases (Dry et al., 2016). Many clinical drug combination successes seem to involve drug pairs with independent effects, rather than synergistic activity within the tumour cells. Using combination therapies to target different cancer cellular processes such as modulating stromal cells that interact with the tumour, strengthening physical barriers that confine tumour growth, epigenetic control, boosting the immune system to attack tumour cells and even

regulating the microbiome to support antitumor responses, could deal with the persistence of cancer chemoresistance (Dry et al., 2016, Klemm and Joyce, 2015)

The process and approaches for dealing with inhibitors such as small molecule inhibitors, monoclonal antibodies and heat shock protein drugs are under constant review to improve cancer treatment. More prodrugs and natural drugs are under development in order to achieve production of more blockers of receptors from overexpression (Fabbro et al., 2002, Barrera et al., 2012).

1.6.1. Small molecule kinase inhibitors

Small molecules are drugs that are developed to inhibit aberrant RTK signalling by selectively interfering with their intrinsic tyrosine kinase activity. This consequently blocks receptor autophosphorylation and activation of downstream signalling pathways (Levitzki, 1999, Levitzki, 2000, Zwick et al., 2002). Most small molecule inhibitors are at present in clinical development for the treatment of human cancers (Kim et al., 2009, Workman and Collins, 2014, Paez et al., 2004, Kris et al., 2003, Herbst et al., 2002, Harrington et al., 2004). These include imatinib, erlotinib, lapatinib, tyrphostin and gefitinib. Inhibitors such as genistein and herbimycin A (isolated from fungal extracts) are the beginning stages for the generation of many types of synthetic small-molecules, especially for the HER family (Noonberg and Benz, 2000). Tyrphostin was the first synthetic RTK inhibitor introduced in 1988 (Levitzki and Mishani, 2006, Kim et al., 2009). Subsequently, another inhibitor, 2-phenylaminopyrimidine, was identified as a lead RTK inhibitor (Kim et al., 2009, Druker et al., 2001, Buchdunger et al., 1996).

Imatinib was one of the novel anti-cancer drugs reported to inhibit kinase activity BCR-ABL fusion protein, which is responsible for constitutive proliferate signalling. Imatinib acts by blocking ATP access to its binding sites in the protein, thus leading to the inhibition tyrosine phosphorylation of the substrate (Fabbro et al., 2002, Manley et al., 2002).

1.6.2. Monoclonal antibodies

Monoclonal antibodies work by blocking ligand–receptor interaction, thereby inhibiting ligand-induced RTK signalling and increasing RTK down-regulation and internalisation. By binding to specific epitopes on cancer cells, monoclonal antibodies induce immune-mediated responses leading to cellular cytotoxicity, and thus apoptosis and growth inhibition (Cragg et al., 1999, Zwick et al., 2002). Monoclonal antibodies such as bevacizumab and cetuximab targeting HER1 (Herbst et al., 2005, Cunningham et al., 2004) and pertuzumab and trastuzumab targeting HER2/HER3 (Goltsov et al., 2014a, Workman and Collins, 2014, Khalil et al., 2016a, Langdon et al., 2010, Langdon and Cameron, 2013), are the inhibitors of RTKs identified as effective drugs for HER receptor overexpressing cancers including ovarian cancer, that suppress dimerization of HER family receptors (Goltsov et al., 2014a, Workman and Collins, 2014). Bevacizumab is a potent, highly selective and orally available HER1 inhibitor that shows higher growth inhibition and promising clinical activity, especially when combined with other drugs (Herbst et al., 2005, Hurwitz et al., 2004, Saltz et al., 2008, Giantonio et al., 2007) to suggest the targeting of oncogenic Ras. Cetuximab binds to HER1 with high specificity and with a higher affinity, thus blocking ligand-induced phosphorylation of HER1 (Cunningham et al., 2004), and its activity tends to be enhanced when combined with other drugs (Cunningham et al., 2004, Van Cutsem et al., 2009, Pirker et al., 2009, Bonner et al., 2010). Pertuzumab, which inhibits HER2 dimerization, has shown improved clinical activity in ovarian cancer (Mullen et al., 2007), and against both breast and ovarian cancer (Langdon et al., 2010). Trastuzumab has also shown an improved treatment activity in patients with HER2 overexpressing cancers (Workman and Collins, 2014). So far, trastuzumab, in combination with pertuzumab, has become part of the routine way of treating patients with known HER2 overexpressing cancers, with an improved pathologic complete response (pCR) of 39% (Workman and Collins, 2014, Goltsov et al., 2014a, Maly and Macrae, 2014). A combination of pertuzumab and trastuzumab has been shown to provide improved anti-tumour activity (Sims et al., 2012a, Baselga et al., 2012, Faratian et al., 2011), thereby suggesting that this combination could be an effective way of dealing with HER2-overexpressing ovarian cancers (Faratian et al., 2011). Monoclonal inhibitors act

by antagonising the binding of ligands to the receptors at the extracellular compartment, thereby resulting in the internalisation of receptors without phosphorylation (Goltsov et al., 2014a, Workman and Collins, 2014).

1.7. Comparisons between small molecules and monoclonal antibodies as RTK inhibitors (RTKi)

Although small molecule inhibitors and monoclonal antibodies are the antagonists of RTK-activated cellular responses, they represent separate drug classes with different mechanisms of action and efficiency (Gui and Shen, 2012, Gharwan and Groninger, 2016, Dassonville et al., 2017). Monoclonal antibodies are specific to RTKs and have a longer half-life than the small molecule RTKi. They act by recruiting immune effector cells, leading to the stimulation of antibody- and compliment-dependent tumour cell cytotoxicity (Gui and Shen, 2012). Small molecule inhibitors are less selective than monoclonal antibodies (Harrington et al., 2004), thus offering the potential of a broader spectrum of activity against other members of the receptor family, such as HER2 and HER4, but probably produce more side effects. Small molecule inhibitors are normally taken orally and are relatively smaller in size than monoclonal antibodies; a characteristic that increases their possibility of easily intruding large tumours, although they are less stable in an in vivo environment (Harrington et al., 2004). The small molecule RTKi has also been reported to have more side effects such as diarrhoea, nausea and vomiting than monoclonal antibodies. This limits incorporating it within standard cancer chemotherapeutic regimes and approaches (Gui and Shen, 2012, Cell, 2016, Imai and Takaoka, 2006). It is left therefore to researchers and clinicians to investigate and decide whether any of these differences between monoclonal antibodies and small molecules are clinically meaningful in cancer treatment (Gui and Shen, 2012, Cell, 2016, Imai and Takaoka, 2006), despite the combination of small molecule inhibitors and monoclonal antibodies being more effective than single treatments with a reported reduced relative risk of death (Slamon et al., 2001).

1.8. NRF2

Moi and colleagues (1994) were the first researchers to describe NRF2 as NF-E2 p45-related factor 2 encoded by NFE2L2, when they identified it as the third human cap 'n' collar (CNC) basic-region leucine zipper (bZIP) transcription factor (Moi et al., 1994). One year later, Masayuki Yamamoto and colleagues (1995) reported an orthologous cDNA from chicken encoding a transcription factor that they called erythroid cell-derived protein with CNC homology (ECH) (Hayes et al., 2016, Itoh, 1997, Itoh et al., 1995). The CNC domain has a unique region located amino terminal to the basic DNA-binding region that defines its identity (Andrews et al., 1993, Bowerman et al., 1992, Mohler et al., 1991). CNC family basic leucine zipper transcription factors play a vital role in the regulation of mammalian gene expression and development (Derjuga et al., 2004). The protein is now designated NRF2 rather than ECH, and in mammalian species NRF2 translocates into the nucleus where it forms a heterodimer with small musculoaponeurotic fibrosarcoma (sMAF), consisting of a 45 kDa NF-E2 and an 18 kDa subunits. It then binds to antioxidant response element (ARE) for subsequent initiation of expression of approximately 250 genes, including cytoprotective and detoxification genes, that contain an ARE with a sequence 5′-TGACNNNGC-3′ in their promoter regions (Hayes et al., 2016).

NRF2 is a member of the bZIP family of transcription factors (Mohler et al., 1995). While the basic region, just upstream of the leucine zipper region is responsible for DNA binding the acidic region is required for transcriptional activation. In mammals, the CNC family is composed of four closely related proteins; p45-NF-E2 (Chan et al., 1993b), NRF1(Chan et al., 1993a), NRF2 (Moi et al., 1994, Itoh et al., 1995) and NRF3 (Kobayashi et al., 1999). Others are two remotely related proteins; BTB and CNC homology 1 (Bach1) (Oyake et al., 1996) and BTB and CNC homology 2 (Bach2) (Muto et al., 1998). The roles of some of the mammalian CNC factors have been extensively studied. These proteins (Figure 1.2), form a heterodimer with other b-ZIP proteins, such as small Mafs (MafK, MafG, MafF), to function as transcription factors (Igarashi et al., 1994). For example, the pattern of heterodimeric association between NRF2 and small Mafs, is that the small Maf protein provides DNA binding activity to NRF2, while NRF2

activates transcription via its transactivation domain (Igarashi et al., 1998). Hence, NRF2 cannot bind to the ARE as a monomer, but requires dimerizaton with one of the small Maf proteins in order to bring about transactivation (Itoh, 1997).

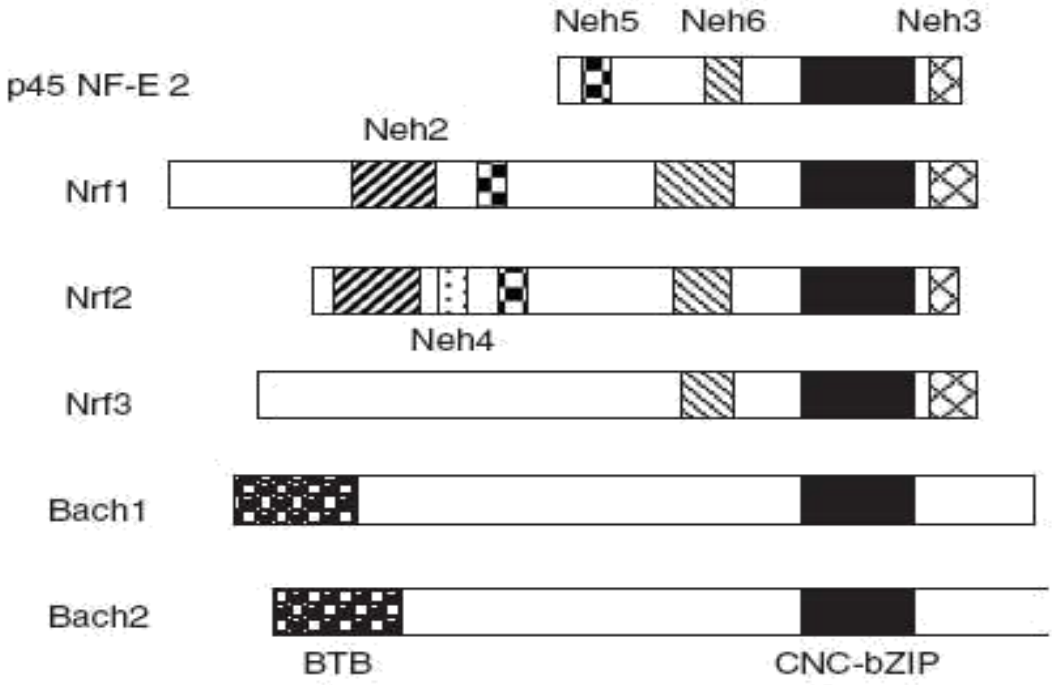


Figure 1.2: Structural properties of CNC protein family members. Six Neh domains are conserved among vertebrate NRF2, while some of them are included in other CNC proteins. Among them, Neh4 is the only domain specific to NRF2. NRF1 also contains a Neh2-like motif, an interaction site for Keap1, but the precise function of NRF1–Neh2 is not known. None of the Neh domains exist in the Bach proteins. Retrieved from (Kobayashi and Yamamoto, 2006)

NRF2 contains seven basic domains, namely Neh1-Neh7 (Figure 1.3). The Neh1 domain is shown to bind to ubiquitin-conjugating enzymes to enhance the stability and the transcriptional activity of NRF2. The second domain, known as Neh2, possess two essential motifs known as DLG, which have less affinity for Kelch-like ECH-associated protein 1 (KEAP1), and ETGE, which has a high affinity for the interaction between NRF2 and KEAP1 (Hayes et al., 2016, O'Mealey et al., 2017). The Neh3 domain contains a carboxy-terminal which associates with transcription co-activators such as chromodomain helicase DNA binding protein 6 (CHD6), which is responsible for the transactivation of ARE-dependent genes. Both Neh4 and Neh5 domains bind with cAMP response element binding (CREB) protein, which facilitates the transactivation of NRF2 target genes. These two

transactivation domains are also reported to interact with the nuclear cofactor known as receptor-associated coactivator 3/ amplified in breast 1/ steroid receptor coactivator-3 (RAC3/AIB1/SRC-3), thereby leading to an improved NRF2-ARE gene expression. The Neh5 domain also possesses a redox-sensitive nuclear export signal that mediates the cellular localisation of NRF2. The sixth domain, known as Neh6, contains a domain that is rich in serine amino acid, and it contains two motifs known as DSGIS and DSAPGS. The Neh6 domain is involved in the degradation of NRF2 even in stressed cells, where the half-life of NRF2 protein is longer than in unstressed conditions. The Neh6 domain also offers stability control of NRF2 when NRF2 is in the NRF2-KEAP1 complex (Ahmed et al., 2017, Namani et al., 2014, Wang et al., 2007). The Neh7 domain is a recent discovery and has been found to specifically interact with RXRα, a nuclear receptor that inhibits the NRF2–ARE signalling pathway (Hayes et al., 2015, Hayes and Dinkova-Kostova, 2014, Hayes et al., 2016, McMahon et al., 2014, Hayes et al., 2010, Krajka-Kuźniak et al., 2017).

1.9. KEAP1

KEAP1, also known as INRF2, is a substrate adaptor that has a MW of a 69-kDa. It is a protein that is rich in cysteine, and most notably cysteine 27 amino acid residues. KEAP1 is normally complexed with E3 ubiquitin ligase (Cul3), an enzyme that under normal basal conditions inhibits the transcriptional activity of NRF2 via its ubiquitination and proteasomal degradation in the cytoplasmic compartment of a cell (Ahmed et al., 2017; Namani et al., 2014). KEAP1 is reported to contain five domains (Figure 1.3): (i) an N-terminal end region; (ii) a BTB dimerization domain; (iii) a cysteine-rich intervening region (IVR); (iv) a double glycine repeat (DGR) domain which possesses six kelch, and (v) a fifth C-terminal end region domain. The BTB domain controls the KEAP1 binding with the Cul3-based ubiquitin E3 ligase that complexes and ubiquitinates NRF2, and also contains a Cys151 residue that plays a vital role in NRF2 stimulation and activation (Krajka-Kuźniak et al., 2017, Hayes and Ashford, 2012). The IVR domain contains the very reactive cysteine residues Cys273, Cys288 and Cys297, which are sensitive to oxidation and are sensors of any agent that induces NRF2 (Hayes and Ashford, 2012, Hayes and McMahon, 2009). Amongst

these cysteine residues, Cys288 and Cys297 residues are primarily responsible as triggers to inhibit the activity of NRF2. The DGR domain, which contains six similar structures of Kelch, has the ability to negatively modulate NRF2 by binding to its Neh2 domain (Hayes and McMahon, 2009, Hayes and Ashford, 2012, Hayes et al., 2016). The DGR and C-terminal region domains, also known as the collectively named DC domain, in association with BTB (BTB-DC) together act as players leading to NRF2 proteasomal degradation (Hayes and McMahon, 2009, Higgins et al., 2009, Hayes and Ashford, 2012, Hayes et al., 2016).

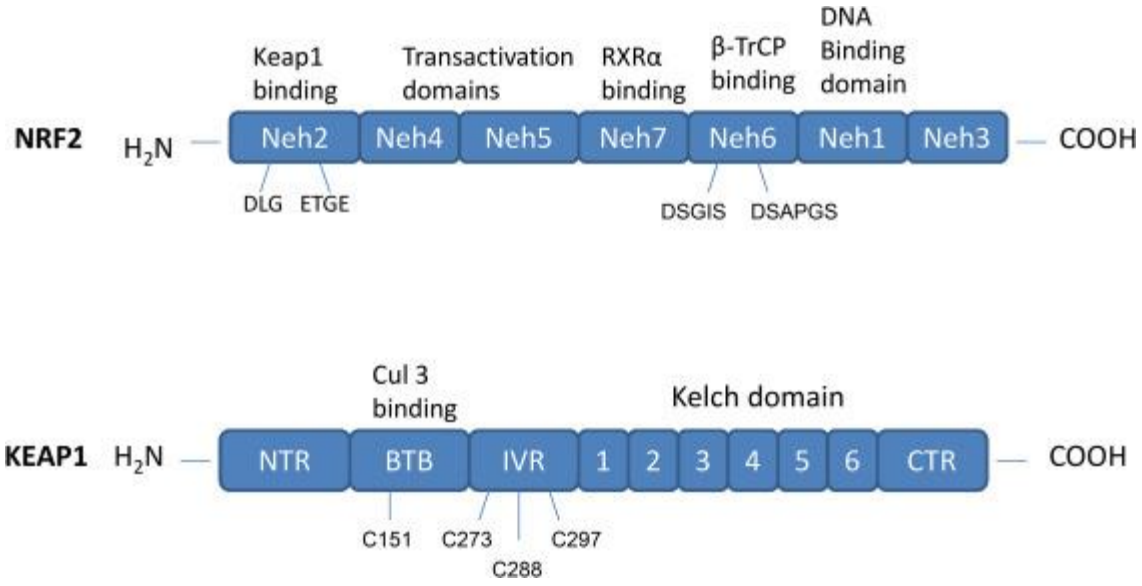


Figure 1.3: NRF2 and KEAP1 domain structures: NRF2 contains seven different domains while KEAP1 has five different domains. Retrieved from (Krajka-Kuźniak et al., 2017).

1.10. NRF2 regulation pattern by KEAP1

NRF2 is maintained in low concentration in the cytoplasm under a normal basal condition, due to control by KEAP1 that targets and presents NRF2 for ubiquitination and subsequent proteasomal degradation (Hayes et al., 2015, Ahmed et al., 2017, Namani et al., 2014). However, since degradation of NRF2 by the 26 S proteasome requires prior ubiquitination of the substrate molecule, recognition and targeting of the NRF2 protein by the ubiquitin ligases may represent a critical rate-limiting step. NRF2 activation has been found to be promoted by oxidative stress in the cells. An increase in the level of NRF2 in response to stress leads to its dissociation from KEAP1, and this being mediated

by a post-transcriptional mechanism rather than an increase in NRF2 mRNA levels. Hence activation of NRF2 has an important role in its stability and transcriptional activity (Huang et al., 2000, Nguyen et al., 2003, Ma, 2013). KEAP1-NRF2 complex is formed in the cytoplasm, where NRF2 is ubiquitinated and degraded in the event of normal basal conditions. In the event of stress state, NRF2 is dissociated from KEAP1 and translocates into the nucleus (Figure 1.4) where it heterodimerise with sMAF and then bind to ARE for initiation of expression of cytoprotective and detoxifying genes (Namani et al., 2014).

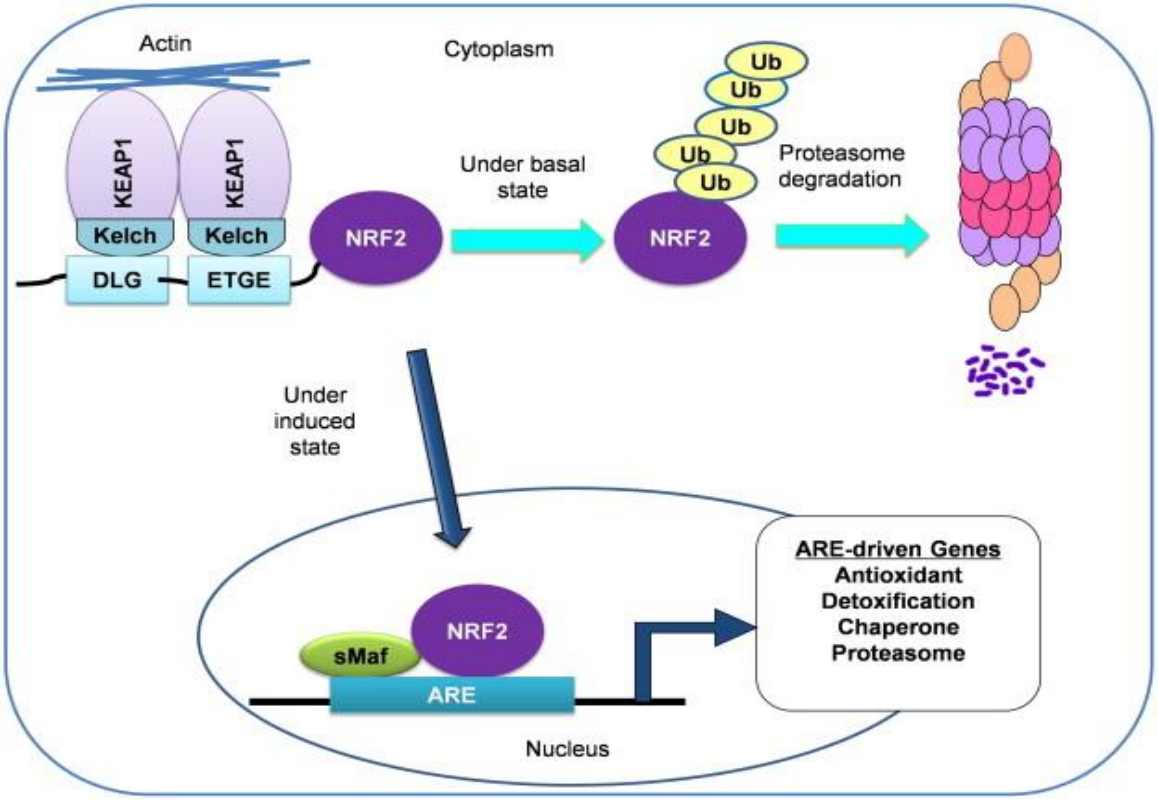


Figure 1.4: KEAP1–NRF2–ARE complex pathway. Illustrates how under normal conditions, NRF2 binds to KEAP1 at its two motifs ETGE and DLG, up to the level of ubiquitination and degradation. The Figure also illustrates the event in the stress state, where NRF2 protected from KEAP1 and obviously protected in the cytoplasm, is released, leading to its translocation into the nucleus where it binds with sMAF for subsequent initiation of cytoprotective, detoxifying and other ARE gene expression. Figure retrieved from (Namani et al., 2014).

Studies have shown that the process where KEAP1 interacts with NRF2 is through a mechanism called “hinge and latch” in which two motifs of NRF2 (DLG and ETGE) bind with the KEAP1 homodimer. The ETGE motif possesses a higher affinity for KEAP1 than the DLG motif and acts as a hinge, whilst DLG acts as a latch (Hayes and Ashford, 2012, Hayes et al., 2016). NRF2 first binds with KEAP1 at the ETGE site where there is high affinity that is through the hinge,

then at the DLG site by the latch. Under normal basal conditions, NRF2 would remain attached to KEAP1 through the hinge and latch interaction until activated by its inducers throwing it into an oxidative stress state, when it would then dissociate from KEAP1 in the cytoplasm. This free NRF2 would then translocate to the nucleus, where it would bind with sMAF proteins to form a heterodimer, and then transactivate ARE-driven gene expression that would lead to the expression of many cytoprotective and detoxifying genes (Hayes, 2000, Hayes et al., 2016, Wang et al., 2007). The phosphorylation of NRF2 by a series of protein kinases is reported to result in changes in the NRF2–KEAP1 complex and subsequent stabilisation of NRF2, which promotes the dissociation of NRF2 from KEAP1 and its accumulation in the nucleus (Namani et al., 2014, Ahmed et al., 2017)

1.11. The basic canonical and non-canonical NRF2 activation pathways

NRF2 activation involves two basic pathways: canonical and non-canonical. The canonical pathway accounts for the primary mechanism of NRF2 activation. This is based on the dissociation of NRF2 from KEAP1 in the cytoplasm leading to the translocation of NRF2 into the nucleus where it dimerizes with sMAF proteins, and then binds to ARE-carrying promoters to subsequently initiate the gene expression of cytoprotective and detoxifying enzymes (Krajka-Kuźniak et al., 2017, Krajka-Kuźniak et al., 2016). The activation of the PI3K/Akt signalling pathway and stresses on the endoplasmic reticulum are some of the mechanisms that can lead to nuclear accumulation of NRF2 and increased ARE-driven gene expression (Xiang et al., 2014, Cullinan and Diehl, 2004, Krajka-Kuźniak et al., 2017). The non-canonical pathways of NRF2 activation involve numerous proteins with motifs similar to the ETGE motif in NRF2 competing with NRF2 for KEAP1 binding. Because of this competition, in this process the NRF2 loses out in the binding to KEAP1 and therefore becomes free, leading to its accumulation in the cytoplasm (Krajka-Kuźniak et al., 2017). This is a harbinger to the eventual ease of activation and translocation of NRF2 to the nucleus. Some of these proteins that compete with NRF2 include p62, a protein that is known to contain

the STGE motif, dipeptidyl peptidase 3 (DPP3) and a partner and localiser of BRCA2 (PALB2) (Krajka-Kuźniak et al., 2017, Krajka-Kuźniak et al., 2016).

1.12 Early evidence involving NRF2 activation in cancer

Earlier NRF2 research has pinpointed the roles of NRF2 in preventing cancers (Praslicka et al., 2016). For instance, a study by Ramos-Gomez et al (2001) reported that NRF2 null mice are more susceptible to carcinogen-induced tumours than their NRF2 wild-type counterparts (Ramos-Gomez et al., 2001). Moreover, another study by Pearson et al., (2008) in which they compared the effects of caloric restriction on NRF2 wild-type and NRF2 null mice, implicated NRF2 as playing an important role in preventing cancer in the caloric restricted mice (Pearson et al., 2008).

Studies by Padmanabhan et al., (2006) and Singh et al., (2006) have reported on the activation of NRF2 in cancer, following their investigations on mutations and polymorphisms in KEAP1 in lung cancer tissues and cell lines (Padmanabhan et al., 2006, Singh, 2006). Increased NRF2 activity in cancer was reported to have a role in increased cancer cell survival and resistance to chemo- and radiotherapy, which could lead to poor prognosis (Solis et al., 2010). Identified differences in the clinical manifestation of tumours suggest that those with sustained NRF2 activation are distinct from those without (Praslicka et al., 2016). Following the findings of the role of NRF2 in chemoresistance, researchers have been focusing on identifying NRF2 inhibitors to modulate NRF2 to overcome chemoresistance (Wu et al., 2014, Chorley et al., 2012, Wang et al., 2007, Khalil et al., 2016b, Khalil and Deeni, 2015, Khalil et al., 2016a, Hayes et al., 2016, Hayes and McMahon, 2009, Yen et al., 2004a, Yen et al., 2004b, Yen and Lamph, 2006, Olayanju et al., 2015, Ren et al., 2011, Chian et al., 2014b, Tang et al., 2011, Gao et al., 2013a, Gao et al., 2013b).

1.13 Chemopreventive and chemoprotective roles of NRF2

A number of studies have reported on the double-edged role of NRF2. It displays a vital role in preventing normal cells from developing tumours and in killing

cancers away from normal cells, yet on the other hand it plays a crucial role in promoting carcinogenesis, drug resistance and cancer protection (Grossman and Ram, 2013, Na et al., 2008, Kim et al., 2008, Li et al., 2008a, Kim et al., 2014, Kim and He, 2014).

The chemopreventive role of NRF2 is reported in many studies, for example, the increased expression of NRF2 effectively yielding a positive result (Thimmulappa et al., 2002, Kwak et al., 2002, Lee and Surh, 2005, Yu and Kensler, 2005). People under treatment with inducers could obtain a high degree of chemopreventive efficacy. Health reflects the ability of an organism, including humans' ability to adapt to stress. Hence the activation of NRF2 as a chemopreventive measure is an adaptive response to environmental and endogenous stresses that serves to render organisms resistant to chemical carcinogenesis and other forms of toxicity (Kensler and Wakabayashi, 2010, Sarkar, 2011, Ahn et al., 2010, Talalay et al., 2007). A wide variety of studies have reported several natural and synthetic compounds such as curcumin, xanthohumol, sulforaphane and oltipraz as inducing NRF2, which in turn leads to chemoprevention in cancers (Grossman and Ram, 2013, Dietz et al., 2005, Lee et al., 2011, Balogun et al., 2003, Farombi et al., 2008, Rushworth et al., 2006, Clarke et al., 2008, Iida, 2004).

In contrast, a number of studies have reported the protective role of NRF2 in cancer leading to increased cancer cell proliferation and survival, a situation that then leads to drug resistance. One of the reasons for NRF2 activation is a loss of proper interaction of the KEAP1 protein, with NRF2 leading to increasing and persistent nuclear accumulation of NRF2. This thereby activates antioxidant and anti-apoptotic gene expression, which in turn leads to drug resistance (McMahon et al., 2014, Kwak et al., 2003, Cho et al., 2008, Wang, 2008, Goldstein et al., 2016, Wu et al., 2017).

Interestingly some studies have reported on ways of overcoming this problematic side of NRF2. For, example, transient transfection of NRF2-siRNA, sensitizes cancer cells to be more susceptible to cisplatin and doxorubicin (Wang, 2008). In addition, the pharmacological inhibition of NRF2 as a way of overcoming chemoresistance and increasing the killing effect of anti-cancer drugs has been

demonstrated (Khalil et al., 2016a, Tsuchida et al., 2017). Therefore, the pharmacological inhibition or genetic knockdown of NRF2 in cancer would help in overcoming chemoresistance (Namani et al., 2014, Ahmed et al., 2017, Khalil et al., 2016a, Khalil et al., 2016b).

1.14 Targeting NRF2 pathway for therapeutic intervention in cancer

Several studies have reported an increased expression of NRF2 which is overexpressed in cancers compared to normal cells, with this being one of the chemoprotective roles of NRF2 in cancers discussed earlier (Lister et al., 2011, Khalil et al., 2015, Hayes and McMahon, 2009, Kweon et al., 2006, Jiang, 2010, Stacy et al., 2006, Kim et al., 2011). Evidence indicates that a dysregulated NRF2/KEAP1 system, for example KEAP1 mutation (Ohta et al., 2008, Padmanabhan et al., 2006) or NRF2 mutation (Shibata et al., 2008), is responsible for NRF2 overexpression in cancers that leads to enhanced cellular proliferation and chemoresistance (Lister et al., 2011, Pandey et al., 2017, Shibata et al., 2008, Ohta et al., 2008, Padmanabhan et al., 2006). NRF2 tends to be overexpressed in cancers when it is freed from KEAP1 anchoring in the cytoplasm at the oxidative state and then translocates to the nucleus, where it heterodimerizes with sMAF and binds to ARE. This, in turn, leads to the expression of cytoprotective and detoxifying genes, such as NAD(P)H dehydrogenase quinone 1 (NQO1) and heme oxygenase-1 (HO-1). This confers protection to cancer cells against reactive oxygen species (ROS)-induced apoptosis and DNA damage, thereby enabling cancer cell survival and growth. Nuclear NRF2 expression due to activation of NRF2-ARE signalling may promote tumour progression and drug resistance, and hence NRF2 inhibition could be a strategic path in cancer treatment (Namani et al., 2014, No et al., 2014, McMahon et al., 2014).

As mentioned earlier, studies have now focussed on the inhibition of NRF2 to overcome the prolonged or uncontrolled activation of NRF2 in causing tissue damage or cancer progression and chemoresistance. However, the screening, discovery and development of specific, potent, and non-toxic NRF2 inhibitors, including retinoids (e.g. RA and bexarotene) remain challenging. The possible

ways of developing specific inhibitors include: (i) transcriptional down-regulation of NRF2; (ii) increased degradation of NRF2 mRNA for subsequently decreased translation; (iii) enhancement of NRF2 degradation, through up-regulation of KEAP1-CUL3 complex, β-TrCP-SCF or HRD1; (iv) blocking the translocation of NRF2 to the nucleus leading to antagonising or blocking the dimerization of NRF2 with sMAF proteins, and (v) blocking the NRF2-sMAF DNA-binding domain (McMahon et al., 2014, Namani et al., 2014, No et al., 2014).

A review by Namani et al (2014) described retinoids as structurally related to vitamin A and other natural and synthetic signalling compounds including retinol, retinal, RA and retinyl esters. They are reported to have an anti-cancer effect because of their proapoptotic and antioxidant activities. Retinoids are stimulated ligands via interaction with two different nuclear receptors called retinoic acid receptors (RARs) and retinoid X receptors (RXRs), and these are members of the steroid/thyroid hormone receptor super-family. The RARs themselves contain the three isotypes RARα, RARβ, and RARγ encoded by the RARA, RARB, and RARG genes, and function as ligand-dependent transcription factors. There are two important isoforms of RARα (α1 and α2) and RARγ (γ1 and γ2) with vital functions; however, RARβ isoform has β1, β2, β3, β4, and β1′) resultant from differential use of promoters and alternative splicing (McMahon et al., 2014, Namani et al., 2014, No et al., 2014).

Mostly RARs form heterodimers with RXRs and in the absence of ligand, an RAR/RXR heterodimer can interact with multiple co-repressor proteins such as the nuclear receptor co-repressor and silencing mediator of RA that regulates the transcription of target genes (McMahon et al., 2014, Namani et al., 2014, No et al., 2014). Also, endogenous ligands such as RAs act as agonists and activate the RAR/RXR heterodimer complex, leading to a reduction in the affinity between the co-repressor and the complex. The coactivator proteins such as steroid receptor coactivators SRC-1, SRC-2, and SRC-3 and proteins that have histone acetyltransferase activity similar to p300-CBP, P300/CBP-associated factor, have general control of amino acid synthesis protein 5-like 2. This will then subsequently interact with high affinity for the RAR/RXR heterodimer, which transactivates the genes targeted by RA through binding to downstream DNA

response elements, known as RA response elements (RARE) (McMahon et al., 2014, Namani et al., 2014, No et al., 2014).

Nuclear receptors have been reported to have a role in cancer, and are hence considered as drug targets in the same way that NRF2 is. Nuclear receptors play vital physiological functions and are mostly found in the nucleus, where they bind to DNA sequences such as hormone-responsive elements and regulate gene expression. The nuclear receptors are regulated either in a ligand-dependent or a ligand-independent manner, for example, RXRα physically interacts with NRF2, forms a protein–protein complex and then negatively regulates ARE gene expression. Studies have reported that nuclear receptors play dual roles in the aetiology of cancer. For example, PPARγ has been reported to play the role of both tumour promoter and tumour inhibitor in cancers (McMahon et al., 2014, Namani et al., 2014, No et al., 2014).

1.15 Silencing NRF2 gene expression using siRNA to improve cancer therapy

Evidence from studies indicates the application of siRNA for targeting cancer to improve treatments so as to overcome resistance to chemotherapy and radiotherapy. This provides a promising therapeutic modality for cancer and other diseases (Huang et al., 2008, Guo et al., 2013, Kanninen et al., 2015, Duong et al., 2017, Esmaeili, 2016). Silencing genes exerts antiproliferative and proapoptotic effects upon cell culture systems, animal models in clinical trials and in most studies. The recognition of the siRNA mechanism and progress in this field has led to several new siRNA-based drugs being applied in clinical trial phases (Mansoori et al., 2014). In combination with standard chemotherapy, siRNA therapy may reduce the chemoresistance of certain cancers, thereby demonstrating the potential of siRNA therapy for treating many cancers (Huang et al., 2008), including ovarian. A combination of siRNA-mediated gene silencing with natural products has been reported to down-regulate the NRF2-dependent response and partly sensitise MCF-7/TAM cells to tamoxifen in a synergic manner (Esmaeili, 2016). Another study by Duong et al. (2017) reported that NRF2-mediated silencing using siRNA reduced the level of aldehyde

dehydrogenase 1 family, member A1 (ALDH1A1) and aldehyde dehydrogenase 3 family and member A1 (ALDH3A1); as well as glutamate-cysteine ligase catalytic subunit (GCLC) expression leading to enhanced antiproliferative effects of the chemotherapeutic agent, 5-fluorouracil (5-FU) in pancreatic cancer cells (Duong et al., 2017). These studies have suggested that siRNA-mediated NRF2 knockdown could increase the efficacy of chemotherapeutic drugs.

1.16 ROS: their dual role in cancer and their relationship with the HER family and NRF2

ROS are ions, molecules or radicals that have a single unpaired electron in their outermost shell of electrons. Because of this characteristic, they are highly reactive, and more so than molecular oxygen (Zhang et al., 2016a, Liou and Storz, 2010). ROS are classified into two groups: free oxygen radicals and non-radical ROS. Free oxygen radicals include superoxide ($O_2^{\bullet-}$), peroxyl radicals (ROO•), hydroxyl radical (•OH), nitric oxide (NO•), organic radicals (R•), alkoxyl radicals (RO•), thiyl radicals (RS•), disulfides (RSSR), sulfonyl radicals (ROS•) and thiyl peroxyl radicals (RSOO•). Non-radical ROS include hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), trioxygen/ozone (O_3), organic hydroperoxides (ROOH), hypochloride (HOCl), peroxynitrite (ONO^-), nitrosoperoxycarbonate anion ($O=NOOCO_2^-$), nitrocarbonate anion ($O_2NOCO_2^-$), dinitrogen dioxide (N_2O_2), nitronium (NO_2^+) and highly reactive lipid-or carbohydrate-derived carbonyl compounds (Liou and Storz, 2010). Amongst these, superoxide, hydrogen peroxide and hydroxyl radicals are the most well studied ROS in cancer (Zhang et al., 2016a, Liou and Storz, 2010).

Just like NRF2, ROS play a vital role in various cellular biological activities including proliferation, growth and apoptosis. They can also facilitate cancer cell proliferation as well as survive and adapt to hypoxia (Zhang et al., 2016b). Cancer cells increase their rate of ROS production in comparison with normal cells to induce the cell signalling pathways necessary for cellular transformation and carcinogenesis (Reczek and Chandel, 2017). Moreover, in order to maintain ROS homeostasis and evade cell death, cancer cells increase their antioxidant capacity relatively. Compared with normal cells, this altered redox environment of

cancer cells may increase their susceptibility to ROS-manipulation therapies (Reczek and Chandel, 2017).

ROS are reported to have dual roles in cancer depending on their concentration in cells. ROS facilitate carcinogenesis and cancer progression with mild to moderately elevated levels, while excessive ROS damage cancer cells dramatically and lead to cell death (Zhang et al., 2016b). Studies have implicated the HER family, NRF2 and ROS in the promotion of cancer cell proliferation, increased detoxification potential and resistance to therapy (Kang et al., 2014b, Manandhar et al., 2012). In particular, the generation of ROS, which are key regulators of the NRF2 pathway (Kang et al., 2005), have been demonstrated as a regulator of the HER family complex and subsequent activation of its functions. Elevation of ROS oxidizes redox sensitive cysteine residues on KEAP1. This results in dissociation of NRF2 from KEAP1, which translocates to the nucleus, heterodimerizes with sMAF and binds to AREs for the initiation of expression of antioxidant genes. Elevation of ROS can trigger signalling pathways such as ERK MAPK and PI3K, which are induced following HER receptor family activation to activate NRF2. These new mechanisms place ROS in a central position where they might act as a point of convergence between the HER receptor family and NRF2. Taken together and in consideration of the dual roles of ROS as a point of convergence between these two cytoprotective pathways, increasing the level of ROS in cancer cells as a strategy could improve treatment.

The foregoing overexpression of HER family receptors and NRF2 are well recognised in cancers and in conferring therapeutic resistance to cancers (Hayes and McMahon, 2009, Hayes and Ashford, 2012, Hayes et al., 2015, Marmor et al., 2004, Bianco et al., 2006, Friedlander, 1998, Psyrri et al., 2005, Phelps et al., 2008, Ledermann and Raja, 2010). In addition, a report by Khalil et al. (2016b) recently demonstrated transcriptional regulation of HER2 and HER3 by NRF2. In addition to demonstrating a relationship between NRF2 function, HER2/HER3 signalling, ROS generation and the sensitisation of ovarian cancer cells to the killing effects of the targeted therapeutics and trastuzumab, pertuzumab or their combination (Khalil et al., 2016b, Khalil et al., 2016a) along with reports that demonstrate the importance of inhibitors of NRF2, it is reasonable to make the following hypotheses:

1. Ovarian cancer develops chemoresistance as a result of overexpression of HER receptor family and NRF2.
2. Since NRF2 is a transcription factor to several hundred genes including proto-oncogenes, it is feasible that HER receptors are transcriptional targets of NRF2 via direct or indirect means involving ROS.
3. Inhibition of NRF2 function could improve the efficacy of HER receptor-targeted chemotherapeutics.

1.17 The aims and objectives of this study

1.17.1 Aim

The main aim of this research is to investigate the regulation of the HER receptor family and NRF2 in ovarian cancer.

1.17.2 Objectives

1. To clone human HER1 and HER4 proximal gene promoter and reporter systems as novel tools for the study of the regulation of HER1 and HER4;
2. To examine the regulations of HER1 and HER4 by NRF2 in ovarian cancer;
3. To screen for potent inhibitors of the NRF2-ARE signalling pathway and then investigate their potential applications in ovarian cancer therapy;
4. To suggest novel interventions that may improve ovarian cancer therapy.

The overall findings of this study may add to the regulation of the overexpression of HER family receptors and NRF2 in cancer, which may be a contributing determinant for the success and/or failure of HER targeted therapies involving RTK inhibitors.

2. CHAPTER TWO

Materials and Methods

2.1. Bioinformatics

2.1.1 Putative NRF2 transcription factor binding sites in promoters HER1 and HER4

Approximately 1.5 kb and 1.3 kb proximal promoter regions of HER1 and HER4 respectively were retrieved from the base (http://www.ensembl.org/index.html) subjected to transcriptional factor binding prediction program (http://consite.genereg.net/) (Sandelin et al., 2004) (Figure 2.1) to predict for putative NRF2 binding sites. The sequences were analysed at 75% stringency.

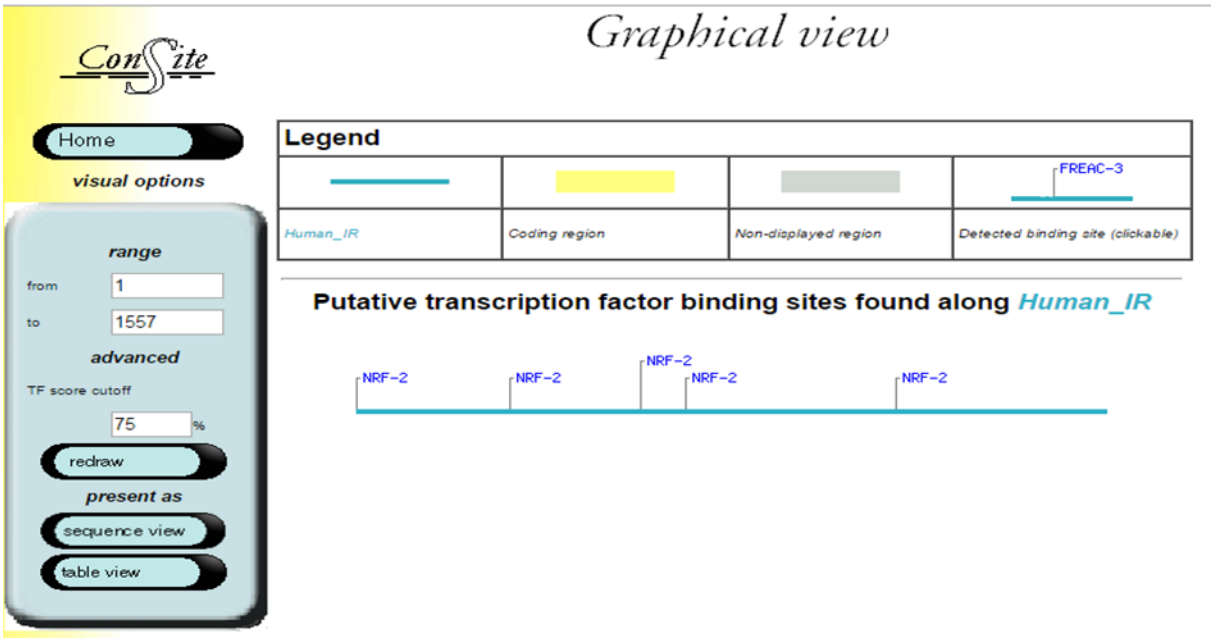


Figure 2.1: Screen shot of the bioinformatics analysis of the retrieved sequences. This is used to predict for putative NRF2 binding sites from the consite tool.

2.1.2 Primer design and analysis

All the primer sequences designed in the current research were first analysed under different criteria before ordering. Primer analysis was performed using the primer analysis software, Oligo-Analyser (Integrated DNA technologies, UK) (Figure 2.2). First of all, it was made sure that the primer sequence designed had a length between 20-40bp, had a minimum GC content of 50%, and that the forward and reverse primers did not have self-complementation. Once, this was established, it was made sure that the primer sequences were specific for the

gene of interest by carrying out basic local alignment search (BLAST) analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Further analysis was performed using the Oligo- Analyzer web resource (https://www.idtdna.com/site/Order/oligoentry/set?seq=CACTCCAGGTACTAGCCAAGG) which determined its melting temperature, a complementary sequence, hairpin formation, and possible self-dimers.

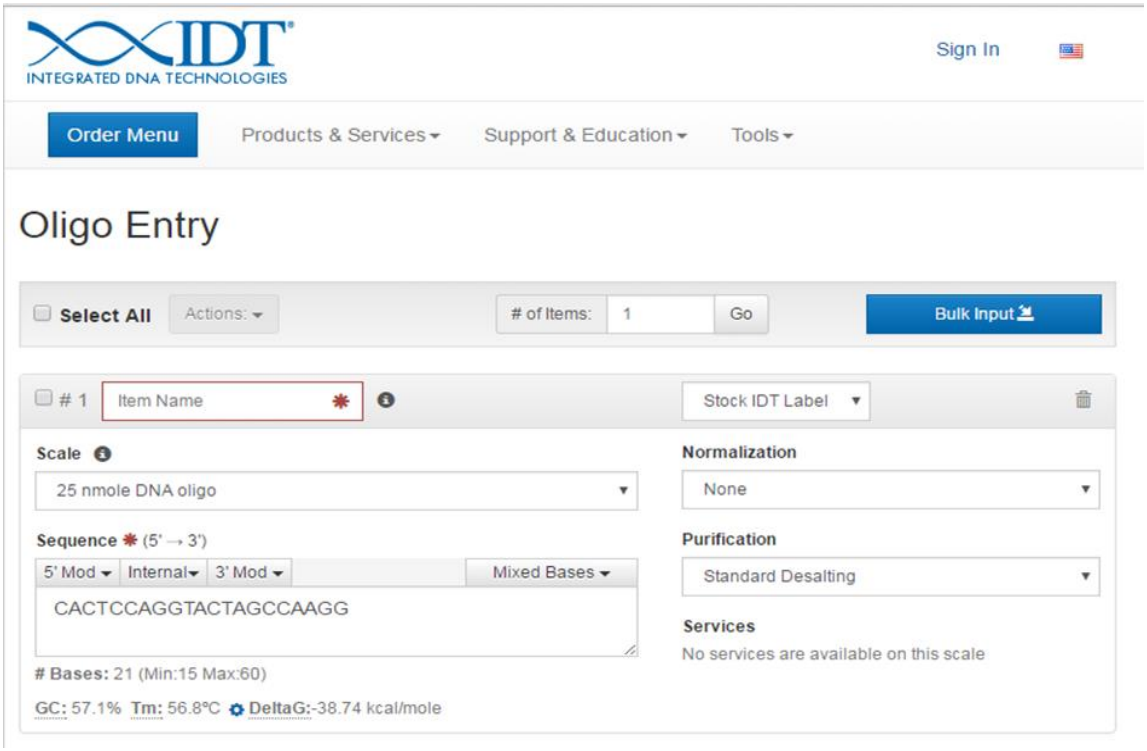


Figure 2.2: Screen shot of the Bioinformatics analysis of the designed primer using the Oligo-Analyser web tool. After the primers were found correct under all the set criteria, they were ordered (Integrated DNA technologies, UK) with PAGE purification. Once the primers arrived in lyophilized form, the primer vials were centrifuged at full speed for 3 min, and re-suspended in Nuclease free water (Invitrogen) to make a stock of 100 µM. 10 µL aliquots of 10 µM final stocks were made and stored at -20°C until used.

2.2. Molecular biology

2.2.1. Isolation of genomic DNA

For the isolation of genomic DNA, buccal swabs were used following the Buccal swab spin® protocol (Qiagen). Buccal swabs were used as a less invasive alternative to a blood sample. The buccal swabs were collected by swabbing the inside of the cheek 5 times with a cotton swab (Whatman bioscience). The cotton

head was ejected in the eppendorf tube (Scientific laboratory supplies) and allowed to air dry for at least 2-3 h. Following this, the manufacturer's instructions were followed to isolate total genomic DNA. The quality of the isolated DNA was examined by running 10 µL of the final DNA solution on 1% agarose gel and genomic DNA was spectrophotometrically quantified.

2.2.2. PCR amplification

In this study, two types of PCR techniques were performed. The first one was the conventional PCR amplification technique for the amplification and clonal manipulation of DNA using a template DNA either from bacterial miniprep extraction or genomic DNA extracted from buccal swabs. The second type was colony PCR where the bacterial colony used as a source of template DNA used to screen for positive clones for a gene of interest. PCR reaction mix was made on ice in PCR tubes (Fisher scientific) using commercially available PCR reagents (Promega). Typically, each PCR reaction volume was 50 µL. The following were added:

GoTaq® flexi buffer (5x).....10 µL

MgCl2 (25 mM).....2.5 µL (final concentration 1.25 mM)

Forward primer (10 µM stocks).....1 µL (final concentration 0.2 µM)

Reverse primer (10 µM stocks).....1 µL (final concentration 0.2 µM)

dNTP mix (10 mM each).....1 µL (final concentration 0.2 mM of each)

DNA template.....0.25 µg

GoTaq® DNA polymerase.....1.5 units (0.3 µL)

Nuclease free water.....To 50 µL.

In the case of colony PCR reactions, these were performed to screen for successful clones by using the bacterial cells directly as a source of template DNA and examining the presence of the gene of interest using primers specific for that gene. Following overnight growth of transformed cells and subsequent to the appearance of colonies, individual colonies were picked with the help of pipette tips and introduced into PCR tubes containing 5 µL of water. The same

tips were also streaked individually from each colony on another plate in order to preserve the clone. Then from that 5 µL cell suspension, 1µL was used as a DNA template in PCR mix. The total volume of PCR mix was set to 25µL in the following set up below:

GoTaq® flexi buffer (5x).....5 µL

MgCl2 (25 mM).....1.25 µL (final concentration 1.25 mM)

Forward primer (10µM stocks).....0.5 µL (final concentration 0.2 µM)

Reverse primer (10µM stocks).....0.5 µL (final concentration 0.2 µM)

dNTP mix (10mM each).....0.5 µL (final concentration 0.2 mM of each)

DNA template.....1 µL of the resuspended colony

GoTaq® DNA polymerase.....1 unit

Nuclease free water.....To 25 µL.

Following completion of PCR reactions, the whole reaction mixes were run on 1% agarose gels using appropriate DNA ladders for analysis and subsequent required applications.

2.2.3. Agarose gel electrophoresis

The agarose gel electrophoresis was performed for the separation, validation, characterization and purification of DNA following PCR amplifications, restriction digestions, genomic DNA isolation, estimation of ligation ratios and other clonal manipulations required in molecular biology works in this study.

Throughout the study, 0.5 to 1% solution of agarose (Sigma) was made in 1x TAE buffer in a conical flask. The percentage of agarose gel used depended upon the size of DNA (for DNA > 10 kb, 0.5 % of agarose was used). Agarose was dissolved in TAE by heating in a microwave for 2-3 min and once the agarose was fully dissolved, the solution was cooled to 40^{o}C at room temperature or sometimes with the help of tap water. For staining the DNA, Gel red nucleic

acid stain (Biotium) was added at 5 µL/100 mL of gel. The gel solution was added to the agarose gel electrophoresis tank after assembling it and a comb-plate was inserted in the gel. After the gel solidified, the comb-plate was carefully removed and 1x TAE buffer was added into the gel tank to fully immerse the solidified gel in the buffer. Following this, 5 µL of 1 kb DNA ladder (Bioline) for estimation of bands was added in the first well and the DNA to be analysed was mixed with the DNA loading dye (Bioline) and carefully loaded into each well. In the case of PCR amplified products, all the content of tube (usually up to 50 µL) were added in each well, and for estimation of ligation ratios, or quality of gel purified DNA, 5 µL of DNA was used and for restriction screening, 15-20 µL of DNA was added. The lid of the tank was closed and the attached cables plugged into the power pack. Agarose gels were usually run for 1 h at 80-100volts.

2.2.4. Visualisation of DNA bands and image capturing

The DNA bands within agarose gels were visualised under UV by placing the gels on UV illuminator (UVP upland, USA). To capture the gel images, gels were placed within the Gel documentation machine (Alpha Innotech, USA), and loading the Alphaimager® software. Depending upon the intensity of bands, appropriate exposure time was set (usually between 500-1000 milli seconds) and image captured through the in-build camera. The images were saved in TIFF file format for later analysis.

2.2.5. Purification of DNA from agarose gels

Purification of DNA involves purifying different DNA molecules from a common mixture (e.g. vector DNA and the insert) or to clean DNA of buffers and salts (e.g. after PCR amplification and restriction digestion), DNA of interest was run on agarose gels and purified from gel using the gel purification kit (Qiagen).

Before doing anything else, first, the gel was placed on UV the illuminator to visualise DNA. Protective equipment (UV face mask, gloves and lab coat) were worn and the UV was turned on. The DNA bands to be purified were carefully cleaved from the gel with the help of a scalpel, and straightaway placed in labelled eppendorf tubes. The rest of the protocols were followed according to manufacturer's instructions (Qiagen). Once the DNA was purified, it was run on

an agarose gel to examine its quality and condition. Its quality and condition were assured when tight bands and less degradable levels (smears) of DNA were observed.

2.2.6. Restriction digestion of DNA molecules

Restriction digestion of DNA involves clonal manipulation of DNA and validating the identity of the successfully cloned constructs. The restriction digestions using restriction endonucleases were performed according to manufacturer's (Promega) instructions. The final volume of digestion mix was made to 20 µL by adding the following:

10x restriction buffer: To final concentration of 1x.

DNA: 1 µg (Usual concentration of DNA was maintained at 1 µg/µL, hence 1 µL used).

The contents were gently mixed by pipetting up and down.

Enzyme-I (between 5-10 units)

Enzyme-II (between 5-10 units)

Sterile de-ionized water, (to the final volume of 20 µL).

This restriction digestion mix was incubated for 1 h at 37^{o}C in a water bath. However, for the restriction digestion of PCR amplified products, the DNA was first run on the agarose gel in order to gel purify it and then subjected to restriction digestion as above. For cloning purpose, the restriction digestion of plasmid vector was usually followed by Shrimp Alkaline Phosphatase (SAP) mediated (Promega) dephosphorylation of the 5' overhangs. For this, after the completion of the above restriction digestion, the reaction mixture was subjected to 65^{o}C for 15 min to inactivate restriction enzymes. This was allowed to cook and 1unit of SAP (Promega) was added and the mixture incubated for up to 15 min at 37° C. SAP enzyme was heat inactivated by incubation at 74^{o}C for 15 -20 min.

2.2.7. Ligation of DNA molecules

Ligation of the DNA molecules includes the gene of interest or insert and linearized cloning vector, both were first run on an agarose gel to have an estimate of their relative molar ratios as determined by their relative intensities and size. Based on those intensities, the following ligation ratios were made: Insert: Vector 3: 1 or 1:1 or 1: 3

In these ratios, the volume of the vector was usually maintained constant while that of the insert was altered to achieve the above molar ratios. After estimating the volume of vector and insert needed, the following were added in the eppendorf tube in order to make a 10 µL ligation reaction:

Insert (volume dependent upon molar ratio)

Vector (volume dependent upon molar ratio)

10x ligation buffer to the final concentration of 1x (Promega)

T4 DNA ligase: 3 Weiss units (Promega)

Nuclease free water, (to final volume of 10 µL).

A non-insert control was also used where nuclease free water was added instead of the insert. The above reaction was incubated either at room temperature for 3 h, or overnight at 4^{o}C. Following this, the next step was bacterial transformation where 5 µL of the ligation mixture was usually used.

2.2.8. Bacterial transformation

Bacterial transformation by heat shock using DH-5α bacterial strain was employed in this study. In this method, chemo-competent cells were employed. For each transformation reaction, 50 µL of competent cells were used. First of all, bench surface was swabbed with 70% ethanol and burner was turned on to maintain a sterile environment. Frozen cells maintained at -80°C were taken out and immediately placed on ice. Cells were allowed to thaw slowly on ice and in the meantime, heat block was set to 42°C. After thawing, the chemo competent cells were very gently mixed with the help of pipette and 50 µL transferred to a pre-chilled and pre-labelled eppendorf tube. The plasmid-vector was next added gently to the tube containing 50 µL chemo-competent cells (either 1 µL or 5 µL of

vector added depending upon application) and the tube placed back on the ice and allowed to incubate for 30 min. After incubation, the tube was placed on the heat block maintained at 42°C and heat shock was given for 45 s during which, bacterial transformation takes place. The tube was put back on the ice for 2 min and then at room temperature. Pre-heated terrific broth (TB) at 37°C was taken out of water bath and 500 µL of that was gently added to the transformation tube. The tube was placed in the shaking incubator set at 37°C and allowed to incubate for 90 min while shaking at 125 revolutions per minute (rpm). In the meantime, TB-agar plates, made with appropriate antibiotic selection (either 50 µg/mL kanamycin or 100 µg/mL ampicillin) were taken out of the fridge, and placed, with lids off, in another incubator set at 37°C.

After the 90 min incubation period, the transformation tube and the TB-agar plates were taken out and placed under the burner. The contents of the transformation tube were carefully transferred to the TB-agar plate and spread evenly with the help of spreader. The plates were taken to incubator maintained at 37°C and placed inverted overnight to allow the transformed bacteria to form colonies.

2.2.9. Screening of the transformed bacterial colonies and making glycerol stocks

Following the bacterial transformation, the growth of transformed bacterial colonies on agar plates made with appropriate antibiotics was checked after 16 h. Following this, using sterile technique, 5 mL of pre-warmed (37°C) TB medium was taken in a 15 mL centrifuge tube and antibiotics (ampicillin 100 µg/mL or kanamycin 50 µg/mL, as appropriate) was added into it.

Based on the number of colonies on the control plate, colonies from the experimental plates were picked up using a sterile loop and inoculated in each of the 15 mL tubes and allowed to grow overnight at 37°C in a shaking incubator at 125 rpm. Next day, the tubes with bacterial suspension were taken out and centrifuged at 5000 rpm at 4°C for 15 min. The media in the supernatant was discarded and the palette dried on a paper towel. After this, to extract the transformed plasmid from bacterial cells, Spin Miniprep kit for plasmid extraction

(Qiagen) was used according to the manufacturer's instruction. To screen and verify the cloned insert, restriction digestions were performed using appropriate restriction enzymes. After successfully transformed bacterial cultures were identified, glycerol stocks were made in the following way. A 50% filtered sterile glycerol solution in TB containing the appropriate antibiotics (100 µg/mL, kanamycin 50 µg/mL) was made. 400 µL of this stock was mixed with 1 mL of the successfully transformed bacterial culture, mixed gently and stored in -80°C. Whenever required, these glycerol stocks were streaked on agar plates and grown overnight at 37°C to get colonies of transformed bacteria.

2.2.10. Bacterial plasmid extraction:

The subsequent day after transformation, once individual colonies of the transformed bacteria appeared on plates with an antibiotic selection, they were grown overnight in the presence of the same selection. The cloned plasmid was isolated from transformed bacteria by either microcentrifuge based plasmid Miniprep (Qiagen) or Midiprep (Qiagen). For techniques like propagation and clonal manipulation, plasmid Miniprep was performed. For transfection of mammalian cells, concentrated and high-quality DNA preparations were used with plasmid midiprep technique. A typical concentration of up to 0.25 mg/mL and up to 1 mg/mL DNA, were achieved by plasmid miniprep and midiprep techniques (Qiagen) respectively.

In the midiprep technique, the glycerol stock sample of successfully transformed bacteria, confirmed to be correct clone following sequencing, was taken out of -80°C freezer and inoculated in 100 mL of TB with antibiotics in a 500 mL conical flask and grown overnight. Next day, the bacterial culture was transferred to individual 50 mL centrifuge tubes and centrifuged at 5000 rpm at 4°C for 15 min. The supernatant was discarded and palette dried on a paper towel. To extract the plasmid, a further procedure was followed according to manufacturer's (Qiagen) instructions.

2.3. Mammalian tissue culture:

2.3.1 Measures taken for the use of cell culture

Measures such as authentication of cell line, cryopreservation, and avoidance of cross-contamination of cell lines were all taken. Furthermore, the cells were checked for microbial contamination (regular mycoplasma test).

2.3.2. Cell lines:

For all the cell lines listed **(Appendix 1, table 9.1.1),** the media was supplemented with filter sterilised 10% foetal bovine serum (FBS), 2 mM glutamine, 1 mM sodium pyruvate, 100 µg/mL streptomycin and 100 U/mL penicillin all purchased from Invitrogen UK. Cells were maintained at 37^{o}C in a tissue culture incubator with an atmosphere containing 5% CO_2.

2.3.3. Sub-culturing the cells

Sub-culturing of cells used in this study was performed regularly to ensure their survival, regular supply for the study and maintain proper growth conditions. It was routinely done with cells that reached 80-90% confluence either in flask or tissue culture plates. All the procedures were done using sterile techniques performed in a sterile tissue culture hood.

Briefly, in the process of sub-culturing, the media, buffers and trypsin were placed in water bath at 37°C at least 30 min prior to subculturing the cells. Tissue culture lab coat and gloves were worn. The gloves were sprayed with 70% ethanol. The laminar flow in the hood was turned and the working surface of tissue culture hood was swabbed with 70% ethanol. Any apparatus, media, trypsin and buffers taken inside the hood was first swabbed by 70% ethanol except for tissue culture flasks and plates. The tissue culture flask needed to be sub-cultured (>80% cell confluence) was taken out of the incubator, observed under the microscope and placed into the hood. With the help of aspirator attached to the suction pump, the old medium was removed from the flask. 4 mL of PBS (Invitrogen) was added to the cells and the flask gently swirled to wash away the old media and dead cells. PBS was aspirated out. 1 mL of pre-warmed 0.25% trypsin (Invitrogen) was

gently added on top of cells with the help of serological pipette, and the flask swirled to ensure spreading of trypsin on the entire surface area of the flask. The flask was taken into tissue culture incubator maintained at 37^{o}C with 5% CO_2 atmosphere and incubated for 5 min (or until the cells were detached as observed under the light microscope). After the cells detached, 4 mL of pre-warmed cell culture media was added to the flask to stop the further action of trypsin and dilute the cells. With the help of serological pipette, the cell suspension was mixed thoroughly by pipetting up and down to ensure total detachment and to break any cell clumps formed. After this, the number of cells in the suspension was determined and either seeded into other flasks for propagation and cell culture maintenance, or cryo-frozen for future use. The type of tissue culture flasks and plates employed, specific to experimental type with the number of cells seeded and the amount of growth media used in the current research study was listed in **(Appendix I, table 9.1.2).**

2.3.4. Cell counting

Throughout the course of this study, human cells were routinely counted for different experiments via counting chamber method. In this method, first of all, the counting chamber and the cover-slip used were cleaned with the help of lens paper and put under the light microscope. The coverslip was placed on top of the gridded area. The grid in the counting chamber was composed of squares of different areas. The cells to be counted were first trypsinized and then diluted in cell culture media. Every time micropipette is used to take 10 µL of cell suspension from the flask and put underneath the coverslip over the counting chamber where the cell suspension spreads quickly. The cells were counted in ten 0.04 mm^2 squares in the grid. Then, the number of cells per µL was calculated with the following formula:

Supposing, the number of cells counted in ten 0.04 mm^2 squares = 20 cells (supposed)

Total area in which 20 cells counted = (10 x 0.04) = 0.4 mm^2

Total volume = 0.4 mm^2 x 0.1 (height of chamber) = 0.04 mm^3

So, 20 cells in 0.04 mm^3.

1 mm3 = 1 µL, hence, 0.04 mm^3 = 0.04 µL

If 0.04 µL has 20 cells, 1 µL has = 20 x 1 µL ÷ 0.04 µL = 500 cells.

Hence, 1 µL has 500 cells.

2.3.5. Cryofreezing cells and reviving frozen cells

In this case, the surplus cells were routinely cryo-frozen for future use. After the cells were trypsinized from a T75 flask, 5 mL of cell media was added to stop the action of trypsin, and the cell suspension was mixed thoroughly with the help of serological pipette attached to the pipette buoy. The cell suspension was transferred to a sterile 15 mL centrifuge tube, capped tightly and taken to the centrifuge machine set at 37°C. The cell suspension was centrifuged at 1000 rpm for 15 min to palette the cells. In the meantime, sterile cryotubes (Thermo Fischer) were labelled with cell details and passage number. After centrifugation, the tube was taken back to the hood, the supernatant was discarded and the cell palette was gently resuspended in 1 ml of freezing media.

Following this, the cell suspension in freezing media was carefully transferred to cryotubes, capped properly and immediately taken for storage in -80°C freezer. When previously frozen cells were needed, the media was first warmed in the water bath at 37°C and 10 ml of media was transferred to the T75 flask. The cryotube with frozen cells was taken out and immediately put in the water bath at 37°C. As soon as the frozen mixture was thawed (usually after 1- 2 min), the cryotube was taken to the tissue culture hood, and with the help of pipette, very gently transferred into the T75 flask with the media. After 16-24 h, old media was replaced by new media to replenish the nutrients and boost cell growth and after every 3-4 days, cell media was usually changed by first aspirating the old media, washing the cells with warm PBS, and transferring fresh media into the flask to boost the healthy condition of the growing cells.

2.3.6 Cell treatments with drugs

The drugs used in this research work involved the preparation of stock solutions of these drugs and stored as instructed by their manufacturers. Working concentrations were achieved by diluting the stock in the media used and were based on prior literature. Prior to treatment with these drugs, cells to be treated were taken out of the incubator; old media removed and pre-warmed PBS added. Pre-warmed media was then transferred to falcon tubes (corning) in the tissue culture hood and the required amount of drug was added to achieve the working concentrations. The PBS over the cells was taken out and the media with the drug carefully added. The amount of media added was dependent upon the type of tissue culture vial in which the cells were grown. For example, the cell lines were maintained in RPMI 1640 media (Gibco® Invitrogen) supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine, 1 mM sodium pyruvate, 100 µg/ml streptomycin and 100 U/ml penicillin in an atmosphere of 5% CO2 and incubated at 37°C. Before experimental treatments, cells were grown for 24 h in RPMI 1640 media prepared as above. Heregulin-β1 (HRG, Sigma) was used by preparing 1 µM stock solution made with 5% trehalose, 10% FBS in PBS and diluted to a final concentration of 1nM with media during treatments. All the drugs were used by directly diluting the drugs in media to desired final concentrations. For drugs that are sensitive to light, stock solutions were made in in amber Eppendorf tubes pre-aired with nitrogen gas. Once the stock solution was made, it was bubbled again with nitrogen gas and closed, stored at -80°C. **(Appendix II, sub-section 9.2.29**) provides the detailed information about the drugs used, their storage and working concentrations and their manufacturers.

2.3.7. Cell transfection

Cell transfections were carried out by liposome mediated gene transfer via the use of commercially available transfection reagent (Lipofectamine 3000, Invitrogen). The transfections aimed at performing Dual-Luciferase based promoter assay or siRNA transfections for gene silencing. Briefly, 16-24 h prior to transfection, cells were seeded desired 24 well plates containing normal cell culture media. Next day, the plates were taken out (with 70-90% confluent cells) and put in the tissue culture hood and old media was removed. Cells were

washed once with warm PBS and 250 µL of pre-warmed serum-free and antibiotic free media (Opti-MEM®, Invitrogen) was added per plate and put back in the incubator. Next was to prepare the transfection complexes for subsequent transfection in the following way:

1. 0.5-1 µg DNA was diluted in 50 µL serum and antibiotic free media in an eppendorf tube labelled tube A.
2. 1-3 µL transfection reagents was slowly added to 50 µL of serum and antibiotic free media in another eppendorf tube labelled tube B.
3. Both tubes were allowed to stand for 5 min.
4. The contents of tube A were gently mixed with the contents of tube B in another eppendorf tube labelled tube C.
5. Tube C was incubated at room temperature (inside tissue culture hood) for 30 min to form the complex.
6. 150 µL of serum free and antibiotic free media was added to a 1.5 mL effendorf tube, and the contents of tube C were gently introduced into it. The total volume in this effendorf tube now was 250 µL and was called the transfection media.
7. The cells to be transfected were taken out of tissue culture hood, old (serum free, antibiotic free) media was aspirated and the 250 µL of transfection media was added on top of these cells gently.
8. The plate was placed back in the tissue culture hood and allowed to incubate for 5 h.
9. After 5-6 h, the transfection media was removed and pre-warmed normal cell culture media was added to the cells. Cells were allowed to grow for 16-24 h. For transfections involving siRNA, cells were allowed to grow for 24-96 h depending on the type of experiment before further studies.

2.4 Biochemistry

2.4.1 Antibodies and the detection systems used in the study:

The different primary antibodies employed in the current study are listed in (**Appendix 1, Table 9.1.1**). To study different HER receptor family and NRF2 proteins, this research employed different biochemical detection systems as a

requirement of the employed biochemical techniques. The secondary antibody detection systems used, its dilution, the name of the technique employed, and the manufacturer are listed in **(Appendix 1, Table 9.1.2)**.

2.4.2 Protein extraction

For western blot analysis of different proteins, total protein content was extracted from cells grown in 60 mm tissue culture plates at 90% confluency ($0.5 \times 10^6 - 1 \times 10^6$) during treatment and protein extraction. The entire extraction process was done on ice as much as possible and cells were never allowed to dry.

Tissue culture plates with cells were taken out of tissue culture incubator and placed on ice. The old media was discarded from the plates and cells were washed three times with 5 mL of ice-cold PBS. After the third wash, 495 µL of ice cold PBS was taken in an eppendorf tube, and 5 µL of 100x protease and phosphatase inhibitor cocktail (Pierce Biotechnology) added to it and mixed. The PBS with the inhibitors added was transferred to the tissue culture plate.

With the help of a tissue culture cell scraper, the cell monolayer was scraped off by gently tilting the plate, and scraping off cells to the bottom. The complete monolayer was scraped off to produce a cell suspension. This suspension was transferred to a pre-chilled eppendorf tube and centrifuged at 800 rpm for 5 min at 4°C to collect the cells in the palette. In the meantime, 1mL of radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Scientific, UK) was taken in another tube and 10 µL of the protease and phosphatase inhibitor cocktail (Pierce Biotechnology) was added into it and placed on ice. After the centrifugation step, the tube with the cell palette and a supernatant was taken out and its supernatant discarded. The cell palette was then resuspended in 200 µL of RIPA lysis buffer (now with inhibitors added) and thoroughly mixed with the help of micropipette. The cell lysate was also subjected vortex for 3 min, followed by centrifugation at 10,000 rpm for 15 min at 4°C. After centrifugation, the cell lysate was placed back on the ice, and 150 µL of supernatant (extracted protein) was transferred into a pre-chilled and labelled eppendorf tubes. This protein lysate was either further processed or quantified or stored in -80°C for future use.

Subsequently, all protein extracts were quantified using the Bradford reagent (Sigma-Aldrich, UK) against BSA as a standard.

2.4.3 Quantification of extracted protein

Protein quantification of the extracted proteins was performed with the help of Bradford assay by using Bradford assay reagent (Pierce Biotechnology) in 96 well-plate formats. Before performing the assay for the extracted protein lysates, a standard curve was established with a protein with known concentration.

The protein lysates prepared were taken out of -80°C freezer and allowed to thaw on ice. The tubes were centrifuged at 10,000 rpm for 5 min at 4°C. In the meantime, the working stock of Bradford reagent (Sigma-Aldrich, UK) was prepared by mixing 1 part in 4 parts of distilled water. After the centrifugation step, 20 µL of each protein was transferred in triplicates to each well of the 96-well plate and mixed with 180 µL of the Bradford reagent per protein sample. The protein lysate and the Bradford reagent mix was allowed to incubate at room temperature for 5-10 min. Next, the 96-well plate was placed in a spectrophotometer (Thermospectronic, USA) and its reading at 595 nm was recorded. Mean of the readings from three wells for each protein sample were taken and quantified by plotting it on the standard curve obtained by performing Bradford assay with a known standard protein **(Appendix 1, Table 9.1.4)**.

Once the concentration of each protein sample was determined, they were all mixed with (4x) lithium dodecyl sulfate (LDS) sample buffer (Nupage, Invitrogen) to the final concentration of 1x. Sample reducing agent (10x) (Nupage®Sample reducing agent, Invitrogen, UK) was added to 1x to the protein lysate boiled at 100ºC for 10-20 min then loaded straight away into the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel or stored at -20°C or -80°C until further use.

2.4.4 Processing of proteins for Immunoblotting

The quantified proteins (1 mg/mL) mixed and stored in LDS-sample buffer were taken out of -20°C freezer and allowed to thaw at room temperature. The samples were then placed on a thermomixer, heated for 15min at 95°C while

shaking. After heating the samples, they were centrifuged at 15000 rpm for 15 min. 15-20 µL (20 µg) of the supernatant of the centrifuged samples were usually loaded (unless otherwise stated) in the SDS-PAGE gels (Novex® Nupage, Invitrogen) for further analysis.

2.4.5 Immunoblotting

Proteins processed for immunoblotting were first subjected to SDS-PAGE by running the samples on precast gels (Novex® Nupage, Invitrogen) using commercially available SDS-PAGE apparatus (XCell SureLock Mini-Cell system, Invitrogen, UK), transferring them to polyvinylidene difluoride (PVDF) using a new and improved Invitrogen™ iBlot™ 2 Dry Blotting System, a fast western transfer which last for only 7 min and processing by enhanced chemiluminescence (ECL) reagent and visualizing proteins by syngene G-BOX visualising machine. For all the proteins blotted in this study, 4-12% gradient, pre-cast gels were used. Also, unless otherwise stated in figure legends, 20µg of protein was loaded per well of these gels. Two pre-cast gels (Novex® Nupage, Invitrogen) were used within the SDS-PAGE apparatus (Novex® Nupage, Invitrogen). The gel apparatus was assembled and two pre-cast gels were installed. The inserted comb plates in the gels were carefully removed and the wells gently washed with distilled water. The SDS-PAGE running buffer was poured into the inner chamber until the wells were submerged and in the outer chamber until the whole base was submerged. In the first well of each gel, 15 µL of pre-stained protein standard (Invitrogen) was loaded followed by loading the samples. The lid of the gel apparatus was fitted at its position and the cables were connected to the power pack. Gels were run at 150-200 V for 1-2 h or until the dye in the protein standard and samples, providing a measure of migration, reached the bottom of the gel. After the run was complete, the lid of the gel tank was taken off, and the gel plates were carefully taken out. The gel was very carefully taken out of the plates and immediately put in the gel trays and given a wash in the transfer buffer to remove SDS and excess buffer. While the gels still in transfer buffer, filter papers of the right size were soaked distilled water, and then all the other set up for iBlot dry transfer system are being made and then finally the gel is carefully placed on the bottom stack containing PVDF with the

support of the filter paper at the back of the gel and the used the roller to remove the bubbles. Following this, the top stack was placed on and the machine was started at 20 V for 7 min transfer.

After the transfer was completed, the machine was disassembled and the PVDF membrane transferred to the tray with the help of forceps. The PVDF membrane was washed gently with distilled water up to three times to wash away any salts and any gel residues. The membrane was examined visually for proper transfer of proteins by looking at the transfer efficiency of pre-stained protein standard. However, to better examine the transfer efficiency and band separation, the PVDF membrane was also stained with Amido black or Ponceau stain (Sigma-Aldrich UK). Next, to prevent non-specific reactivity, the membrane was blocked with either, 5% non-fat dry milk or 5% BSA prepared in 1X TBST (washing buffer), a mixture of tris-buffered saline (TBS) and Polysorbate 20 (also known as Tween 20) in based blocking buffer for 1h at room temperature with mild shaking. Primary antibodies were diluted in antibody blocking buffer according to manufacturer's instructions, typically at 1 in 1000 dilution in 8-10 mL of blocking buffer.

Following 1 h blocking, the blocking buffer was discarded off the membrane and primary antibody was added. Primary antibody incubation was performed at 4°C overnight with gentle shaking. The subsequent day, the membrane was washed three times with washing buffer, each wash for 5 min with gentle shaking. In the last washing step, 1 in 1000 dilution of anti-mouse or anti-rabbit IgG, the HRP-linked secondary antibody was made in washing buffer. The washing buffer in the last washing step was discarded from the membrane and the diluted HRB-conjugated secondary antibody was added. The membrane was incubated for 1-2 h at room temperature with mild shaking. After incubation with secondary antibody, the membrane was washed three times with washing buffer for 5 min each with gentle shaking. In the last washing step, pierce ECL 2 western blotting substrate (Thermoscientific) was taken out of fridge and equal volume each of solution A and solution B on the membrane and incubated for 1-2 min and then the membrane was taken and place on clean, clear and transparent flat bag and removed the bubbles, the membrane while inside the flat bag was taken straightaway to the gel documentation machine for the visualization of bands.

Syngene G-BOX was used to capture the images with the attached camera. The images were saved in TIFF file format for later analysis typically involving the calculation of relative abundance via integrated optical densitometry analysis of each protein band. To establish a loading control, the same blot was stripped off from previous antibodies by incubating the membrane with stripping buffer and then blocked for one hour and reprobing with the β-actin antibody.

2.4.6 Densitometry

Densitometry was calculated using Image J software and Densitometry 1 Channel plugin. All values shown are the protein of interest divided by the respective β-actin loading control value.

2.4.7 Cell viability assay

The CellTiter-Glo® 2.0 assay kit (Promega) was used to determine cell viability, as described by the manufacturer. Briefly, cells were seeded in a luminometer compatible 96-well plate and allowed to adhere for 18-24 h. Following treatments using different concentrations of the compounds, the plate and its contents were equilibrated to room temperature for approximately 30 min, a volume of CellTiter-Glo 2.0 reagent equal to the volume of cell culture medium present in each well the contents were then mixed for 2 min on an orbital shaker to induce cell lysis and the plate was then incubated at room temperature for 10 min to stabilize the luminescent signal and finally the luminescence was recorded using luminometer (MODULUS, Promega). The luminescent signal is proportional to the amount of ATP in the sample, which indicates the presence of metabolically active cells.

2.4.8 siRNA transfection

siRNA was used to knockdown NRF2 (Qiagen). For siRNA transfection, cells were seeded in triplicate either in 96-well plates in triplicate (2×10^4) or in 24-well plates (0.5×10^5 cells) or in 60 mm plates (0.5×10^6 cells), and allowed to grow for 24 h. Following this, cells were co transfected using either 7 pmol of siRNA (96-well plate) or 20 pmol siRNA and 1 µg of different PGL3 promoter constructs (24-well plates) or 75 pmol siRNA only (60 mm plates) or and incubated for a further 24 h or 48 h or as required. Cells transfected in 96 well plates were

processed for either cytotoxicity assay, ROS assay or total GSH assay, those in 24-well plate were further processed for dual luciferase assay while those in 60 mm plates were harvested for immunoblotting. In all cases, scrambled siRNA was used as a control while transfection was performed using Lipofectamine 3000 (Life Technologies) according to manufacturer's instructions.

2.4.9 Luciferase activity assay

Luciferase activity was measured using the Luciferase Assay System (Promega), according to the manufacturer's instructions to determine NRF2 dependent transcriptional antioxidant response following different treatments, stable clones of MCF7 cells carrying PGL3 vector with a cloned 8 copies of cis- AREs reporter construct (AREc32). Briefly, MCF7-AREc32 cells were seeded in luminometer-compatible 96 well plates at a density of 1.5×10^4 cells per well and allowed to attach for 18 h. Next day, cells were washed with pre-warmed PBS and 100 µL of media containing the required treatments was added and further allowed to incubate for 24 h, at the end of treatment, cells in each well of a 96-well plate were washed with PBS and lysed in 30 µL of the lysis buffer provided with the kit. A 5 µL portion of the lysate was mixed with 25 µL of Luciferase assay reagent and the luminescence was quantified (McMahon et al., 2014) using luminometer (MODULUSTM, Promega). To normalise the luminescence signal, 2 µL portion of the lysate was mixed with 98 µL Bradford reagent assay to estimate protein content using to Bradford assay.

2.4.10 ROS Detection

ROS detection assay was performed by using 2′,7′-Dichlorofluorescin diacetate (DCFDA) staining (Sigma). Briefly, cells were seeded in triplicate at a density of 0.2×10^5 cells/well of opaque flat-bottom 96-well tissue culture plates in 100 µL media without phenol red and allowed to grow for 18 h. Following this, cells were either left untreated or treated at a desired time points. A 50 mM stock solution of DCFDA was added to each well containing 100 µL pre-existing media to achieve a final concentration of 25 µM and incubated for 45 min at 37°C. Fluorescence signal intensities indicating ROS levels were recorded by taking readings using 96-well fluorescent multiplate reader (MODULUS, Promega) using excitation and

emission spectra of 485 nm/535 nm. To normalise the fluorescence signal, cells in the same wells were stained with Coomassie brilliant blue stain (Sigma) for 1 h and washed with distilled water and 10% SDS solution was added to release the absorbed dye for 10 min while shaking. The absorbance values at 595 nm were then recorded using a multiplate absorbance reader (MODULUS, Promega) data used after normalising the fluorescence values.

2.4.11 Dual-Luciferase-based promoter Assay

To perform Dual-Luciferase based HER family promoter assay, the HER1 and HER4 promoter region was first amplified from the human genomic DNA using promoter-specific primers, cloned into the luciferase reporter vector, PGL-3 basic, and transfected into human epithelial cell lines, PEO1, OVCAR3 and SKOV3 (For all the data of HER1 and HER4 promoter cloning, see CHAPTER 3). Transfection of the HER1 and HER4 promoter reporter vector was carried out using Lipofectamine 3000 (Thermoscientific, UK) and the vector pRL-CMV was used as an internal control for transfection efficiency. Each transfection for a particular experiment was performed in triplicates. Transfection was carried out in 24-well format by first seeding the cells at a density of 5 x 10^4 for 24 h prior to transfection. At the time of transfection, the cells were 70-90% confluent.

Old media was removed and cells were gently rinsed with 1 mL of pre-warmed PBS. 0.5 mL of serum free and antibiotic free media (Opti-MEM®, Invitrogen, UK) was added per well and the plate placed back in the incubator. For transfection, the DNA to be transfected i.e. pHER1/PGL3 and prHER4/PGL3, the empty PGL3 basic vector and the internal control vector (pRL-CMV) and the transfection reagent (Lipofectamine 3000, Invitrogen, UK) were taken out and placed in the hood. For each well, 0.5 µg of DNA to be transfected (either prHER1/PGL3 or prHER4 or empty PGL3 basic vector) and 0.1 µg of pRL-CMV were mixed together with 25 µL of serum free and antibiotic free media Opti-MEM®. Next, 1 µL of the transfection reagent was mixed with 25 µL of Opti-MEM® in a separate eppendorf tube. The tubes were incubated for 5 min and their contents mixed together and further incubated for 20 min to allow the DNA complex to form.

After the incubation, 0.4 mL of Opti-MEM® was added to the tube containing the DNA complex and gently mixed together. The contents of this tube were added to each well and the plate incubated for 5 h for transfection to take place. After incubation, the old media was removed and 0.5 mL of pre-warmed cell culture media was added and plates incubated for at least 16 h before further experiments and analysis.

After 16-24 h of transfection, the old media was discarded, cells rinsed once with pre-warmed PBS and treatments with the required drug solutions in media were performed. On the day of performing the luciferase assay, 1x passive cell lysis buffer (Dual Luciferase assay kit, Promega, UK) was prepared. The 24-well plate was taken out of incubator, old media discarded, each well gently rinsed with 0.5 mL of PBS, and 125 µL of the 1x passive lysis buffer added to each well. The plates were placed on the shaker and gently rocked (50 rpm) for 30 min to form the cellular protein lysates. In the meantime, the Luciferase assay reagent (LAR-II, Promega) was reconstituted and by means of the multi-channel pipette for faster and accurate pipetting, 50 µL of this reconstituted reagent was added per well of black bottom, opaque 96-well plate (Sigma-Aldrich). The LAR-II reagent provided a substrate of luciferase activity generating a stabilised luminescent signal with the addition of the cell lysate.

Next, 20 µL of the cell lysates from each transfection (done in triplicates) were transferred (in quadruplets) to each well of the 96-well plate with the added LAR-II reagent with the help of multi-channel pipette. Hence, four independent readings were taken in 96-well plate for each transfection done in triplicates. After the addition of cell lysate, the plate was immediately placed in the 96-well Luminometer (Modulus template® Turner biosystems) for measuring the luminescence.

Dual luciferase assay program was set up with 2 s of pre-measurement delay followed by measuring luminescence via the inbuilt luminescence filter at 0.5 second measurement period per well. After the reading, the plate was taken out and 50 µL of the reconstituted Stop and Glo® was added per well using a multichannel pipette. The addition of the second reagent stopped the luminescence generated by Firefly luciferase (sourced from prHER1/PGL3 or

prHER4/PGL3) and provided a substrate for Renilla luciferase (sourced from pRL-CMV). The plate was returned to the luminometer and another reading was taken as above. The first reading (After LAR-II addition) was normalised to the second reading (after Stop and Glo® addition). The means of the luminescence signals acquired from the quadruplet treatments were taken, with each transfection done in triplicates. The mean values were normalised to the mean value of untreated controls for that experiment and expressed as 1.

2.4.12 Measurement of Total Glutathione (GSH)

The measurement of total GSH was performed using GSH/GSSG-Glo™ Assay (Promega) according to manufacturer's protocols. Briefly $1x10^4$ cells where seeded in luminometer-compatible 96-well plates and allow to grow overnight at 37°C in a 5% CO_2 culture incubator. The following day, the cells were treated accordingly. Following this, the contents in the cell were removed. Then 50 µL per well of GSH Lysis Reagent, that was prepared no longer than 30 min before use, was added. The lysate was shaken at room temperature for 5 min on a plate shaker. Following this, 50 µL per well of Luciferin Generation Reagent was added to all wells. The Luciferin Generation Reagent was prepared within 30 min before use. The plates were then shaken briefly and incubated at room temperature for 30 min. Finally, 100 µL per well of Luciferin Detection Reagent was added and the plates were shaken briefly, waited for 15 min to stabilize the luminescent signal and finally the luminescence was recorded using luminometer (MODULUS, Promega).

2.4.13 Statistical Analysis

All statistical analyses were performed using GraphPad Prism Software version 6. Test for normality of data was determined by Shapiro-Wilk and D'Agostino-Pearson tests. The significance (value) of differences of pooled results was determined by independent t-tests, one-way or two-way analysis of variance (ANOVA) followed by post hoc Tukey's tests. The reason for choosing these types of analysis in this study is that, the study wanted to find out the differences between two unrelated groups or the means of three or more independent (unrelated) groups or compares the mean differences between groups that have

been split on two independent variables so as understand if there is an interaction between the two independent variables on the dependent variable, hence the choice of the independent t-test (also called the two sample t-test), one-way ANOVA and two-way ANOVA respectively. Independent t-test is an inferential statistical test that determines whether there is a statistically significant difference between the means in two unrelated groups. The one-way ANOVA is used to determine whether there are any statistically significant differences between the means of three or more independent (unrelated) groups. It compares the means between the groups that are of interest and determines whether any of those means are statistically significantly different from each other. It is important to note that the one-way ANOVA is an omnibus test statistic and cannot tell which specific groups were statistically significantly different from each other only that at least two groups were. To determine which specific groups differed from each other post hoc tests need to be applied. Post hoc tests attempt to control the experiment wise error rate (usually alpha = 0.05) in the same manner that the one-way ANOVA is used instead of multiple t-tests. Post hoc tests are termed a posteriori tests that are performed after the events; the events in this case are after independent t-test and one-way ANOVA. The two-way analysis of variance is an extension to the one-way analysis of variance. The Assumptions in two-way ANOVA are when the populations from which the samples were obtained are normally or approximately normally distributed, the samples are independent, the variances of the populations are equal and the groups must have the same sample size. Data presented are the means ± S.D. of n = 3 independent experiments performed either in triplicates or in quadruplicates. Significant difference was defined as (^{ns}: P > 0.05*: p<0.05, **: p<0.01, ***: p<0.001 and ****: p<0.0001), ns= not statistically different, *= statistically significant (*< **<***<****). For western blots, images are representative of at least two independent experiments for each condition.

2.4.14 Imaging and Analysis

Images were camera captured using Syngene G-BOX Chemi-XX6 Gel Documentation System (Synoptics, UK). Syngene has unique flatfix technology which eliminates saturation from images by using an Auto Aperture feature. The

images were saved in TIFF file format and analysed typically involving the calculation of relative abundance via integrated optical densitometry analysis of each protein band. Densitometry was calculated using Image J software and Densitometry 1 Channel plugin (NIH, USA). The band intensities of all the untreated/scrambled siRNA and treatment groups were normalized on the band intensities of their corresponding β-actin loading controls. The normalized data were then processed into bar charts and presented for easy interpretation.

3. CHAPTER THREE

Cloning of the Proximal Promoter Regions of Human *HER1* and *HER4* Genes

3.1 Abstract

Human HER1 and HER4 are members of the HER family receptors, including HER2 and HER3. The HER receptor family of RTKs are one of the most extensively studied for their role in aetiology and development, human cancers. The cloning of proximal regulatory regions of *HER1* and *HER4* genes is reported here. Approximately 1.5 kb and 1.3 kb DNA fragments encompassing the proximal promoters of *HER1* and *HER4* genes, respectively, were each placed upstream of the luciferase reporter gene in the pGL3 vector to be prHER1 and prHER4, respectively. These were used to transiently transfect a panel of ovarian cell lines (OVCAR3, PEO1 and SKOV3), in order to examine their constitutive expression. Both prHER1 and prHER4 showed a high and differential basal level of expressions in all the cell lines tested when compared to the control pGL3 vector. This suggests the functionality of the promoter constructs and their feasibility as tools to study the regulation of expression of *HER1* and HER4 receptors.

3.2 Introduction

The uniquely close relationship between the members HER receptor family suggests that they have evolved from a common ancestral gene. All the genes have been linked to cancer transformation. Both HER1 and HER4 are the cellular homolog of the oncogene v-erbBB and are all reported to be overexpressed in cancers including ovarian. The majority of HER1 and HER4 activities involves transcriptional activation, tumour cell proliferation, growth suppression (Jones, 2008, Sheng and Liu, 2011).The analysis of mechanisms controlling the regulation of expression of genes for growth factor receptors is therefore important for understanding of the malignant state (Tal et al., 1987).

Studies have reported that identification of promoter sequences and transcription factors essential for transcriptional regulation of the gene may greatly contribute to understanding its molecular mechanisms of regulation (Takakura et al., 1999, Li et al., 2010). Expression of a target gene in both prokaryotic cell and eukaryotic cell has been a common research goal of researches of genes of interest (Cao et

al., 2017), since initiation of the genetic engineering by Herbert Boyer and Stanley N. Cohen in 1972 (Cohen et al., 1973). PCR has been essential for gene cloning because it can provide target gene fragments in vitro in large amount and short time without the limitation of restriction sites. The established strategy for the construction of recombinant expression vector includes two cloning steps: PCR cloning (cloning of PCR amplified target gene into cloning vector) and restriction enzyme mediated sub-cloning of the target gene from cloning vector into expression vector (Cao et al., 2017). Here in this chapter, the core proximal promoter regions of HER1 and HER4 genes were cloned and their activity was examined in ovarian cancer cells. Thus, the study in this chapter was undertaken to address the following aim and objectives.

3.3 Aims and objectives

3.3.1 The aim

The main aim of this chapter is to clone human HER1 and HER4 gene proximal promoters as gene reporter systems.

3.3.2 The objectives are:

1. To retrieve approximately 1.5 kb and 1.3 kb of the proximal promoter regions of human HER1 and HER4 genes respectively
2. To analyse and identify the putative NRF2 binding sites present in each promoter
3. To design the primers and then clone the promoter regions of human HER1 and HER4 genes
4. To generate functional HER1 and HER4 proximal DNA promoter gene reporter constructs as novel tools for the study of the transcriptional regulation of HER1 and HER4.

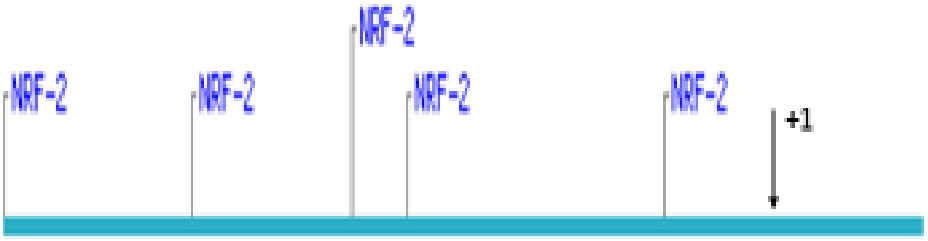
The generation of functional proximal core promoters of human HER1 and HER4 gene reporter constructs would be valuable novel tools in examining NRF2 as an important transcriptional regulator of the HER family genes in ovarian cancers. The findings of such a study may partly explain the overexpression of HER family receptors in cancer and may have implications to the success and/or failure of

HER targeted therapies involving RTK inhibitors. Chemotherapies are limited when tumour cells circumvent action of therapeutic agents' due to the readjustments in coexpression of the receptors, their ligand binding dynamics, or changing preference for the dimerizing partner (Nagumo et al., 2009a, Goltsov et al., 2014b) suggest that the anticancer effect of these agents might be better predicted by effectively limiting HER receptor family expression at the DNA level or at least identifying a common regulatory centre of their transcription. Thus, the identification of factors such as NRF2 that coordinately mediate or modulate the transcriptional expression of HER receptors will be vital (Khalil et al., 2016b) . Consequently, the functionality of the promoter constructs was examined for basal activity, when the cloned promoters were transiently transfected in a panel of ovarian cell lines with a differential basal level of NRF2, PEO1>SKOV3>OVCAR3 (Khalil et al., 2015).

3.4 Results

3.4.1 Putative NRF2 transcription factor binding sites found along 5' upstream region of in promoters HER1 and HER4 at 75% cut off score

Approximately 1.5 kb and 1.3 kb of the proximal promoter regions of HER1 and HER4 gene sequences respectively were retrieved from Ensembl Genome database (http://www.ensembl.org/index.html). The sequences were subjected to bioinformatics analysis (Figure 3.1) insilico; (http://consite.genereg.net/cgi-bin/consite) (Sandelin et al., 2004) to ascertain the putative NRF2 binding sites present in each of the promoters. The sequence was analysed at 75% stringency. The 75% stringency was chosen as a fine balance between the identification of a putative and likely functional binding sites as against the possibility of predicting false positive or non-functional putative NRF2 binding at lower stringency.



Transcription factor	Human_IR				
	Sequence	From	To	Score	Strand
NRF-2	CACTCCAGGT	1	10	6.292	-
NRF-2	AAATTCCTGT	321	330	6.335	-
NRF-2	GCAGGAAGCA	591	600	6.091	+
NRF-2	AAAGGAACAG	683	692	6.514	+
NRF-2	AGCTTCCGCG	1119	1128	7.655	-

1.5kb HER1 promoter

```
   1 CACTCCAGGT ACTAGCCAAG GACTACAAAA TCAACGTGAA TGTCAGCTTT
  51 TGTATCAAAA GCTCAAAGGA GAAACTCAAA CTTTACATAG ATGTCCCATG
 101 AAGATGTTCA GCAAACCCAT TCTTCTCTGT TCCCTGGAAT CCATCCCAGT
 151 ATTGTGCTAT GTGTGTGTCT AGTAATTCTT TACAAAAAGC TCTGTTTCTT
 201 GTGATGCTAT CAGATCACAT TGAAGAATAT ACAAGCCGTA CTATGAAGGC
 251 TGTTGTCTCA TATAGTCCTA ACGTAGTGAG AACTGATGTT CTTACATGCT
 301 GTCTTTTTGG GCACTCAAAG AAATTCCTGT ACAGTCTTAC AAATCAGTTG
 351 TAGCTTAAAT TGATTTGTGT TGTGACTTGT ACACACAGGT CACATTCCCT
 401 TGACAGAAAA TATAGTTTAA AACCAAATTT GCAGCCCTTG TTAAGTGAAT
 451 GCACAGGACT TTATTGTATT CAGGTCTTTT ATTGTAAGAC TCACTCCTGT
 501 CTTCATTTTA TGTTCCACTG TTGTGCTTCC CATTTGCCTT TCTCTAGTTT
 551 TGTTTTCTGT GTTTCTACGG ACTGCTCTCA GCCCAGGTGT GCAGGAAGCA
 601 CACACATGCC TGCAGAGCCT TCATGGCCTC TGCATTCAGG GCATGACTTC
 651 AACGCACAGT GGCTGTACTG ATTTGTTAAA ACAAAGGAAC AGATTACTTC
 701 TCCTAATTCA CAGGGAAGTT CCAGGTTGTG CGGGCAGTGA GCAGACCTGT
 751 GTCTGTCTGC GCTTGCCCTG GTGAAAAACC CCACCGTTCA GGCTGCAGGG
 801 TGCGAGACCC AGGCACAAAC ATTTTGCTGG ATGAGGAGGA AAGATGTAAG
 851 GTTGCTCCCC TTCAGAGACA GCAAAGGGCA GGTCTGTAGC TTCACTTACT
 901 TCAGGATTGT GATTTTTGAC AGAGCCGAGA GATCAGGGTT GTTGAACCAG
 951 GCCTGAAGGT CCTAGTGAAT CTCGTGAAGA GAGGAGGGGT CTGGCTGTAA
1001 CATGGACCTA GAGGACATTT TTACTGCAGG AGAAGGAACA GTGGGGATGG
1051 GGTGGACTTG CCAAAGGAAT ATAGCTCAAG TTCCTGCAGC CCAAAAAAGC
1101 TCAGTTTCTT TTGGCCAAAG CTTCCGCGAG TTTCCCTGGC ATTTCTCCTG
1151 CGGGAGCTAC AGGGGCAGTG GGACACTTAG CCTCTCTAAA AGCACCTCCA
1201 CGGCTGTTTG TGTCAAGCCT TTATTCCAAG AGCTTCACTT TTGCGAAGTA
1251 ATGTGCTTCA CACATTGGCT TCAAAGTACC CATGGCTGGT TGCAATAAAC
1301 ATTAAGGAGG CCTGTCTCTG CACCCGGAGT TGGGTGCCCT CATTTCAGAT
1351 GATTTCGAGG GTGCTTGACA AGATCTGAAG GACCCTCGGA CTTTAGAGCA
1401 CCACCTCGGA CGCCTGGCAC CCCTGCCGCG CGGGCACGGC GACCTCCTCA
1451 GCTGCCAGGC CAGCCTCTGA TCCCCGAGAG GGTCCCGTAG TGCTGCAGG
```

(a) Putative transcription factor binding sites found along *HER1* promoter

Transcription factor	Human_IR				
	Sequence	From	To	Score	Strand
NRF-2	CAAGGAAGGG	334	343	7.140	+
NRF-2	TCCGTAAGCG	875	884	6.049	+
NRF-2	GAGGGAAGAC	939	948	6.142	+
NRF-2	AGCTTCCCCT	989	998	6.091	-

1.3kb HER4 promoter

```
   1 GAGTGGGAAA TGGAGATCAA GGTCTCAAAT GAAAGGCAGG GTGGTTTGAT
  51 TTTTAAGCTC CCCTGATACT TTTTATTTAT ATATTTGCTG CTTCTAGAAA
 101 AACCAAAAAA GGAAAGTGAT TCATACATAG AATGCCTGTA CAAATAGCAA
 151 ACCAATAACT ACCCCCAGCA GATTCTGGCC CTTGCCAGGG TGCGGTTTCA
 201 GAGCTGTAAA AGGCAAGGGA TATGTACATT GAAATGACCC AGTCCCAGAA
 251 TATTTTAGAT TCAGAAAAGG TCAGAGACTT TCGTAACTAG CAATGACCTC
 301 CAGATTAGGG ACCATGAATG TGCAGAGTGA GGACAAGGAA GGGGCCTAAA
 351 GCTGGGTCTA CTGTAGAATA AACAGAATCA TTTAAGGTTA ATTATGTTCT
 401 GATGTGGCCT CCTTAAATTC TTTCAGGCTG GGGACAATTT CTAGCCCTTT
 451 AAGATCAAAT CAGTGGCTTT GGACTAGAAT CTGCTTAATA AGAATGAACT
 501 CCAAAGGTGA AAAAAAAACA TAAAATCTAA AAATCAAAGG ATATGCCTTT
 551 ATAAATGTGA AAGTTTAGTT TGGCTCAATG TTGGTCAGTT TAGAAATGTG
 601 GGAGTAATCG GAGAGATGGC ATAACTGGGG GCGGGAGGCG GGGCGGTGAT
 651 TATACCCCTA CCACTACCTA ATCCTCAGGG GCTACCAGTT ACTTGCTGTA
 701 CACACTTTTA AAGTACTTTC TAATTGTGGA CCATGAGTGG CAGAGTACTG
 751 CGGGCGGAGG CTTTTTGCTG AAAGCAACAT TGACTTTTAT TTCTAGGGAA
 801 AGTCTTGTCT GTAGATGAGC ACCGTGGGGC AACGGAGAAA ATGCTGACTT
 851 TCTTGTTTAA AAAAAAAAAA AATTTCCGTA AGCGGCCCTC CAATGGTCCG
 901 TCCACTCAGC AGGCCAGCTG TCCCAGGCCT CTGCCAGGGA GGGAAGACGA
 951 GTGCTTCAGT GCATGGCTTC GGTGAGCCCT TTAAATCCAG CTTCCCCTGC
1001 CCCCACCAAG AGGCTCCCTT TCCCTGACAG GTGTTTTCCC AACTCCGGAG
1051 GCCCAGGATT TTGGGGAGTG GCTGAGCCAA TAAGATTTCC CTTTGGAAGG
1101 GGGGGGGAAG GGCGTGGCTA GTTGGGTCAG AGCATGGGCG GGGTTTGCGC
1151 GGCTGAGCAG AGCCGGCCTG GTTGCGTGGA GGGTTGTTTT ATTCCCACCG
1201 CCCCCCGCAC CTTTTTTTTT TTTTTTTCTG GAGTCTTATT AATTTCTCTG
1251 TGGGCTGCAG CTGGAGACCG CGGAGCGCTG GAAATGACGC TCGGAGCTTT
1301 AATTACCGCA GCCGCCGGAC AAGTGTGAGG AAAGCTGAGA G
```

b. Putative transcription factor binding sites found along *HER4* promoter

Figure 3.1: An in-silico analysis of HER1 and HER4 promoter sequences. (a) 1.5 kb promoter region of HER1 gene was obtained from the database (ensemble.org) and subjected to transcriptional factor binding prediction program (http://consite.genereg.net/cgi-bin/consite) to predict for putative NRF2 binding sites as indicated. (b) The same analyses were performed for HER4 promoter. In (a) and (b), +1 indicate the transcriptional start site (predicted from (http://www.cbs.dtu.dk/services/Promoter/) sequences highlighted in blue show NRF2 binding sites as predicted by ConSite, and sequences in bold brown represent transcription start sites. The sequence was formatted using EMBOSS Seqret at (http://www.ebi.ac.uk/Tools/services)

3.4.2 Designing primers for PCR

Following the in-silico analysis where the putative NRF2 binding site was analysed from the promoter sequence of the *HER1* and *HER4* gene, specific primers to amplify this region were designed and analysed for physical properties using primer 3.0 (https://www.ncbi.nlm.nih.gov/gquery/?term=primer+blast) and OligoAnalyzer 3.1((https://eu.idtdna.com/calc/analyzer). In order to maximise the chance of capturing the promoter elements upstream of the promoters, the reverse primer was constrained to be within the 5' UTR and situated as close as possible to the transcription start codon. The forward primers were constrained to be within a tRNAlys sequence approximately 1500 bp 5' of *HER1* and 1300 bp 5' of *HER4* genes, respectively. In order to ensure primer specificity, stringent primer design parameters were utilised. The Nucleotide BLAST tool from Human Genome at National Centre for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/projects/genome/guide/human/) was used to perform a genome-wide homology searches and alignment.

HER1 promoter primers:

Forward: CACTCCAGGTACTAGCCAAGG

GC: 57.1, Tm: 56.8

Kpn1 forward: GG-GGTACC- CACTCCAGGTACTAGCCAAGG

Reverse: GTG CCA TTA TCC GAC GCT G

GC: 57.9, Tm: 56.4

XhoI Reverse: GTG CCA TTA TCC GAC GCT G-CTCGAG-CGG

HER4 Promoter Primers:

Forward: GAGTGGGAAATGGAGATCAAGGTC

GC: 50, Tm: 57.2

XhoI Forward: CCG- CTCGAG-GAGTGGGAAATGGAGATCAAGGTC

Reverse: GGA CAA GTG TGA GGA AAG CTG AGA G

GC: 52, Tm: 59.2

NcoI Reverse: GGA CAA GTG TGA GGA AAG CTG AGA G-CCATGG-CATG

3.4.3 Polymerase chain reaction (PCR)

Human genomic DNA was used as a template to obtain PCR amplified products using the specific primers designed for *HER1* and *HER4* genes. The expected size 1.5 kb and 1.3 kb (Figure 3.2) were extracted and gel purified using the Qiagen extraction kit. The PCR products were run and extracted from agarose gel (Qiagen), digested using the restriction enzymes (Promega) *Kpn1* and *XhoI* for HER1 and *XhoI* and *NcoI* for HER4, and ligated into PGL3 vector (Promega) to create HER1 and HER4 promoter constructs prHER1 and prHER4 respectively. These are HER1 and HER4 gene reporter constructs driving the expression of luciferase gene for utilisation in dual luciferase reporter assay (Promega) (Figure 3.3). More details of the cloning, verification and evaluation of the functionality of these gene reporter constructs are given in next following sections.

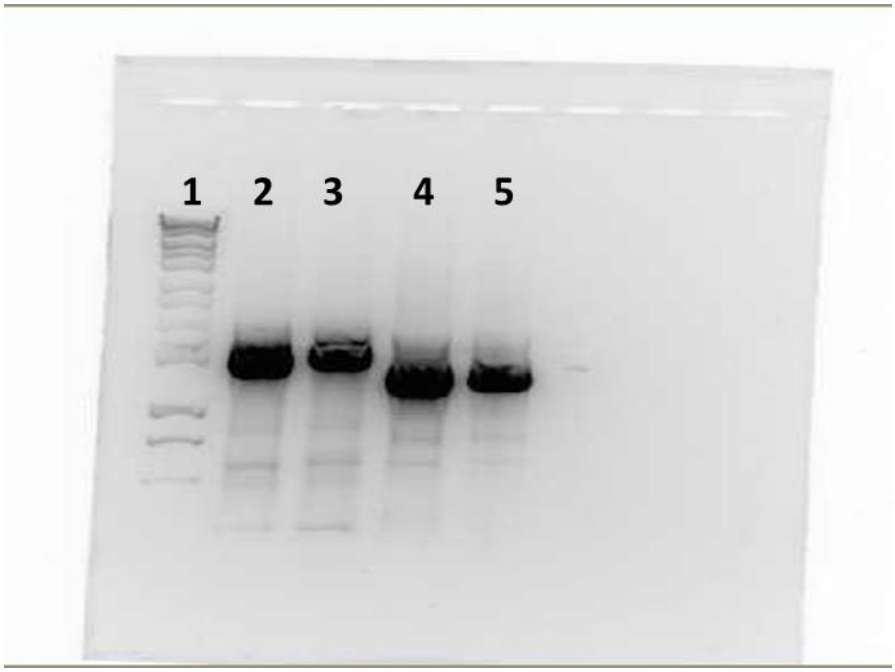


Figure 3.2: A 1% agarose gel purification of amplified prHER1 and HER4. 25 µL of PCR products were run on gel and 5 µL of DNA hyperLadder 1 kb was used, the gel was run at 100 V for 1 h (Note: lane 1 is a ladder, 2 and 3 are for prHER1 and 4 and 5 are for prHER4)

3.4.4 Excision of HER1 1.5 kb and HER4 1.3 kb gene promoter sequence bands from Agarose Gel

The amplified *HER1* and *HER4* gene promoter fragments were excised from their respective gels, and subsequently weighed prior to gel purification, in accordance with the QIAquick Gel Extraction Kit Protocol according to manufacturer's instructions and as described in the (Chapter 2)

3.4.5 Ligation, transformation, clonal selection, glycerol stock and Miniprep of pGem-T Easy-HER1 1.5 kb and pGem-T Easy-HER4 1.3 kb

In order to explore and propagate the cloning of (*HER1* 1.5 kb and *HER4* 1.3 kb) PCR product, the promoter sequences were respectively ligated into the pGEM-T-Easy Cloning Vector (Promega) (Figure 3.3) in accordance with the methodology. Successful growth of bacterial colonies following bacterial transformation (with the respective ligation mixtures), spread plating and 24 h of incubation were observed. Both blue and white colonies were present. White colonies denoted successful ligations as transformed bacteria that have assimilated the pGEM-T Easy Vector (Promega) contain the respective insert DNA. Blue colonies denoted unsuccessful ligations as transformed bacteria that have assimilated the pGEM-T Easy Vector (Promega) do not contain the respective insert DNA.

A single white bacterial colony with respect to each transformation was inoculated into 15 ml centrifuge tube (Corning Incorporated) containing 5 ml of LB-Media with Ampicillin selection. Inoculated cultures were grown in a shaking incubator at 37^{o}C for 24 h. Following 24 h of incubation, duplicate glycerol was made and stored at -80^{o}C in accordance with the protocol. Remaining bacterial culture was used to isolate and purify high-quality plasmid DNA from the bacteria using Miniprep (Isolate Plasmid Mini Kit - Bioline).

3.4.6 Restriction digests of pGem-T Easy-HER1.5 kb and pGem-T Easy-HER4 1.3 kb constructs and DNA sequencing

The restriction digest was carried out on the Miniprep DNA samples to further verify the successful ligation and cloning of the gel purified DNA fragments of

HER1 and *HER4* genes. The *EcoR1* restriction enzyme (Promega) was used to perform a single enzyme digest of the pGEM-T Easy-HER1 and -HER4 gene constructs. This was possible owing to multiple EcoR1 recognition sites on both sides of the multiple cloning regions in the pGEM-T Easy Vector. Figures 3.4A and 3.4B show successful restriction digest. Linearised pGEM-T-Easy vector fragments are clearly visible on the agarose gel.

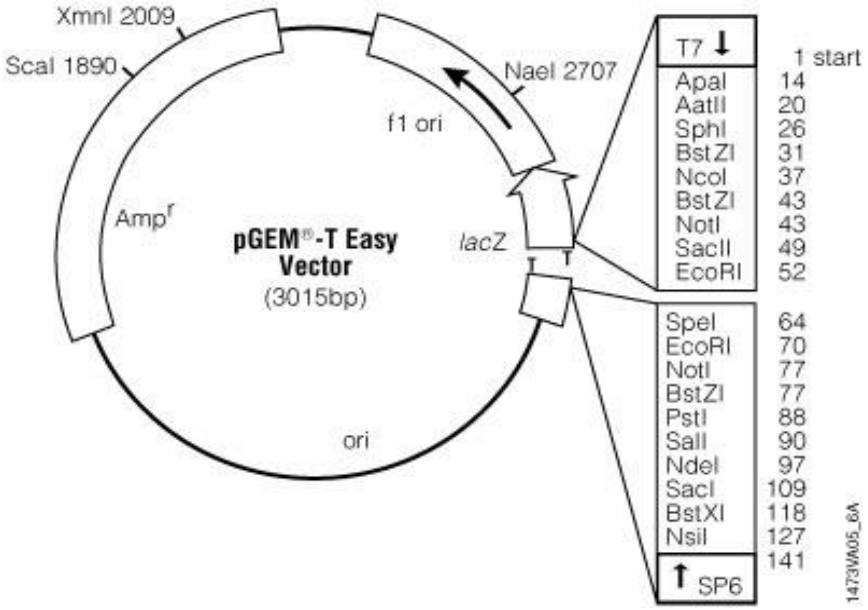


Figure 3.3: pGEM®-T Vector Map and Sequence Reference Points. pGEM®-T Easy Vectors which are linearized vectors with a single 3´-terminal thymidine at both ends. The T-overhangs at the insertion site greatly improve the efficiency of ligation of PCR products by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases (Maps)

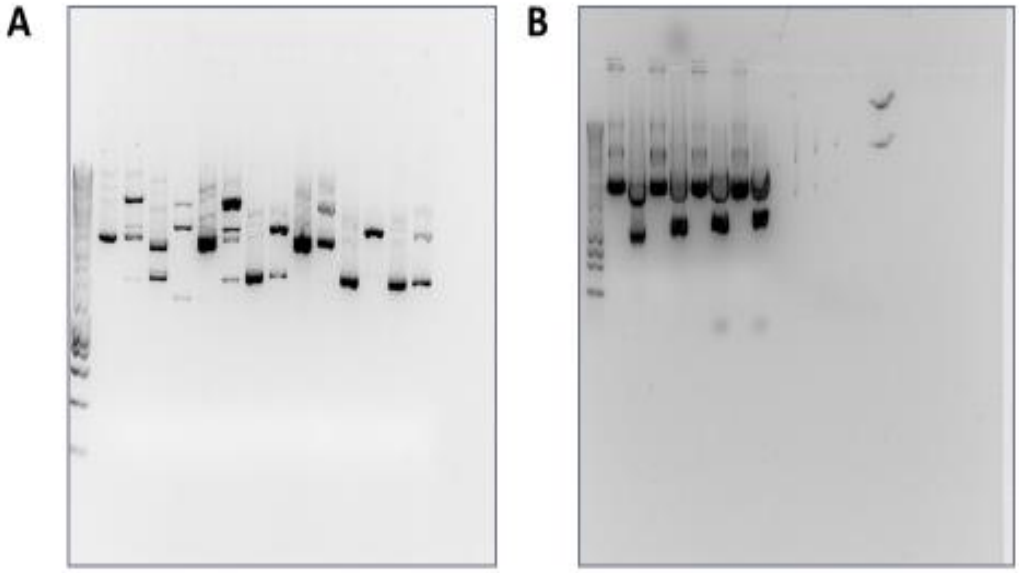


Figure 3.4: EcoR1 restrictions digest of purified pGEM-T Easy-HER1 and HER4 (1.5 kb and 1.3 kb) plasmid DNA. Gel was ran for 45 min at 80 volts (a) Lane 1, is 2 µL of 10 kb DNA Hyper ladder I (Bioline). Lanes 3, 5, 7 etc, 5 µL of pGEM-T Easy-HER1 1.5 kb restriction digest mixture. (b) Lane 1, is 2 µL of 10 kb DNA Hyperladder I (Bioline). Lanes 3, 5, 7 and 9, are 5 µL of pGEM-T Easy-HER4 1.3 kb restriction digest mixture.

Thus, the successful restriction digest of the Miniprep samples signified that insert ligation into pGEM-T Easy Vector (Promega) was also successful.

3.4.7 Restriction digests of PGL3 prHER1.5 kb and prHER4 1.3 kb for sub-clone to PGL3 vector

The restriction digest was carried out on the DNA samples of PGL3, prHER1 and prHER4 in order create to a sticky end compatible for its respective PGL3 vectors. HER1 promoter was digested using KpnI and XhoI restriction enzymes at the 5' and 3' ends of the HER1 promoter respectively and HER4 was digested using XhoI and NcoI restriction enzymes at the 5' and 3' ends of the HER4 promoter respectively. Two sets of PGL3 vector were digested with either KpnI and XhoI restriction enzymes suitable for ligating HER promoter or XhoI and NcoI restriction enzymes suitable for ligating HER4 promoter successful ligation and cloning. It is worth knowing that PGL3 vector (Promega) contain all these restriction enzymes used (Figure 3.5)

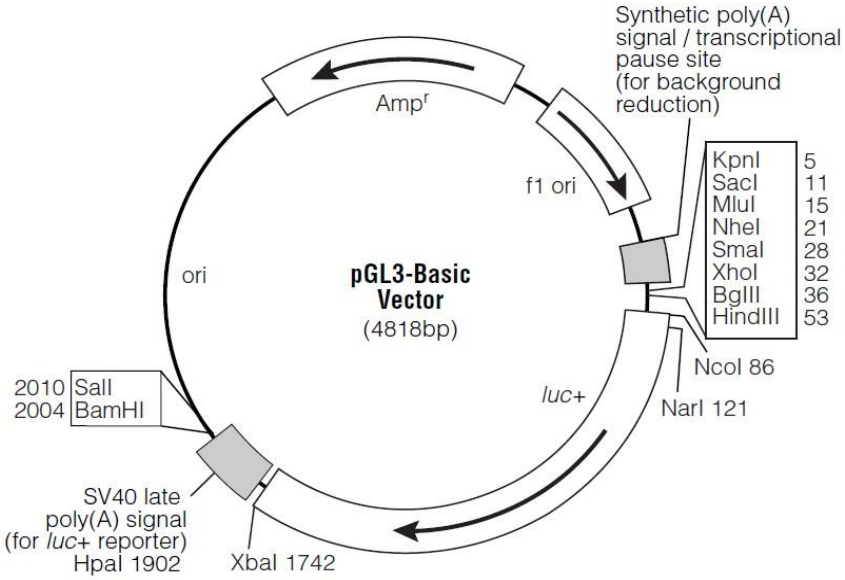


Figure 3.5. PGL3-Basic Vector circle map. Additional description: luc+, cDNA encoding the modified firefly luciferase: Ampr, gene conferring ampicillin resistance in E. coli; f1 ori, origin of replication derived from filamentous phage; ori, origin of replication in E. coli. Arrows within luc+ and the Ampr gene indicate the direction of transcription; the arrow in the f1 ori indicates the direction of ssDNA strand synthesis. (Figure from Promega)

3.4.8 Ligation, transformation, clonal selection, glycerol stock and Miniprep of PGL3-HER1 1.5 kb and PGL3-HER4 1.3 kb

In order to explore and propagate the cloning of (*HER1* 1.5 kb and *HER4* 1.3 kb) PCR product, the promoter sequences were respectively ligated into the PGL3 cloning vector (Promega) in accordance with the methodology. Successful growth of bacterial colonies following bacterial transformation (with the respective ligation mixtures), spread plating and 24 h of incubation were observed. A single bacterial colony with respect to each transformation was inoculated into 15 ml centrifuge tube (Corning Incorporated) containing 5 ml of TB-Media with Ampicillin selection. Inoculated cultures were grown in a shaking incubator at 37^{o}C for 24 h. Following 24 h of incubation, duplicate glycerol was made and stored at -80^{o}C in accordance with the protocol. Remaining bacterial culture was used to isolate and purify high-quality plasmid DNA from the bacteria using Miniprep (Isolate Plasmid Mini Kit - Bioline).

Subsequently, the successful restriction digest of the Miniprep samples (Figure 3.6) signified that insert ligation into PGL3 vector (Promega) was successful. Therefore, two eppendorfs each containing 10 µL elution buffer and a 5 µL

Miniprep sample (HER1 1.5 kb HER4 1.3 kb) were prepared and sequenced (DNA Sequencing and Services, College of Life Sciences, University of Dundee, Scotland). Following this, the Nucleotide BLAST tool from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cg) was used to perform a genome-wide homology search to verify the cloned and sequenced gene promoter (HER1 1.5 kb and HER4 1.3 kb) as being HER1 and HER1.

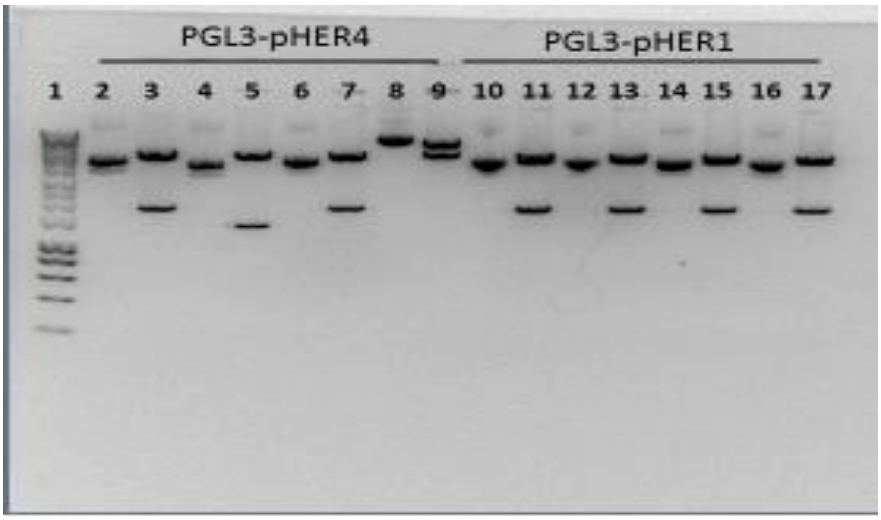


Figure 3.6: Restrictions digest of purified PGL3-HER1 and PGL3- HER4 (1.5 kb and 1.3 kb) plasmid DNA. Gel was ran 45 min at 80 volts (a) Lane 1, is 2 µL of 10 kb DNA Hyper ladder I (Bioline). Lanes 2-9 are for PGL3-pHER4, digested with NcoI/XhoI and DNA in lane 5 i.e the digested of lane 4) was chosen as the successful clone. Lanes 10-17 are for PGL3-pHER1, digested with KpnI/XhoI and all of them were successful clones.

3.4.9 Verification of sequenced DNA by BLAST analysis of cloned and sequenced HER1 and HER4 gene promoters

The Nucleotide BLAST tool from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cg) was used to perform a genome-wide homology search to verify the cloned and sequenced gene promoter (HER1 1.5 kb and HER4 1.3 kb) as being HER1 and HER4.

3.4.7.1 Pairwise alignment of the cloned and sequenced DNA with retrieved HER1 and HER4 promoter sequences from Ensemble

The EMBOSS needle nucleotide alignment tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html) was used to examine the homology between the sequenced DNA (*HER1* 1.5 kb and *HER4* 1.3 kb) and the retrieved *HER1* and *HER4* promoter sequences from Ensembl (Ensemble Genome Browser, 2016; European Bioinformatics Institute, 2012). Figures 3. 7 (A and B)

shows a base pair identity and a 99.8% sequence similarity and the homology demonstrated by the sequence alignments verified that the HER1 and HER4 promoters have successfully been cloned and sequenced.

A

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EMBOSS_001         1 CACTCCAGGTACTAGCCAAGGACTACAAAATCAACGTGAATGTCAGCTTT     50
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EMBOSS_001         1 CACTCCAGGTACTAGCCAAGGACTACAAAATCAACGTGAATGTCAGCTTT     50

EMBOSS_001        51 TGTATCAAAAGCTCAAAGGAGAAACTCAAACTTTACATAGATGTCCCATG    100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
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                     |||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001      1301 AATTACCGCAGCCGCCGGACAAGTGTGAGGAAAGCTGAGAG   1341
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Figure 3.7: Pairwise alignment of the cloned and sequenced: HER1 1.5 kb and HER4 1.3 kb gene promoter with retrieved HER1 1.5 kb and HER4 1.3 kb sequence from Ensemble (Ensemble Genome Browser, 2016), (A) is for HER1 and (B) HER4.

3.4.10 Midiprep of PGL3, pGL3-HER1 and pGL3-HER4 and spectrophotometric analysis

A single white bacterial colony with respect to each transformation (PGL3, pGL3-HER1 and pGL3-HER4) was inoculated into 15 mL centrifuge tubes (Corning Incorporated) containing 5 mL of LB-Media with Ampicillin selection. Inoculated cultures were grown in a shaking incubator at 37ºC for 24 h. The following day, one ml of each remaining bacterial culture was used to inoculate 3 separate sterile conical flasks containing 100 mL TB media with Ampicillin selection. The bacterial cultures were grown in a shaking incubator for 24 h. These bacterial cultures were subsequently used to isolate and purify high-quality plasmid DNA from the bacteria using Midiprep **(**Refer to PureYield Plasmid Midiprep System - Promega).

Table 3.1: Plasmid DNA was produced form midiprep. The concentration of vector or vector/insert was quantified spectrometrically

MidiPrep DNA	Purity Ratio (260/280)	Concentration (ng/µl)
PGL3-Vector	1.897	690
pGL3-HER1 1.5 kb	1.890	900
pGL3-HER4 1.3 kb	1.880	800

3.4.11. Examining the functionality of the cloned promoter constructs

Previous study by our group has reported a significant high basal level and differential expression of HER2/HER3 among the cell lines (Khalil et al., 2016b). Here similar transcriptional reporter assays to demonstrate basal and differential expression of HER1/HER4 alongside the already studied promoters HER2/HER3 (Khalil et al., 2016b), was investigated. To examine the functionality of cloned promoters, 1.5 kb and 1.3 kb DNA fragments encompassing the *HER1* and *HER4* gene proximal promoters, as well as that of *HER2* and *HER3* clone by Khalil et al., 2016b were each placed upstream of the luciferase reporter gene in the pGL3 vector to be prHER1, prHER2, prHER3 and prHER4 accordingly. These were used to transiently transfect a panel of ovarian cell lines (OVCAR3, PEO1 and SKOV3) in order to examine their constitutive expression. All prHER1, prHER2, prHER3 and prHER4 showed a high and differential basal level of expressions in all the cell lines tested when compared to the control pGL3 vector (Figure 3.8). This suggests the functionality of the promoter constructs and their feasibility as tools to study the regulation of HER family receptors.

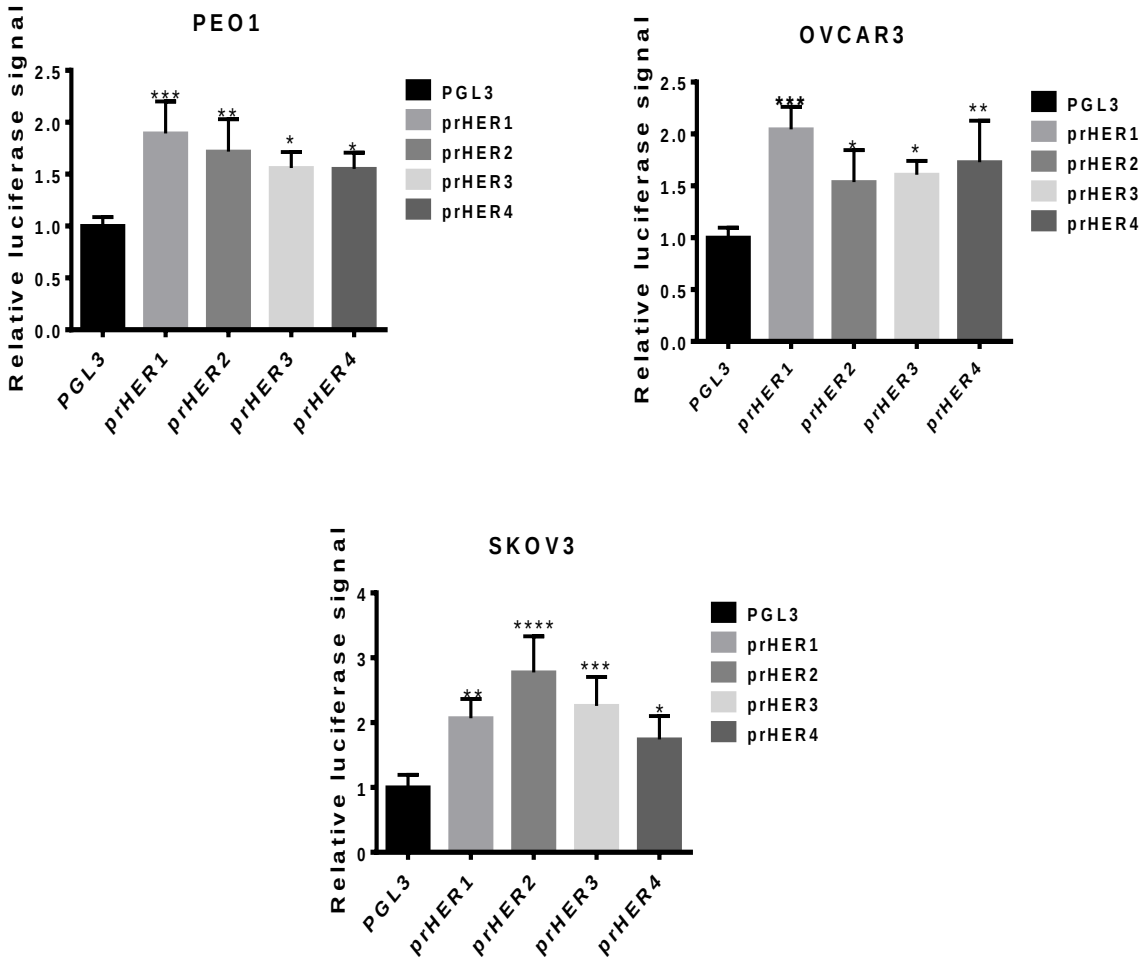

Figure 3.8: Examining the basal activity of the promoters. All the promoters showed a high and differential basal level of expression in all the cell lines tested when compared to the control pGL3-basic vector. Briefly SKOV3, OVCAR3 and PEO1, cells were transfected with either empty PGL3 basic vector or 1 μg PGL3 basic vector with cloned fragments of either HER1 (prHER1) HER2 (prHER2) HER3 (prHER3) or HER4 (prHER4) promoter driving the expression of luciferase gene. Co-transfection with 0.2 μg pRL-CMV plasmid was performed as an internal transfection control. Following 24 h of transfections the lysates were prepared and luciferase activity was measured using Dual luciferase reporter assay (Promega) in multiplate reader (MODULUSTM, Promega). Data presented are the means ± S.D. of n = 3; independent experiments performed in quadruplicates and expressed in fold change to PGL3 with statistical significance determined by ONE WAY ANOVA followed by Tukey's post hoc test (∗: $p < 0.05$, ∗∗: $p < 0.01$, ∗∗∗: $p < 0.001$ and ∗∗∗∗: $p < 0.0001$). Note that, the already studied prHER2 and prHER3 (Khalil et al., 2016b) are used as positive controls here. This study cloned only pHER1 and pHER4.

3.5. Key findings

This chapter has reported the information about the cloning of promoter regions of *HER1* and *HER4,* and indicate differences in relative basal activity of the promoter regions. The computer-based sequence analysis performed had revealed that both HER1 and HER4 promoters contain putative NRF2

transcription factor response element (Figure 3.1). Following this, the chapter has reported the successful clones (Figure 3.6) and sequences (Figure 3.7) of the promoter regions of *HER1* and *HER4* gene, and then finally determine their functionality using dual luciferase assays by transfecting their respective DNA constructs into the ovarian cancer cell lines (3.8). The presence of conserved cis elements for these factors suggests possible additional ways of HER1 and HER4 regulation and expression.

Taken together, this section has reported the cloning of the promoter regions of the human HER1 and HER4 genes, and testing them, confirmed their activity and functionality. This chapter has provided novel tools for the study of the regulations of the expression of *HER1* and *HER4* genes by NRF2, which will be investigated further in the next chapter.

4. CHAPTER FOUR

Regulation of HER1 and HER4 by NRF2 in Ovarian Cancer Cells

4.1. Abstract

The HER family cell surface receptors dimerize upon ligand binding to activate their tyrosine kinase domains which subsequently lead to the phosphorylation of tyrosine residues in the intracellular domain of the receptors. These receptors are the regulators of normal cellular proliferation, differentiation and survival, as well as determinants of cancer initiation, maintenance, and progression. NRF2 is an essential transcription factor for multiple genes encoding antioxidants and detoxification enzymes. NRF2 is implicated in promoting cancer therapeutic resistance by its detoxification function and link with proliferative pathways. However, the linkage between NRF2 and HER family growth factor-induced proliferative pathway remains poorly understood. Here, it is demonstrated that pharmacological activation of NRF2 by tBHQ or pharmacological and genetic inhibition of NRF2 by RA and siRNA upregulates or downregulates HER1 and HER4 expression at both transcriptional and protein levels. Also, inhibition of NRF2 led to reduced total GSH and an elevation of ROS in all the cell lines used in this study. Hence, NRF2 regulates both HER1 and HER4 receptors, via direct or indirect means involving ROS, suggesting that regulation of both *HER1* and *HER4* gene expression could be both at the transcriptional and translational levels.

4.2. Introduction

The HER receptor kinase family and NRF2 are the regulators of cellular proliferation, differentiation and survival as well as the casual factors leading to cancer initiation, maintenance, and progression (Yarden, 2001, Yarden and Sliwkowski, 2001, Normanno et al., 2006, Gschwind et al., 2004, Ritter and Arteaga, 2017, Marmor et al., 2004, Gui and Shen, 2012, Cao et al., 2008). Overexpression of the HER receptor family and NRF2 acting in synergy with various molecular pathways have been shown to correlate with poor survival outcomes in cancers including ovarian (Marmor et al., 2004, Bianco et al., 2006, Friedlander, 1998, Psyrri et al., 2005, Phelps et al., 2008, Ledermann and Raja, 2010, Alečković and Kang, 2015, Clayton et al., 2004, Hough et al., 2000, Barbosa et al., 2014, Gomperts et al., 2009, Allard et al., 2016, Anuranjani and

Bala, 2014, Akhdar et al., 2009, Chen et al., 2013, Ahmed et al., 2017, Namani et al., 2014).

tBHQ, the major metabolite of butylated hydroxyanisole, induces an antioxidant response through NRF2. However, the mechanism by which tBHQ induces NRF2 activity is not entirely understood (Imhoff and Hansen, 2010, Farhoosh and Tavassoli-Kafrani, 2010). A rich body of evidence has demonstrated that tBHQ is effective in protecting against cellular dysfunction induced by oxidative stress inducers, such as alcohol, dopamine, hydrogen peroxide, and glutamate, in various cell types (Li et al., 2014). It has been well-established that tBHQ exerts its antioxidant function through a mechanism whereby it increases NRF2 protein stability via repression of KEAP1-mediated ubiquitination (Turley et al., 2015, Alarcón-Aguilar et al., 2014, Li et al., 2014). Based on these observations, tBHQ has become one of the most widely employed NRF2 activators in a number of studies

Retinoids such as RA and bexarotene, on the contrary, are chemopreventive and chemotherapeutic agents that inhibit NRF2. RA regulates cell proliferation, differentiation, and morphogenesis (Garattini et al., 2014, Wang et al., 2007, Heo et al., 2016). It inhibits tumorigenesis through suppression of cell growth and stimulation of cellular differentiation (Garattini et al., 2014). Also, RA promotes apoptosis and this property may contribute to its antitumor properties. The effects of retinoids are mediated by specific nuclear receptors, namely, retinoic acid receptors (RAR-α, -β, and -γ) and retinoid X receptors (RXR-α, -β, and -γ). RXRs form heterodimers with RARs or other nuclear hormone receptors and function as transcriptional regulators. RA, for example, activates RAR-RXR heterodimers and exerts its biological actions by binding to retinoic acid response elements (RAREs). In addition, retinoids can either activate or repress gene expression through RAR/RXR heterodimers interacting with other transcription factors, such as AP-1, estrogen receptor α, and NF-κB activities (Heo et al., 2016, Hayes et al., 2016, Hayes and McMahon, 2009, Wang et al., 2007).

Based on the previous reports, it is assumed that tBHQ, via activating NRF2, may confer protective effects on cancer cells leading to chemoresistance and therefore modulating NRF2 could be a great strategy in overcoming the

resistance (Khalil et al., 2016b, Li et al., 2014). Both HER receptors and NRF2 are recognised as agents in cellular proliferation and adaptation to ROS leading to therapeutic resistance to cancers. Moreover, NRF2 activation and KEAP1 inactivation mutations leading to permanent constitutive adaptive activation of the NRF2 pathway are often observed in cancers (Khalil et al., 2016b). Number therapeutic approaches such as anti-cancer radio- and chemotherapy greatly depend on ROS manipulation to induce cytotoxicity. Studies have implicated HER family, NRF2, and ROS in the promotion of cellular proliferation and therapeutic resistance in cancer cells. (Khalil et al., 2016b, Lu et al., 2017, Roy et al., 2016, Chen et al., 2016, Leone et al., 2015, Kovac et al., 2015, Jayakumar et al., 2015, Li et al., 2015). This study aims to investigate and identify the link between NRF2 and the HER family receptors signalling pathway, in order to determine a way of improving responses to therapies (Khalil et al., 2016a, Khalil et al., 2016b).

In this chapter, gene transcriptional reporter assays were generated, followed by pharmacological activation and inhibition or siRNA knockdown of NRF2.

4.3. Aims and objectives

The main aim of this chapter is to examine the regulations of the HER1 and HER4 by NRF2 in ovarian cancer.

4.3.1 The aim

4.3.2. The objectives are:

1. To test the effect of pharmacological activation of NRF2 on HER1 and HER4 expression in ovarian cancer cells.
2. To test the effect of pharmacological and genetic inhibition of NRF2 on HER1 and HER4 expression in ovarian cancer cells.

4.4. Results

4.4.1. Treatment with tBHQ causes transcriptional and translational upregulation HER1 and HER4

Several studies have shown that NRF2 promotes resistance to chemotherapeutic agents and contributes to general cytoprotection, metabolic reprogramming, and cell survival (Abdullah et al., 2012, Ahmed et al., 2017, Hayes et al., 2016, Hayes and Ashford, 2012, Hayes and Dinkova-Kostova, 2014, McMahon et al., 2014). Conversely, RTK targeted therapies involving inhibitory monoclonal antibodies and small molecules against HER receptors has generated interest in recent years as a potential strategy to overcome ovarian cancer cell therapeutic resistance (Khalil et al., 2016a, Khalil et al., 2016b, Langdon et al., 2010, Langdon and Cameron, 2013). Here a panel of ovarian cells transfected with the prHER1 and prHER4 for reporter assays were exposed to increasing concentrations of tBHQ to explore the nature of this transcriptional regulation. Interestingly, all the cell lines exhibited significant dose-dependent transcriptional upregulation of HER1 and HER4, as well as the other members of HER family when tested with varying concentrations of tBHQ (Figure 4.1).

Furthermore, tBHQ treatment caused upregulation of both total NRF2 and phospho Akt alongside total HER1 and HER4 levels (Figure 4.2) in all the ovarian cancer cell lines tested (PEO1, OVCAR3 and SKOV3). These results demonstrated that pharmacological activation of the NRF2 protein caused transcriptional upregulation of HER receptors pathway with concomitant translational induction of total HER1 and HER4 proteins. This implied that the HER receptor pathways might be subjected to coregulatory mechanisms by the antioxidant response pathway and NRF2.

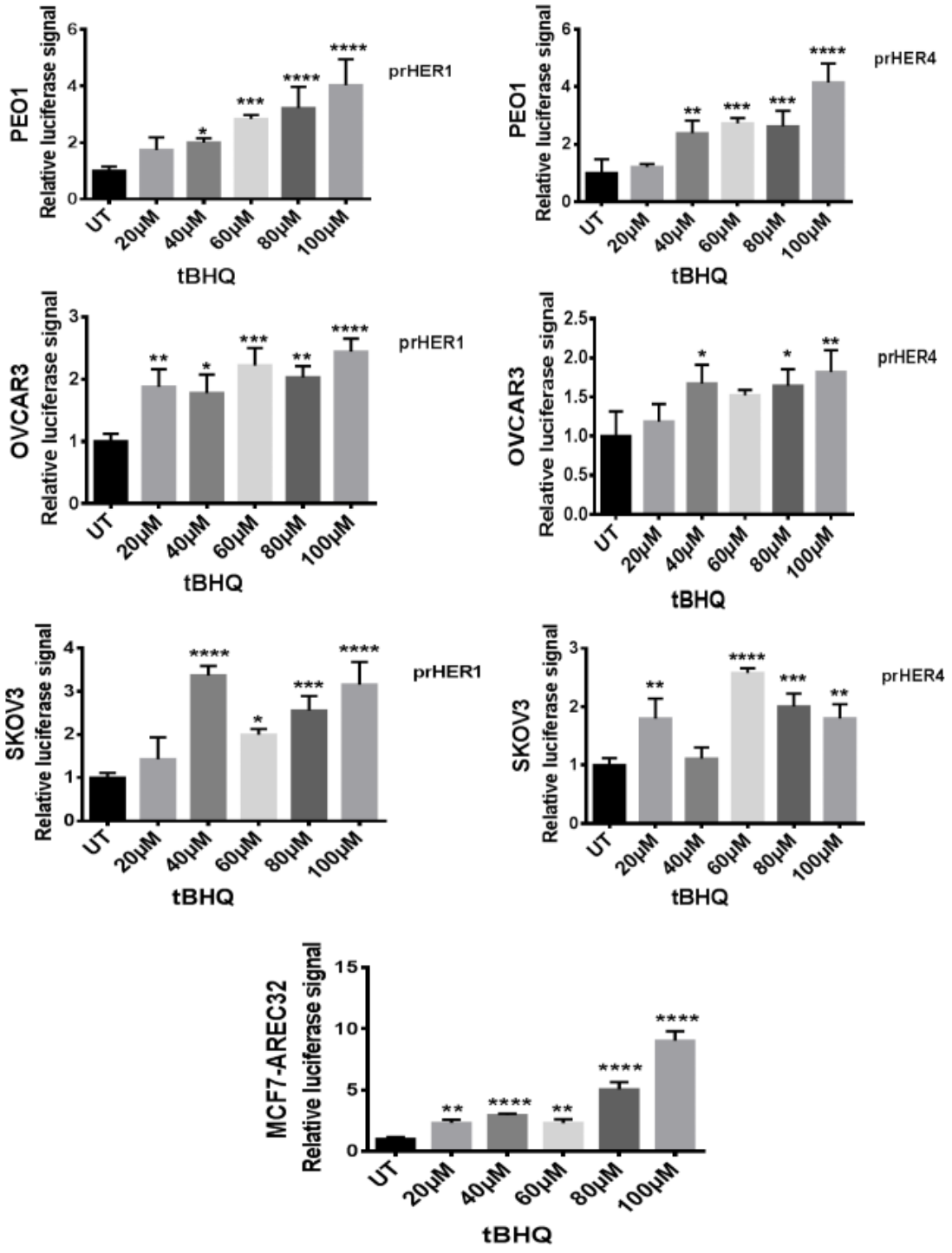

Figure 4.1: Treatment with tBHQ causes transcriptional and protein induction of HER1 and HER4. tBHQ causes transcriptional induction of HER1 and HER4 and induction of ARE in a concentration dependent manner. MCF7-AREc32 which already contains stably cloned 8 x cis-AREs driving NRF2 dependent expression of luciferase gene was left without any transfection while PEO1, OVCAR3 and SKOV3 cells were transfected with either empty PGL3 basic vector or 1 µg PGL3 basic vector with promoters of HER1 cloned driving HER1 expression of luciferase gene. Co-transfection with 0.2 µg pRL-CMV plasmid was performed as an internal transfection control. Where required PEO1, SKOV3 and OVCAR3 cell lines and MCF7-AREc32 stable cell line were treated in quadruplicate with different concentrations of tBHQ as indicated at normal condition for 24 h. Data shown are the means ± S.D. of n = 3 independent experiments. Each independent experiment consists four replicates. Statistical significance determined by ONE WAY ANOVA followed by Tukey's post hoc test (*: p<0.05, **: p<0.01, ***: p<0.001 and ****: p<0.0001).

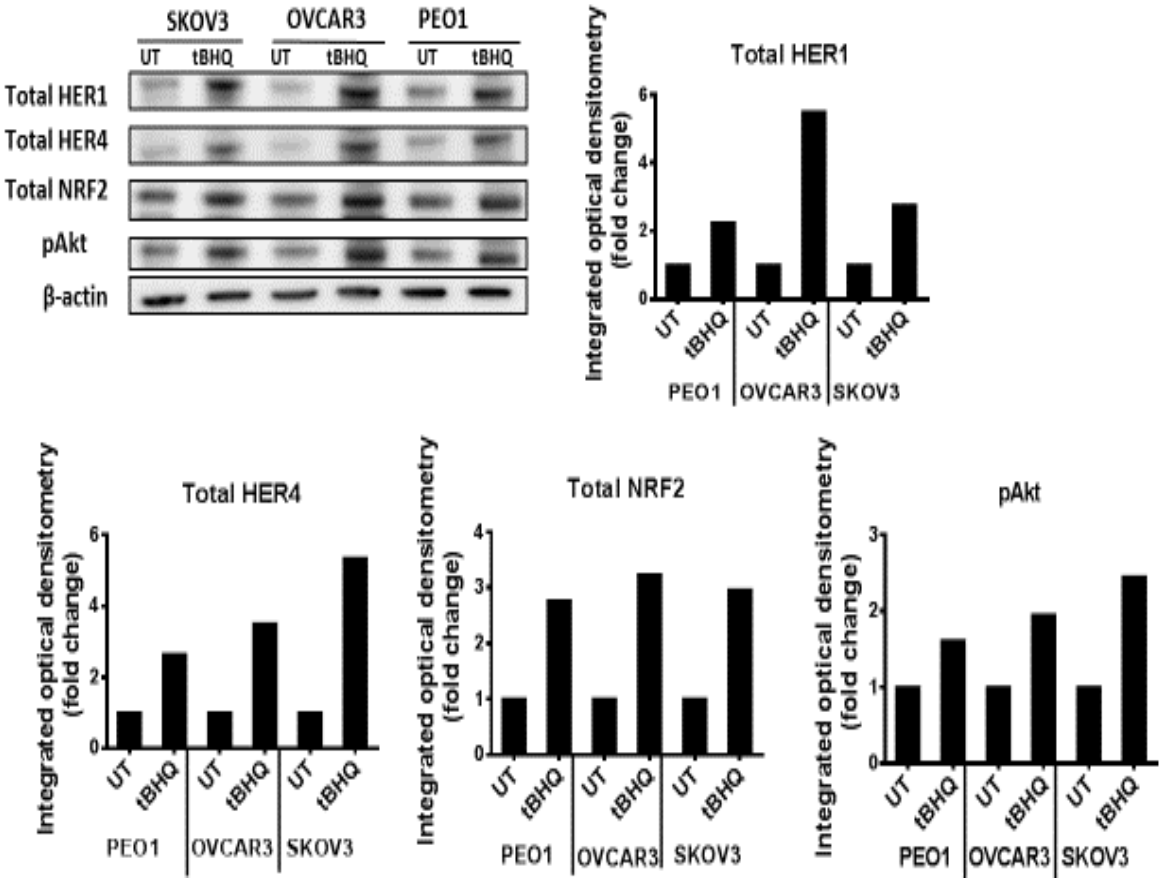

Figure 4.2. Treatment with tBHQ causes protein induction of HER1 and HER4. Immunoblot analysis following treatment with tBHQ demonstrated protein induction of HER1 and HER4 receptor and also activation of total and with an increase of pAKT. Briefly exponentially growing cells were either left untreated (UT) or treated with 100 μM tBHQ for 24 h before being harvested and processed for immunoblotting using relevant antibodies. Bar chart shows total NRF2, total HER1, HER4 and phospho-AKT levels in PEO1, OVCAR3 and SKOV3 cell lines by quantifying immunoblot signal intensities obtained and normalised to the value of UT and expressed as fold change. Images are representative of at least two independent experiments performed.

4.4.2. Treatment with RA inhibited NRF2 pathway leading to downregulation of HER1 and HER4

To further delineate the role of NRF2 in the regulation of HER1 and HER4 receptors expression, the expression of HER1 receptor following the antagonism of NRF2 and its function was examined. Previous studies have used RA to pharmacologically inhibit NRF2 and its function (Wang et al., 2007, Wu et al., 2014, Khalil et al., 2016a). To confirm this, the ovarian cancer cells were treated with RA to pharmacologically inhibit NRF2. The generated gene transcriptional reporter *HER1* and *HER4* and western blot analysis of HER1, HER4 and NRF2 levels were used to delineate the role of NRF2 in the regulation of HER1 and HER4 receptor expression. The cell lines were individually transfected with the constructs of prHER1 and prHER4, except MCF7-AREc32 stable cell line with

served as positive control. This time, the cells were either left untreated or treated with 2.5 µM RA for 24 h. The results revealed a significant transcriptional downregulation of HER1 and HER4 in all the cell line, PEO1, OVCAR3 and SKOV3 as well as MCF7-AREc32 cell lines (Figure 4.3). Moreover, the protein level has shown a significant protein repression of total NRF2, HER1, HER4 as well as pAKT (Figure 4.4). These results demonstrated that pharmacological inhibition of NRF2 protein caused inhibition of HER receptor pathway and transcriptional and translational downregulation of HER1 and HER4. This supported the hypothesised cross-link between NRF2 and HER receptors.

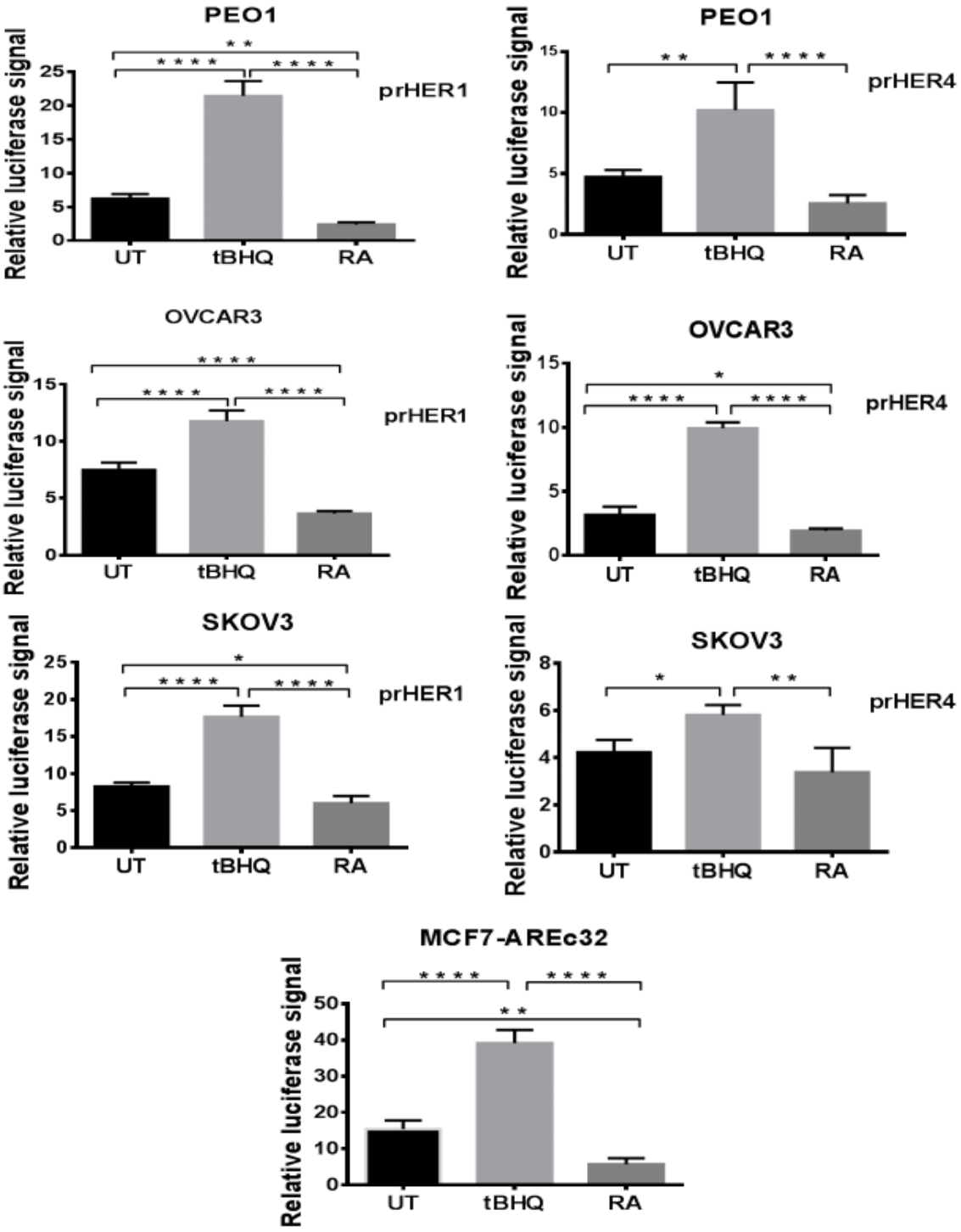

Figure 4.3: Treatments with RA causes inhibition of NRF2 and transcriptional downregulation HER1 and HER4. Luciferase assay showing transcriptional downregulation of HER1 and HER4 following NRF2 inhibition by RA in PEO1, OVCAR3 and SKOV3 cell lines. Exponentially growing PEO1, SKOV3 and OVCAR3 excluding MCF7-AREc32 cell lines were transfected with either empty PGL3 basic vector or 1 µg PGL3 basic vector with cloned NRF2-AREs driving the expression of luciferase gene. Co-transfection with 0.2 µg pRL-CMV plasmid was performed as an internal transfection control as described in the materials and methods. At 24h post-transfection, cells were either left untreated or treated with 100 µM tBHQ or 2.5 µM RA for 24 h. Following treatments, lysates were prepared and luciferase activity was measured using Dual luciferase reporter assay (Promega) in multiplate reader (MODULUSTM, Promega). Data shown are the means ± S.D. of n=3 independent experiments performed in quadruplicates triplicates with statistical significance determined by ONE WAY ANOVA followed by Tukey's post hoc test (*:p<0.05, **:p<0.01, ***:p<0.001 and ****:p<0.0001).

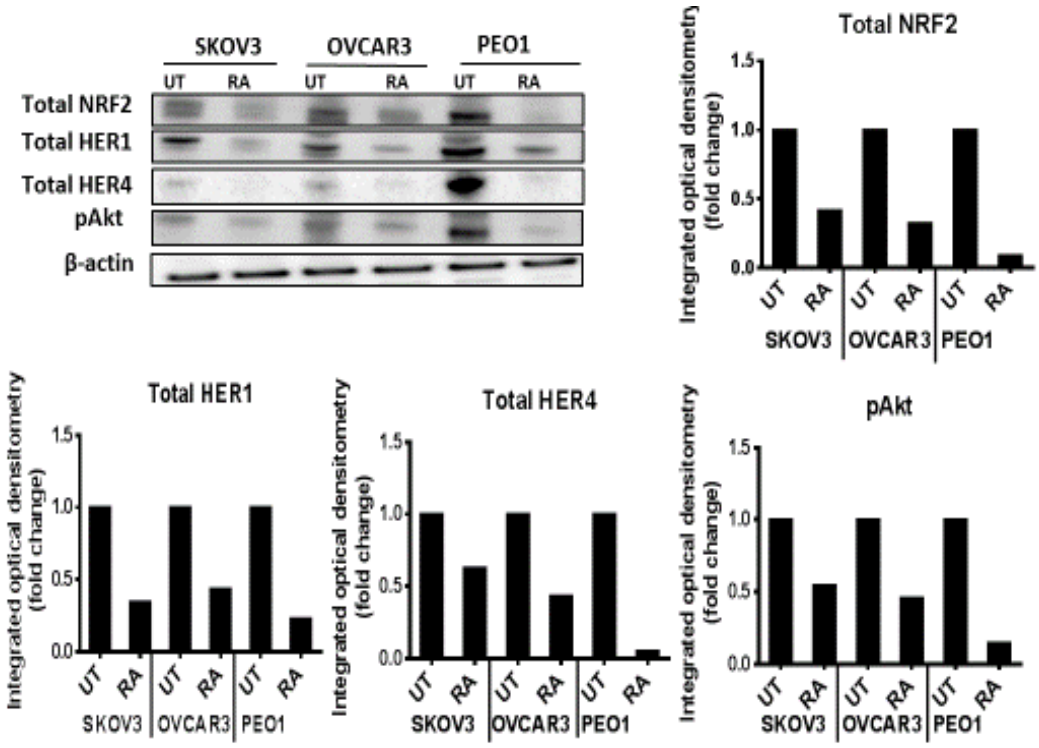

Figure 4.4: Treatments with RA causes repression of NRF2 as well as downregulation HER1 and HER4. Immunoblot analysis following treatment with RA demonstrated protein downregulation of both HER1 and HER4 receptors and decrease of NRF2, pNRF2, HO-1 and pAKT. Exponentially growing cells were either left untreated (UT) or treated with 2.5 μM RA for 24 h before being harvested and processed for immunoblotting using relevant antibodies. Bar chart shows total NRF2, phosphor-NRF2, HO-1, total HER1, total HER4, and phospho-AKT levels in PEO1, OVCAR3 and SKOV3 cell lines by quantifying immunoblot signal intensities obtained. Images are representative of at least two independent experiments performed.

4.4.3. Genetic knockdown of NRF2 causes downregulation HER1 and HER4

Studies have used siRNA to genetically inhibit NRF2 and its function (Huang et al., 2008, Guo et al., 2013, Kanninen et al., 2015, Duong et al., 2017, Esmaeili, 2016). To assess the effect of siRNA on NRF2 and HER1 and HER4 receptors expression, the expression of HER1 and HER4 receptors following the siRNA-mediated knockdown of NRF2 was examined. The generated gene transcriptional reporter for *HER1* and *HER4* and western blot analysis of HER1, HER4 and NRF2 levels were investigated. The PEO1, OVCAR3 and SKOV3 cell lines were individually transfected with the constructs of prHER1 and prHER4. Following this, cells were co-transfected with 75 pmol NRF2 specific siRNA for 24 h. The results revealed a significant transcriptional downregulation of HER1 and HER4 (Figure 4.5). At the protein level, following the knockdown with siRNA for 24 h and 48 h there was a significant protein repression of HER1, HER4 as well as pAKT, and with a significant inhibition of total NRF2 (Figure 4.6). Taken together,

these interesting results demonstrated that inhibition of NRF2 protein caused inhibition of HER receptor pathway and transcriptional and translational downregulation of HER1 and HER4. This also supported the implication that both antioxidant response and HER receptor family pathways might be subjected to coregulatory mechanisms and that NRF2 regulates HER1 and HER4.

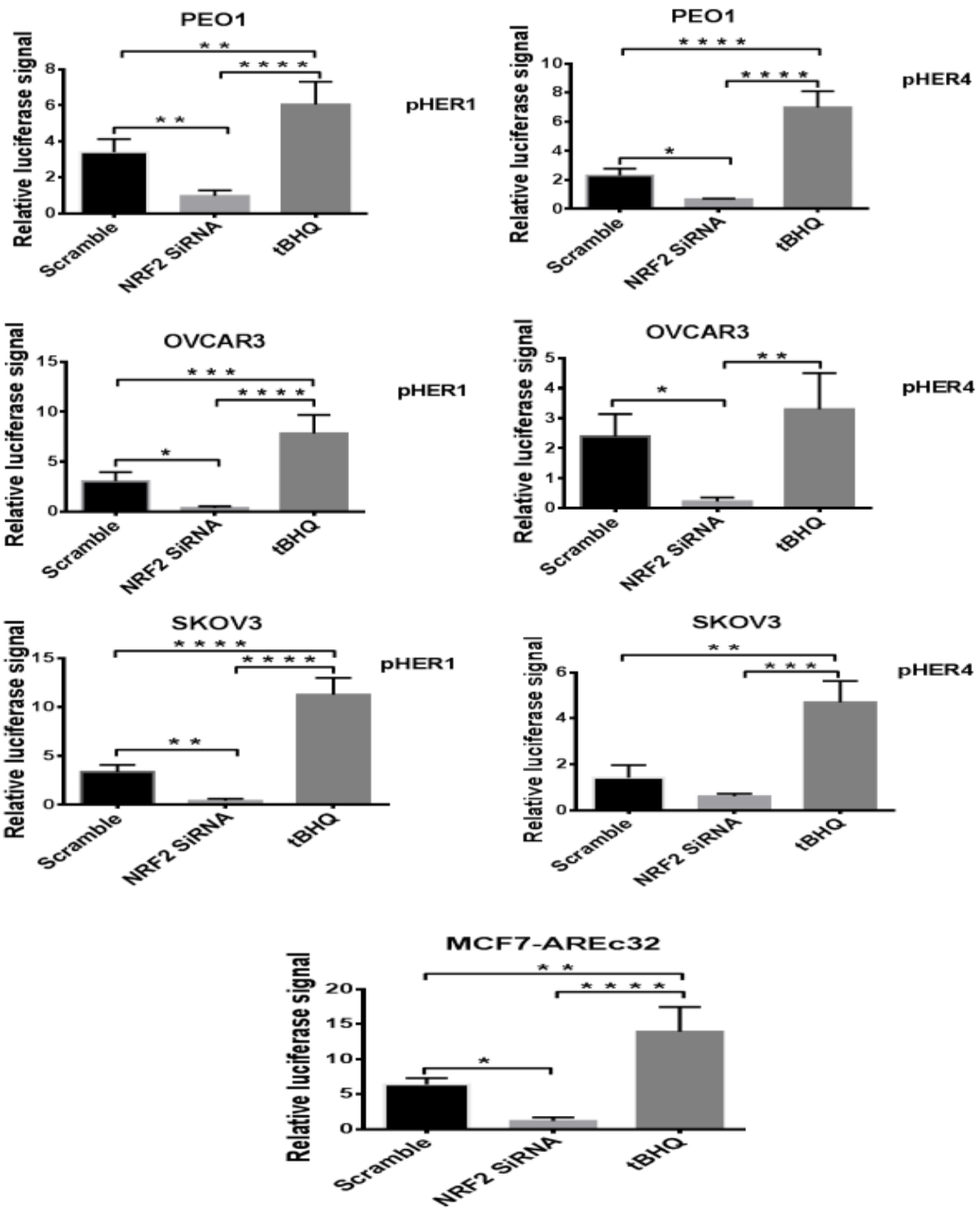

Figure 4.5: Genetic knockdown of NRF2 causes transcriptional downregulation HER1 and HER4 Knockdown of NRF2 causes inhibition its transcriptional antioxidant program and the transcriptional level of HER1 and HER4 in both constitutive and tBHQ induced states. MCF7-AREc32 which already contains stably cloned 8 x cis-AREs driving NRF2 dependent expression of luciferase gene was left without any transfection while PEO1, OVCAR3 and SKOV3 cells were transfected with either empty PGL3 basic vector or 1 µg PGL3 basic vector with promoters of HER1 and HER4 cloned driving HER1 and HER4 expression of luciferase gene. Co-transfection with 0.2 µg pRL-CMV plasmid was performed as an internal transfection control. Where required, cotransfection with either scrambled RNA (Sc) or NRF2 siRNA was performed using 20 pmol siRNA. At 24 h after transfection, treatment with 100 µM tBHQ was performed where indicated for 4 h following which, cells were processed for dual luciferase reporter assay (Promega) to record luciferase activity in multiplate reader (MODULUS, Promega). Data are the means with ±S.D. of n=3 independent experiments in triplicates, with statistical significance determined by ONE WAY ANOVA followed by Tukey's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$

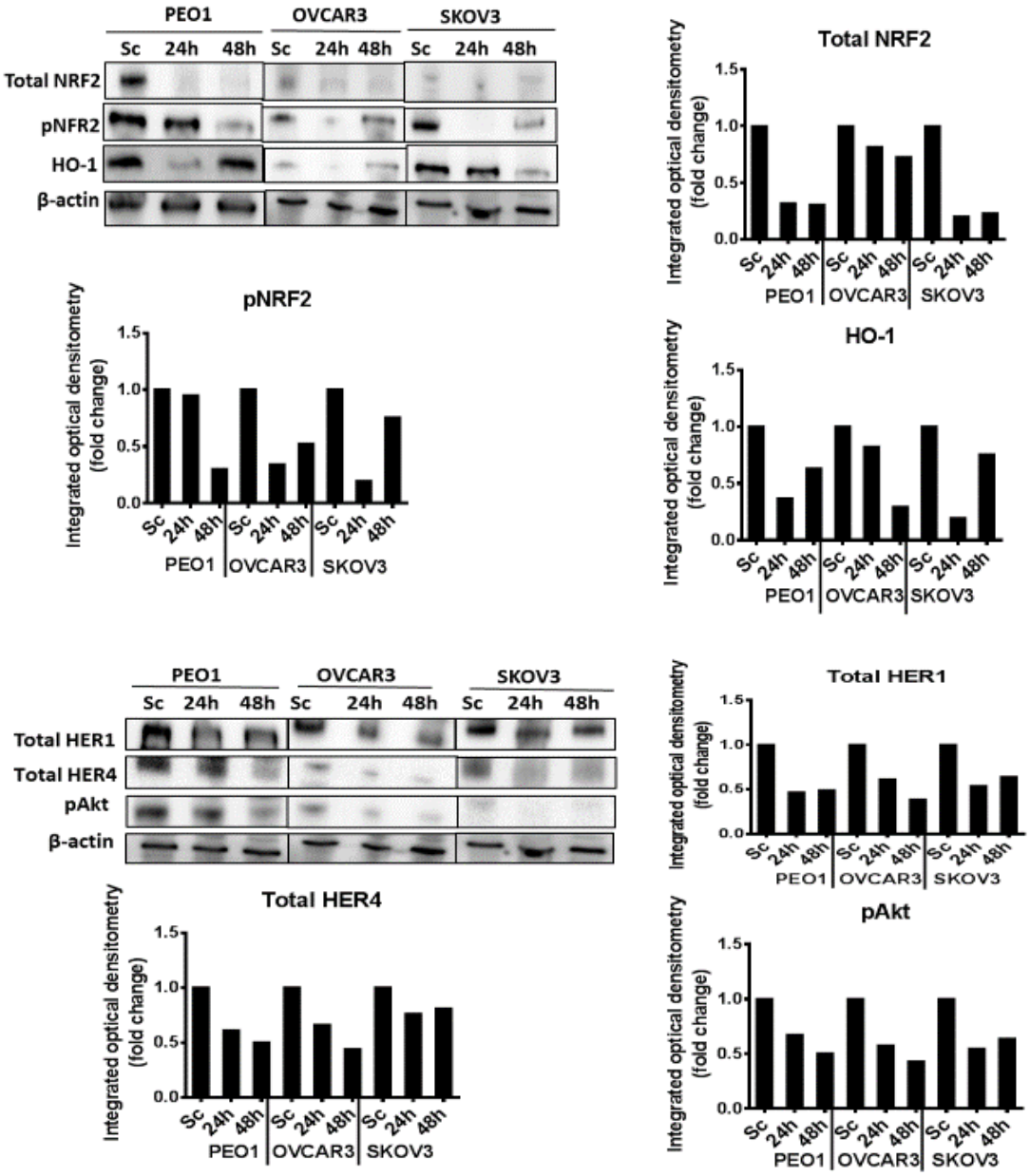

Figure 4.6: Genetic knockdown of NRF2 causes protein downregulation HER1 and HER4. Immunoblot analysis following knockdown of NRF2 demonstrated protein downregulation of both HER1 and HER4 receptors and decrease of NRF2, pNRF2, HO-1 and pAKT in PEO1, OVCAR3 and SKOV3 cell lines. Cells were either transfected with scrambled siRNA (Sc) or transfected with 75 pmol of NRF2 siRNA (Si). After 24h and 48 h, cells were being harvested processed for immunoblotting using relevant antibodies. β-actin of the same blot was used as loading control. Bar chart shows the levels relevant proteins by quantifying immunoblot signal intensities obtained and expressed as fold change. Images are representative of at least two independent experiments performed.

4.4.4. Treatment with tBHQ reduces the knocking down effect of siRNA

Based on the results obtained following activation and inhibition of NRF2 by tBHQ and siRNA respectively (Figures 4.5 and 4.6), it is thought that tBHQ dependent induction of antioxidant pathways in the cell lines would disrupt the depletion of

NRF2 following knockdown by siRNA and to confirm this, cells were exposed either genetic inhibition of NRF2 with siRNA or pharmacological activation of NRF2 by tBHQ following the genetic depletion of NRF2. The evidence to implicate NRF2 in the regulation of *HER1* and *HER4* expression was obtained when co-treatment of ovarian cancer and MCF7 AREc32 cells with tBHQ and NRF2-siRNA compromised the tBHQ-dependent induction of either HER1 or HER4 promoters-driven or ARE-driven luciferase gene expression in the ovarian cancer cells or in MCF7 AREc32 cells (Figure 4.7), respectively. This inhibitory effect of siRNA on the tBHQ-dependent induction of gene expression (Figure 4.8) appeared to be concomitant with decreased total NRF2 levels in all the cells tested, thereby supporting the earlier conclusions drawn from Figure 4.7.

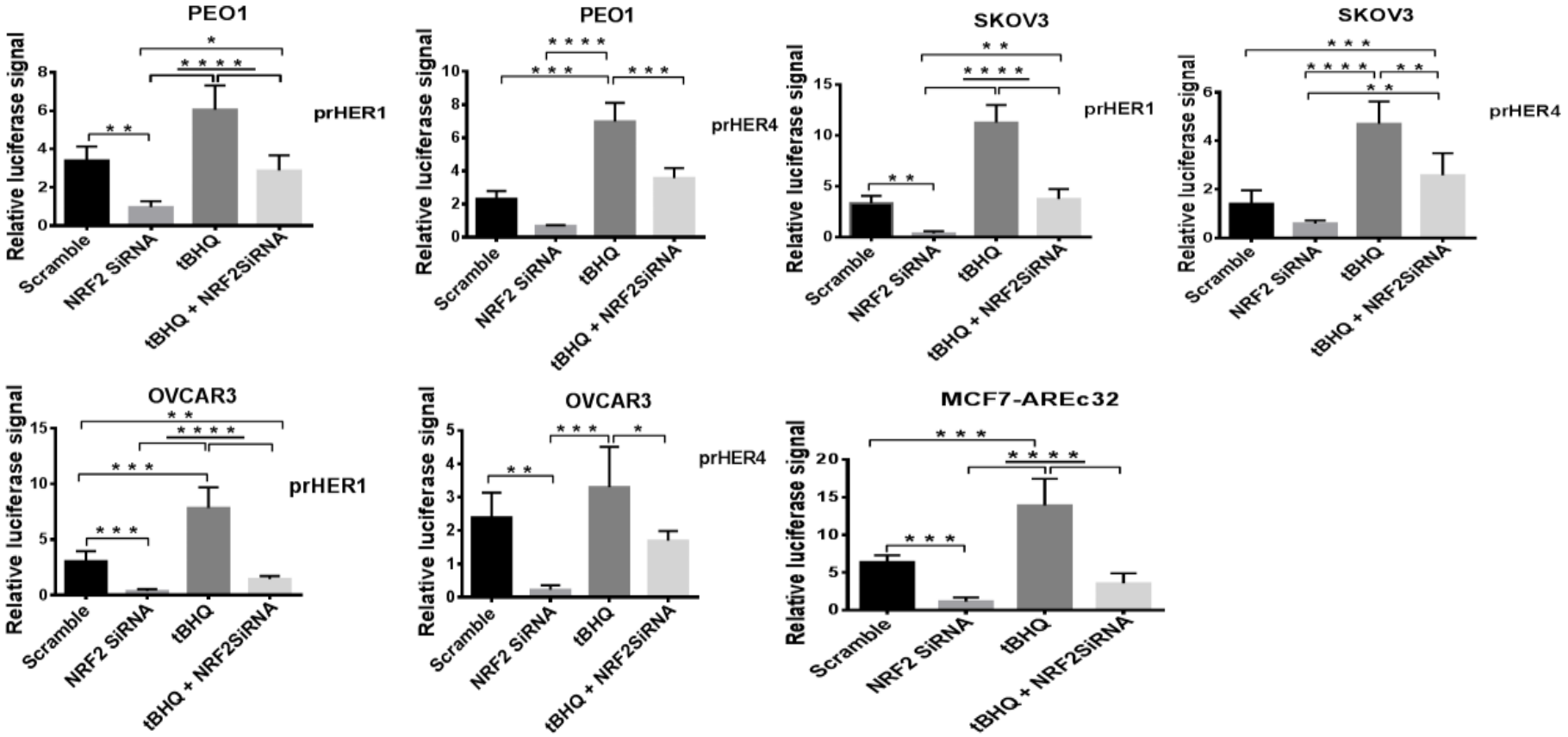

Figure 4.7: Treatment with tBHQ reduces the knocking down effect of siRNA. Knockdown of NRF2 cause inhibition its transcriptional antioxidant program and the transcriptional level of HER1 in both constitutive and tBHQ induced states. MCF7-AREc32 which already contains stably cloned 8 x *cis*-AREs driving NRF2 dependent expression of luciferase gene was left without any transfection while PEO1, OVCAR3 and SKOV3 cells were transfected with either empty PGL3 basic vector or 1 μg PGL3 basic vector with promoters of HER1 cloned driving HER1 expression of luciferase gene. Co-transfection with 0.2 μg pRL-CMV plasmid was performed as an internal transfection control. Where required, cotransfection with either scrambled RNA (Sc) or NRF2 siRNA was performed using 20 pmol siRNA. At 24 h after transfection, treatment with 100 μM tBHQ was performed where indicated for 4 h following which, cells were processed for dual luciferase reporter assay (Promega) to record luciferase activity in multiplate reader (MODULUS, Promega). Data are the means with ±S.D. of n=3 independent experiments performed in quadruplicates, with statistical significance determined by ONE WAY ANOVA followed by Tukey's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$

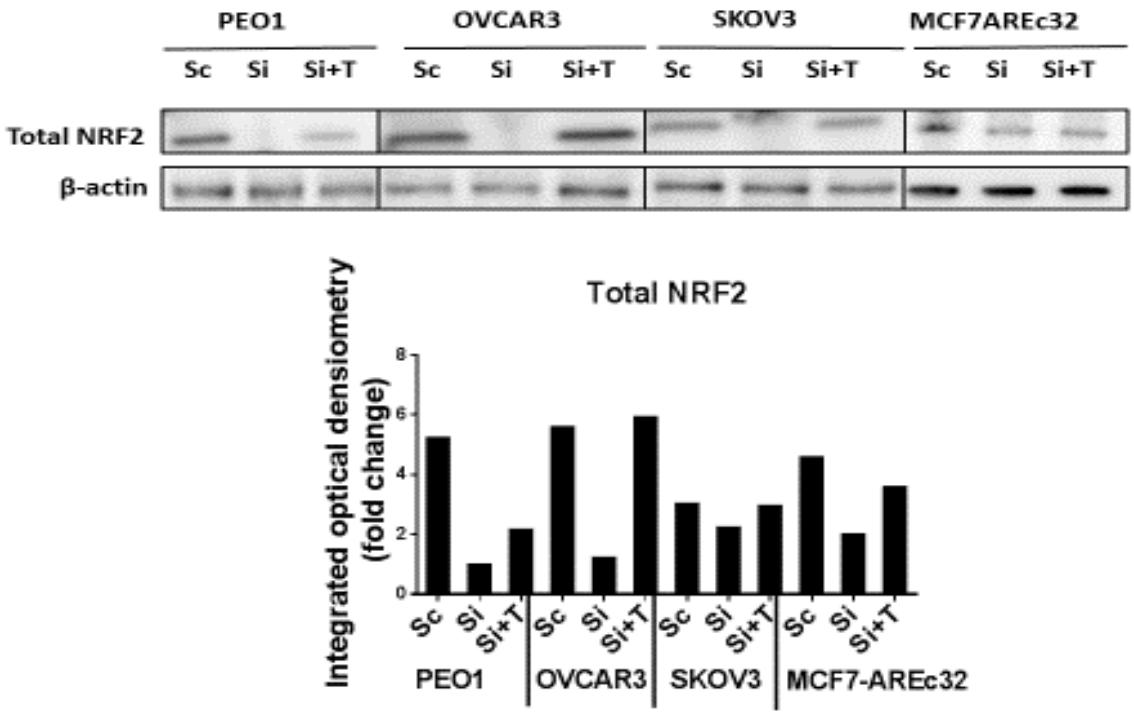

Figure 4.8: Action of tBHQ reduces the knocking down effect of siRNA. Immunoblotting analysis showing repression of NRF2 following NRF2 knockdown by siRNA in PEO1, OVCAR3 and SKOV3 cell lines. Cells were either transfected with scrambled siRNA (Sc) or transfected with 75 pmol of NRF2 siRNA (Si). After 24 h, cells were either left untreated or treated with 100 μM tBHQ (T) for 4 h, before being processed for immunoblotting using relevant antibodies. β-actin of the same blot was used as loading control. Bar chart is the average of n=3 independent experiments performed showing NRF2 levels by quantifying immunoblot signal intensities obtained and expressed as fold change. Images are representative of at least two independent experiments performed.

4.4.5. Pharmacological inhibition and Genetic knockdown of NRF2 by RA siRNA respectively elevates the level of ROS

Based on the above results describing the effects and mechanisms of NRF2 knockdown and inhibition by siRNA and RA respectively. ROS are expected to accumulate as consequential to the loss of NRF2 function. In order to confirm the direct relationship between of NRF2 and ROS, the total basal ROS was quantified following NRF2 inhibition and knockdown to determine whether NRF2 depletion caused elevation of ROS. To achieve this, the treated cells were loaded with 2′,7′-Dichlorofluorescin diacetate dye which is a fluorescent marker of intracellular ROS. The results confirmed an elevation of ROS resulting from both NRF2 inhibition and knockdown (Figure 4.9).

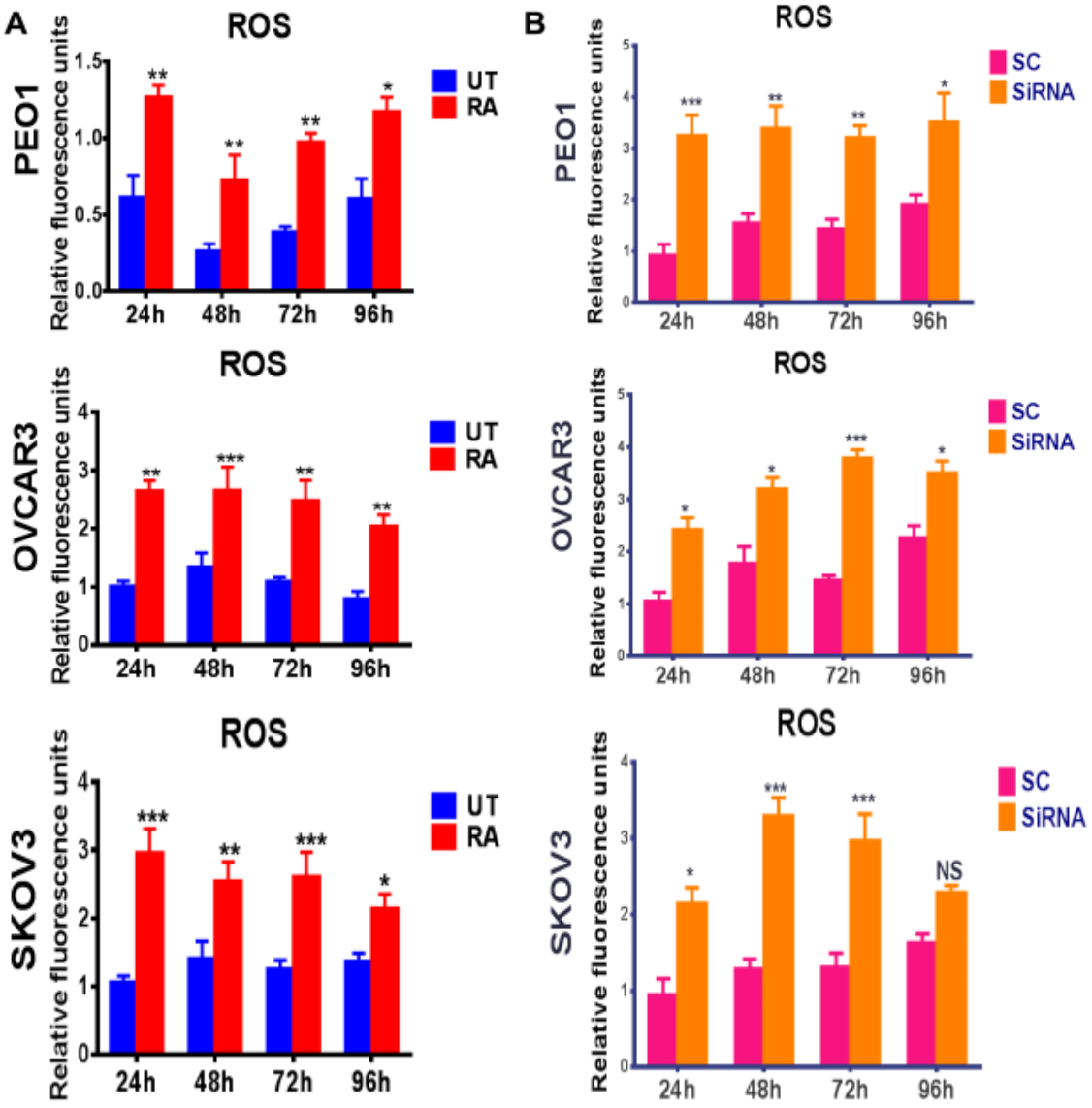

Figure 4.9: Inhibition and Knockdown of NRF2 by RA and siRNA respectively elevates the level of ROS. (A) RA treatment and **(B)** knockdown of NRF2 by siRNA causes increase in ROS levels. Exponentially growing cells were seeded in triplicates in opaque flat bottom black walled 96-well plates for 24 h. Following this, cells were either left untreated (UT)/Scramble (SC) or treated with 2.5 μM RA or 7 pmol of siRNA for different time points as indicated. Following incubations, cells were loaded with DCFDA fluorescent stain for 45 min and assayed for ROS by measuring fluorescence as described in Materials and Methods. Data are the mean ± SD of n=3 independent experiments performed in triplicates and shown as fold change of RA or siRNA treated cells to UT with statistical significance determined by TWO WAY ANOVA followed by Tukey's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

4.4.6. Action of RA and siRNA on NRF2 causes depletion of total GSH

Following the observed inhibition of NRF2 and increase in ROS in these cell lines following treatment with RA and siRNA, next, it is asked whether NRF2 knockdown and treatment with RA would cause a depletion of total cellular GSH.

To address this, cells were either left untreated or treated with RA or siRNA. Here, HRG which is reported to induce total cellular GSH was used as positive control. At 24 h, treatment with HRG induced total cellular glutathione, while both RA and siRNA significantly reduced this level in all cells (Figure 4.10). This study demonstrates that whatever happens to NRF2 also could affect enzymes it controls.

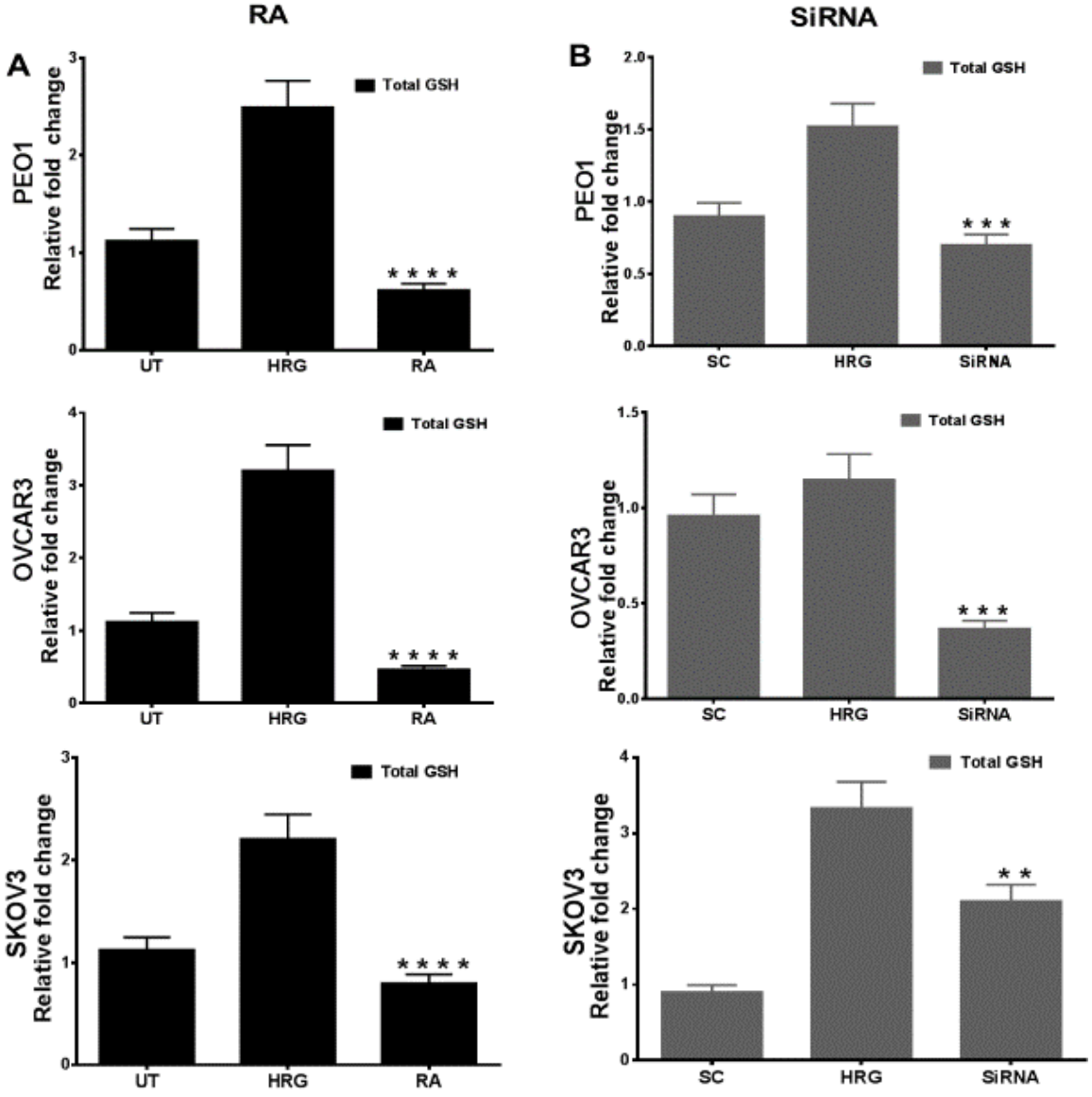

Figure 4.10: Pharmacological treatment with RA and Knockdown with siRNA on NRF2 causes depletion of total GSH level. **(A)** RA and **(B)** siRNA causes depletion of total GSH. Exponentially growing cells where seeded in luminometer-compatible 96-well plates and allow to grow overnight at 37°C in a 5% CO_2 culture incubator. The following day, the cells were either left untreated (UT)/Scramble(SC) or treated with media containing 1nM Heregulin alone (HRG) or with co-treatment 2.5 µM RA or 7 pmol siRNA for 24 h before being processed for glutathione assay. Data is the mean ± SD of n=3 independent experiments performed in triplicates and were normalized and expressed as fold change to the UT and compared to HRG. Statistical significance was determined by independent t-test according to the scale * p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

4.5. Key findings

This chapter has confirmed the transcriptional and translational regulatory role of NRF2 on HER1 and HER4 receptors. It started by indicating that, NRF2 activation by tBHQ not only induced the NRF2 dependent antioxidant response pathway as expected but interestingly also induced both transcriptional (Figure 4.1) and protein levels of HER1 and HER4 (Figure 4.2). NRF2 inhibition by RA (Figures 4.3 and 4.4) and knockdown by siRNA (Figures 4.5 and 4.6), also repressed NRF2 dependent antioxidant response pathway as well as downregulated both transcriptional and protein level of HER1 and HER4. The disruption of the knockdown effect of siRNA was observed when tBHQ was introduced in the treatment (Figures 4.7 and 4.8).

Also in order to examine the consequence of NRF2 inhibition and confirm the direct link between of NRF2 and ROS, the total basal ROS as well as total GSH, following NRF2 inhibition and knockdown were quantified. This will determine whether NRF2 depletion caused elevation of ROS. The data indicated that NRF2 repression led to the elevation of ROS (Figure 4.9) and depletion of total GSH (Figure 4.10) in all the cell lines. Thus, these results confirmed the transcriptional regulatory role of NRF2 for HER1 and HER4 receptors and illustrated alteration of protein abundance as a result of such transcriptional regulation. The data presented in this chapter confirm the regulation of HER1 and HER4 by NRF2.

5. CHAPTER FIVE

Screening for Inhibitors of the NRF2-ARE Signalling Pathway and their Potential Applications in Ovarian Cancer Therapy

5.1. Abstract

NRF2 is a transcription factor that governs the expression of a battery of genes to combat oxidative and electrophilic stress. However, modification of KEAP1 by ROS stabilises NRF2 by escaping from degradation in the cytoplasm to the nucleus. NRF2 then binds to AREs in the promoter region of the cluster of various genes. NRF2-ARE pathway hyperactivation can protect cancer cells from oxidative stress and promote cell proliferation. Moreover, activation of the NRF2 pathway is critical for resistance to chemotherapeutic agents. In this chapter, a total of ten potent NRF2 inhibitors were screened and then the most potent among them were used to provide a molecular and rational basis for the use of NRF2 inhibitors in overcoming chemoresistance in ovarian cancer. This would give a possible NRF2-inhibiting mechanism of these compounds, their effects of sensitizing cancer cells to chemotherapeutic agents, and the prospect of applying them in translational and clinical cancer therapies.

5.2. Introduction

Drug screening is the process by which potential drugs are tested, identified and optimized before selection of a candidate drug to progress to clinical trials. It can involve screening large libraries of chemicals for a particular biological activity in high-throughput screening assays. The screening would help to find small molecular compounds that possess the potential to interact with specific biomacromolecules, mostly proteins, thereby bringing the desired effect in the functioning of the target molecules (Malik et al., 2017). Cell-based screenings are essential for the identification and characterization of drug candidates (Agus and Janovjak, 2017). These assays are used to measure cell proliferation, viability and cytotoxicity and are commonly used to monitor the response of cells in culture after treatment with various stimuli. The luciferase assays and Intracellular ATP-based luciferase assays would be suitable for the screening of chemical libraries because they allow for simple, efficient, rapid-evaluation of the activities of test compounds with signal detection within a short period of time (Suganuma et al., 2017).

NRF2 activity is tightly controlled, suggesting that opportunistic stimulation of NRF2 signalling by drugs used in the treatment of cancer is undesirable. NRF2 contribute to the survival and proliferation of cancer cells leading to chemo resistance because of its ability to induce drug detoxifying genes. While transient activation of NRF2 in normal cells is desirable, persistent activation of NRF2 is detrimental (McMahon et al., 2014). Interrupting the NRF2 activity may be an effective way to sensitize cancer cells to chemotherapy and radiotherapy (Arlt et al., 2013). In this chapter, cell based assays such as luciferase assay, ATP-dependent assay, ROS assay as well as total GSH assay were employed. The contemporary chemical inhibitors of the NRF2 signalling pathway, their effects in cancer cells and their potential clinical applications were examined.

5.3. Aims and objectives

5.3.1 The aim

The main aim of this chapter is to screen for potent inhibitors of the NRF2-ARE signalling pathway and then investigate their potential applications in ovarian cancer therapy

5.3.2 The objectives are:

1. To firstly, perform the cytotoxicity assay of all the compounds to be screened in order to obtain the low-lethal concentration
2. To screen the compounds using MCF7-AREc32 luciferase reporter stable cell line to obtain the most potent modulators of NRF2/ARE-dependent activity
3. To use any of the most potent compounds obtained for further investigations of its potency in ovarian cancer therapy.

5.4. Results

5.4.1. Cytotoxic screening of the spectrum collection the compounds as inhibitors of NRF2 in cancerous cells.

Firstly, the cytotoxicity of all the compounds was investigated to evaluate the less toxic concentration of the compounds to be used for further screening as NRF2 inhibitors. To address this, an exponentially growing MCF7-AREc32 cell line stably expressing 8xcis-ARE were either left untreated (DMSO vehicle) or treated with different concentrations of the tested compounds (Figure 5.1). It was noticed that almost all the compounds were less toxic in range of 0-10 µM and therefore that range of concentration was chosen for further studies.

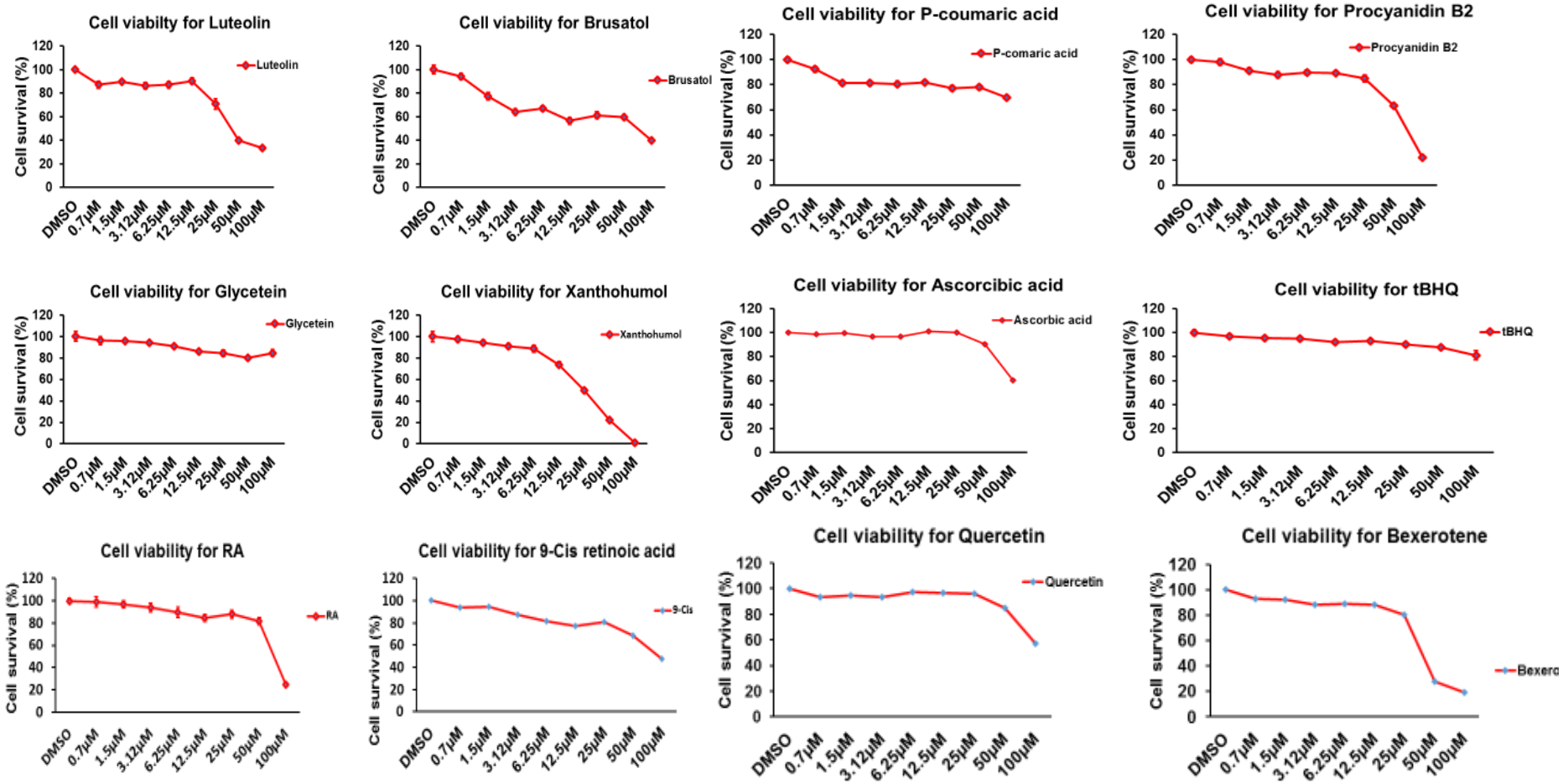

Figure 5.1: Cytotoxic screening of the spectrum collection the compounds. Briefly, exponentially growing MCF7-AREc32 cells were seeded in a 96-well plate and allowed to adhere for 18-24 h. Following 72 h treatments using different concentrations of the compounds, the plate and its contents were equilibrated to room temperature for approximately 30 min, a volume of CellTiter-Glo 2.0 reagent equal to the volume of cell culture medium present in each well the contents were then mixed for 2 min on an orbital shaker to induce cell lysis and the plate was then incubate at room temperature for 10 min to stabilize the luminescent signal and finally the luminescence was recorded using luminometer (MODULUS, Promega). The luminescent signal which is proportional to the amount of ATP in the sample, also which indicates the presence of metabolically active cells is expressed in %.

5.4.2. Determination of activities and effects of compounds on NRF2-ARE signalling pathway

To identify novel small molecule inhibitors that will disturb the NRF2/ARE binding pathway, a group of compounds, of which some were reported to influence NRF2- KEAP1 pathway (McMahon et al., 2014, Kwak and Kensler, 2010), were examined. MCF7-AREc32 stable cell line which is known to contain low levels of NRF2 (McMahon et al., 2014, Wang et al., 2007), and therefore low levels of luciferase expression were used in this investigation. This makes it an excellent cell model with which to identify drugs that activate or inhibit NRF2 signalling (McMahon et al., 2014, Wang et al., 2007). A total of 10 drugs, which target a variety of biochemical pathways were investigated The selection of most of the compounds, however, just as reported by Machmahon et al (2014), was made with no foreknowledge or preconception of their likelihood to activate or inhibit NRF2 signalling (McMahon et al., 2014) except those used as control. The screening confirmed that some of the drugs elevated and some inhibited luciferase activity in the MCF7-AREc32 cell line (Figure 5.2). Collectively, these data showed that all the compounds tested altered NRF2-ARE signalling either by activating or by inhibiting it. The improved ability to identify potent inhibitors of NRF2 through in vitro screening described here improves the speed and cost associated with screening NRF2-ARE inhibiting compounds for drug development.

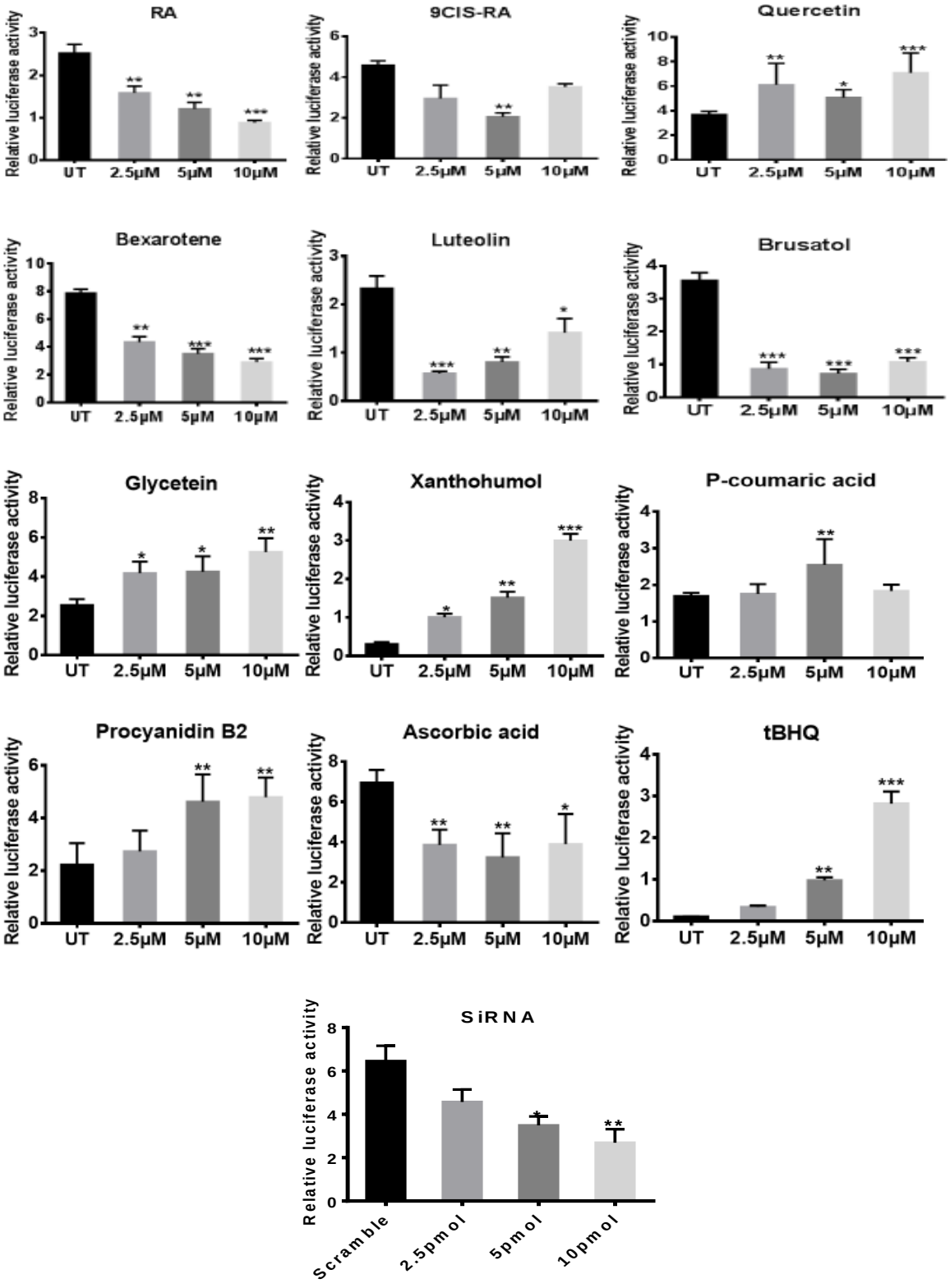

Figure 5.2: Screening of panel of compounds gives rise to activation and inhibition of NRF2 dependent antioxidant response pathway. Exponentially growing MCF7-AREc32 cell line stably expressing 8xcis-AREs driving the expression of luciferase gene in an NRF2 dependent manner were either left untreated (UT) or treated with different compounds at 2.5 µM, 5 µM or 10 µM. For siRNA treatment, cells were treated with scrambled or with NRF2 siRNA (2.5 pmol, 5 pmol, 10 pmol) for 24h. Following this, cell lysates were prepared and assayed for Luciferase activity (BrightGlo Luciferase system, Promega). Data are the mean values ± S.D of n=3 independent experiments performed in quadruplicates with statistical significance determined by ONE WAY ANOVA followed by Tukey's post hoc test according to the scale ** P <0.05, **P <0.01 ***P <0.001.

MCF7-AREc32 stable cell line carries a luciferase reporter gene under the control of the ARE. Both the basal and the inducible ARE-luciferase activities in these cells are primarily regulated by NRF2. Therefore, this cell line provides an ideal cell-based assay for screening for antagonists of NRF2.

Based on the results obtained in (Figure 5.2) and the context of the aim of screening to identify the compounds that show more potency towards inhibiting NRF2-ARE pathway, the compounds that appeared to be most promising were selected for further investigations as described below.

5.4.3. Potent NRF2-ARE inhibitors repress NRF2, pNRF2 and HO-1 and generate ROS

Although the beneficial effects of NRF2 activation have been demonstrated in various healthy cell systems, there is cumulative evidence demonstrating the dark side of NRF2 in cancers. NRF2 is reported to contribute tumour resistance to a variety of anti-cancer drugs through detoxification of drug-induced ROS thereby preventing cellular accumulation of drugs and reducing the efficacy the drugs (Choi and Kwak, 2016). Furthermore, ROS which is reported to be a regulator of NRF2 stability and activity have also been shown to trigger the ARE pathway, implicating the modulation of ROS level by NRF2 function (Khalil et al., 2016b). To investigate this matter, the compounds that inhibited the antioxidant transcriptional program, were further examined in MCF7-AREc32 cell lines for effects at the protein level and then determined their level of ROS generation capacity. Exposure of MCF7-AREc32 cell lines to these compounds at 2.5 µM caused a decrease in pNRF2, total NRF2 and HO-1 levels (Figure 5.3). Interestingly the decrease was more significant in the presence of bexarotene. The compounds-induced reduction in NRF2 levels suggests that they can be used in targeting NRF2. Next, using the same set of compounds, it was found that they also elevated ROS levels (Figure 5.4). These findings suggested that while these compounds inhibit NRF2 dependent ARE pathway, such treatment also elevates cellular ROS levels in MCF7-AREc32 cell lines. Importantly, treatment with bexarotene was found to significantly induce more ROS when compared to all the other compounds tested.

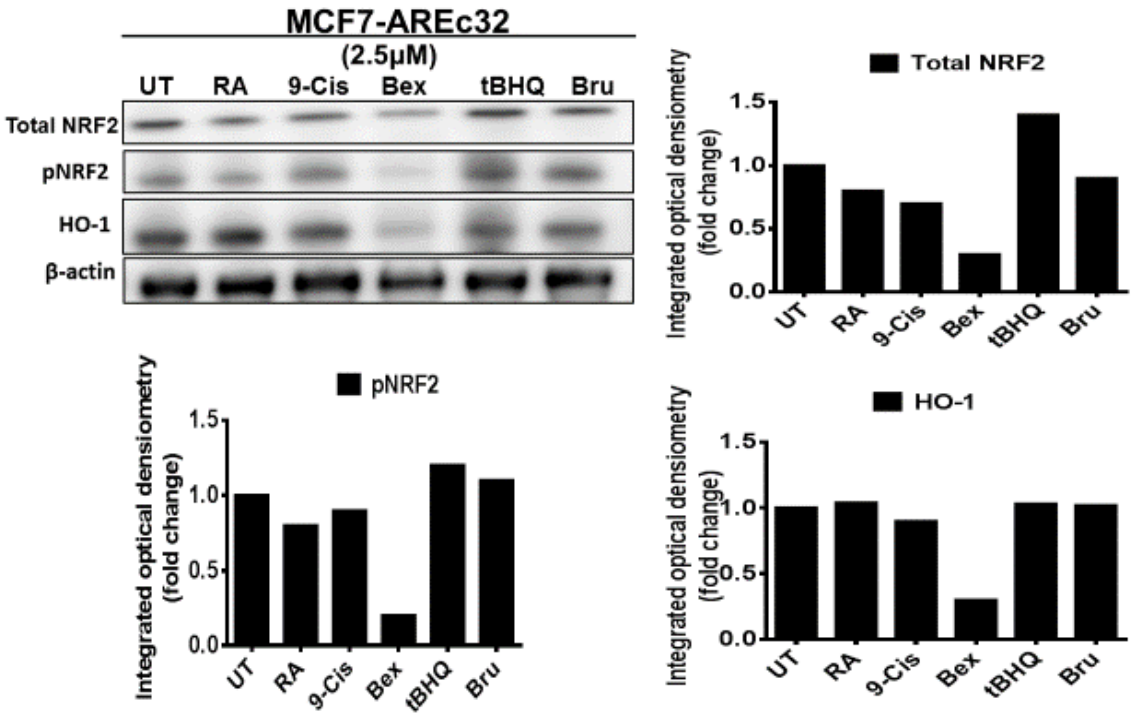

Figure 5.3: Treatments with panel of compounds causes inhibition of NRF2 dependent antioxidant response pathway and generates ROS. (A) Western analysis showing repression of NRF2, pNRF2 and HO-1 levels following the compounds treatment in MCF7-AREc32 cell lines. Exponentially growing cells were either left untreated or treated with 2.5 μM RA or 9-Cis or bexarotene or tBHQ or Brusatol for 24 h before being harvested to prepare protein lysates or processed as described in Materials and Methods. Beta-actin (β- actin) was used as loading control and bars indicate total NRF2, phosphor-NRF2 and HO-1 levels following quantification of immunoblot signal intensities obtained in (A) and normalized to the value of UT and expressed as fold change. The signal intensities of bands were quantified through integrated optical densitometry measurement. Images are representative of at least two independent experiments performed.

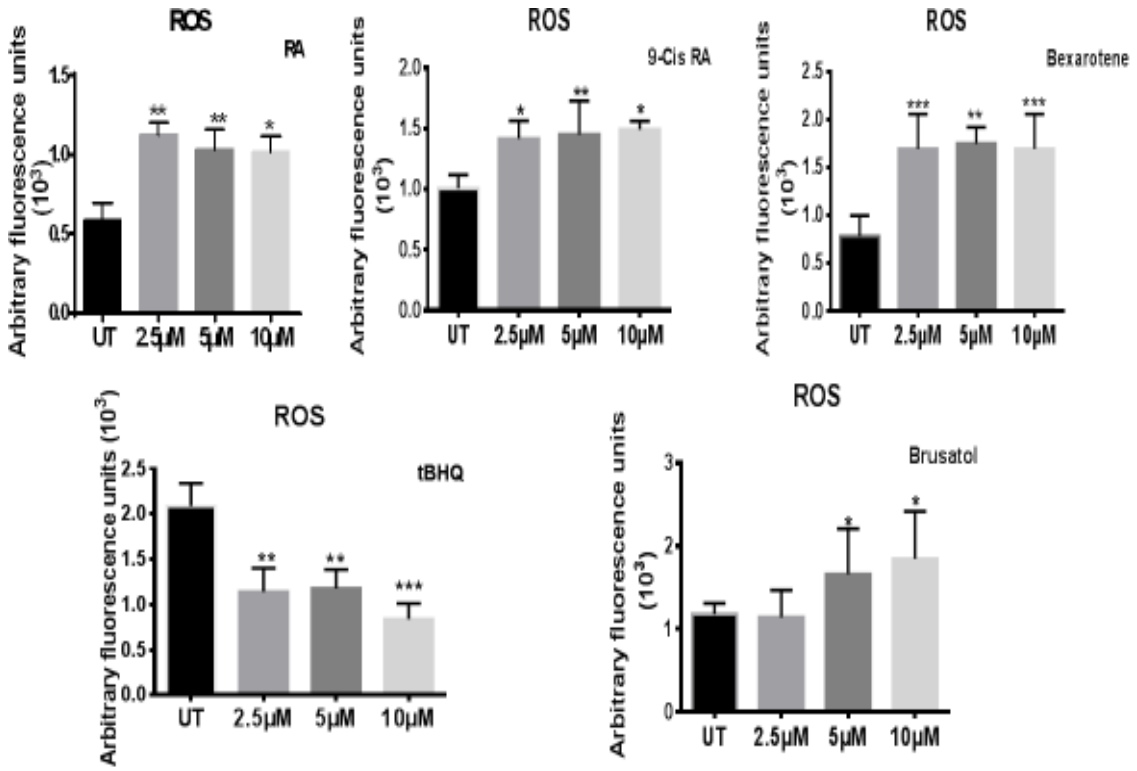

Figure 5.4 Treatment with panel of compounds causes persistent elevation of ROS in MCF7-AREc32. Exponentially growing MCF7-ARE cells were seeded in triplicates in opaque flat bottom black walled 96-well plates for 24 h. Following this, cells were either left untreated (UT) or treated with 2.5 μM RA or 9-Cis or bexarotene or tBHQ or Brusatol for 24h. Following incubations, cells were loaded with DCFDA fluorescent stain for 45 min and assayed for ROS as described in Materials and Methods. Data are the mean values ± S.D of n=3 independent experiments performed in quadruplicates with statistical significance determined by ONE WAY ANOVA followed by Tukey's post hoc test according to the scale ** P <0.05, **P <0.01 ***P <0.001.

5.4.4. Low-lethal dose of lapatinib and erlotinib causes repression of NRF2-dependent transcription and generation of ROS

Following the observation of the effect of the tested compounds on NRF2, the widely known RTK inhibitors lapatinib and erlotinib that inhibits HER receptor family pathway activation (Ryan et al., 2008, Rolfo et al., 2014, Geyer et al., 2006, Kuang et al., 2010), were then investigated after establishing their IC50 (15 μM and 20 μM respectively) to further ascertain whether they will inhibit NRF2-dependent transcription. To address this the MCF-AREc32 cell line, containing a stably expressing 8 copies of NRF2 dependent cis-regulatory AREs, as a luciferase reporter was used here as well. The results indicated that both lapatinib and erlotinib were toxic at the highest concentration (10 μM) (Figure 5.5A), and were able to inhibit the NRF2/ARE dependent transcription (Figure 5.5B) and generate ROS (Figure 5.5C) both at the low-lethal dose of 0-10 μM.

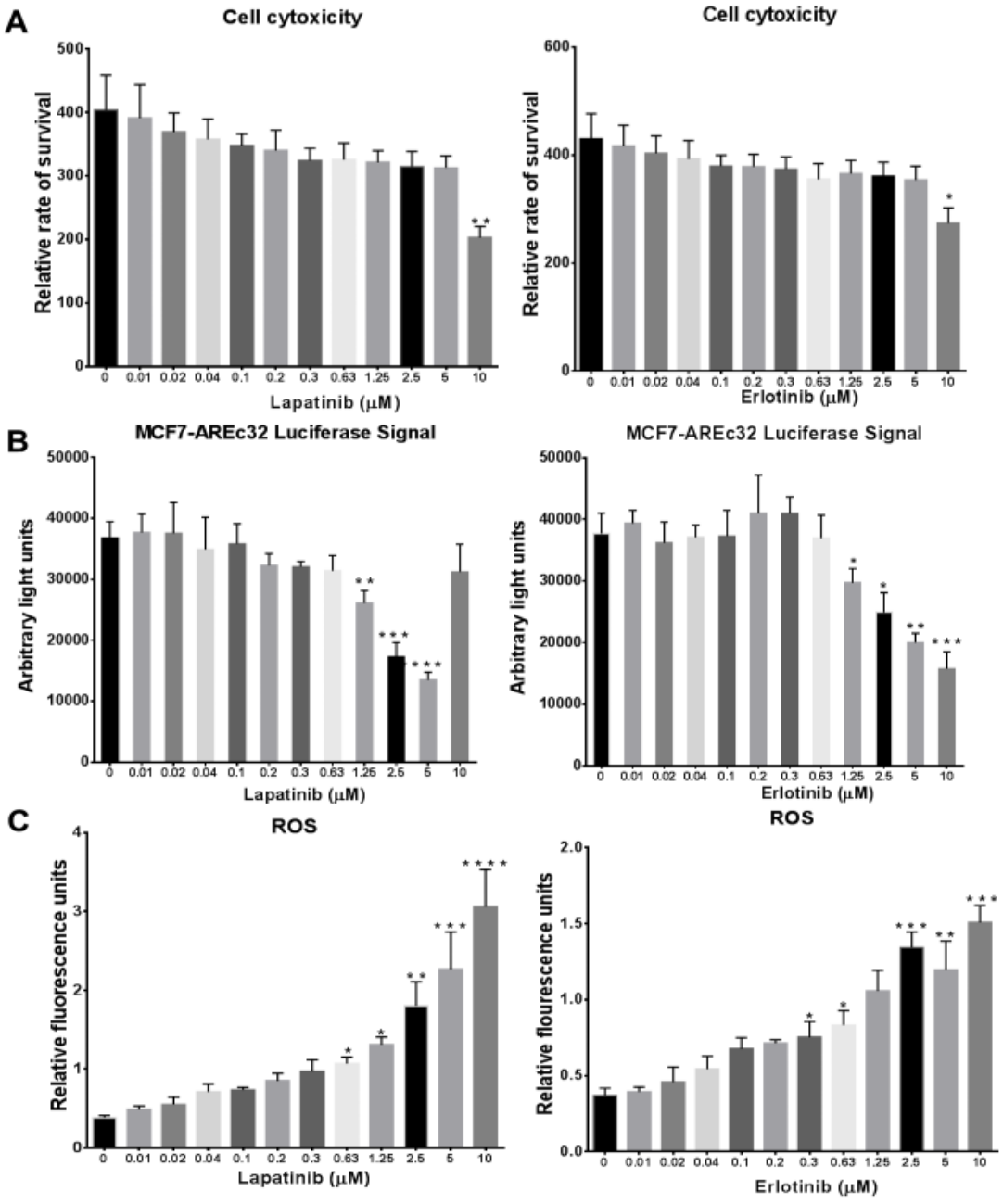

Figure 5.5: Treatment with low-lethal dose of lapatinib and erlotinib involves repression of NRF2-dependent transcription and generation of ROS. (A) Treatment with low-lethal dose of lapatinib and erlotinib in cell survival. Exponentially growing MCF7-AREc32 cells were seeded in triplicates in 96-well plates for 24 h, Following this, were treated with different concentrations of lapatinib and erlotinib (0-10 μM) as indicated, following this, cell number was assessed indirectly by use of the cell titre glo assay as described in Materials and Methods. (B) Treatment with low-lethal dose of lapatinib and erlotinib involves repression of NRF2-dependent transcription. Exponentially growing AREc32 cell line stably expressing cis-regulatory AREs driving the expression of luciferase gene in an NRF2 dependent manner were treated with different concentrations of lapatinib and erlotinib (0-10 μM). Following this, cell lysates were prepared and assayed for Luciferase activity as described in materials and method. (C) Treatment with sub-lethal dose of lapatinib and erlotinib involves generation of ROS. Exponentially growing MCF7-AREc32 cells were seeded in triplicates in opaque flat bottom black walled 96-well plates for 24 h. Following this, were treated with different concentrations of lapatinib and erlotinib (0-10 μM) as indicated. Following incubations, cells were loaded with DCFDA fluorescent stain for 45 min and assayed for ROS as described in Materials and Methods. Data shown are mean values ± S.D of n=3 independent experiments performed quadruplicates, normalized to untreated (UT) and expressed as fold change with statistical significance determined by ONE WAY ANOVA followed by Tukey's post hoc test according to the scale *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

Based on all the information obtained in (Figures 5..2 and 5.3), in addition to being in clinical use (Hatton and Yee, 2008, Farol and Hymes, 2004), bexarotene is chosen as the compound for further studies. Further, the cell viability assays of lapatinib and erlotinib (as RTK inhibitors) and bexarotene (as NRF2 inhibitor) were performed in PEO1, OVCAR3 and PEO1 ovarian cancer cells in order to choose the required concentrations for subsequent studies.

5.4.5. Cell viability of lapatinib, erlotinib and bexarotene in ovarian cancer

To determine the required concentration of these drugs (lapatinib, erlotinib, and bexarotene) for further use, exponentially growing PEO1, OVCAR3 and SKOV3 cell lines were either left untreated (DMSO vehicle) or treated with different concentrations of the tested compounds, lapatinib (Figure 5.6A), erlotinib (Figure 5.6B) and bexarotene (Figure 5.6C). It was noticed that almost all the compounds are less toxic to the range between 0-10 µM and therefore that range of concentration was chosen for further studies.

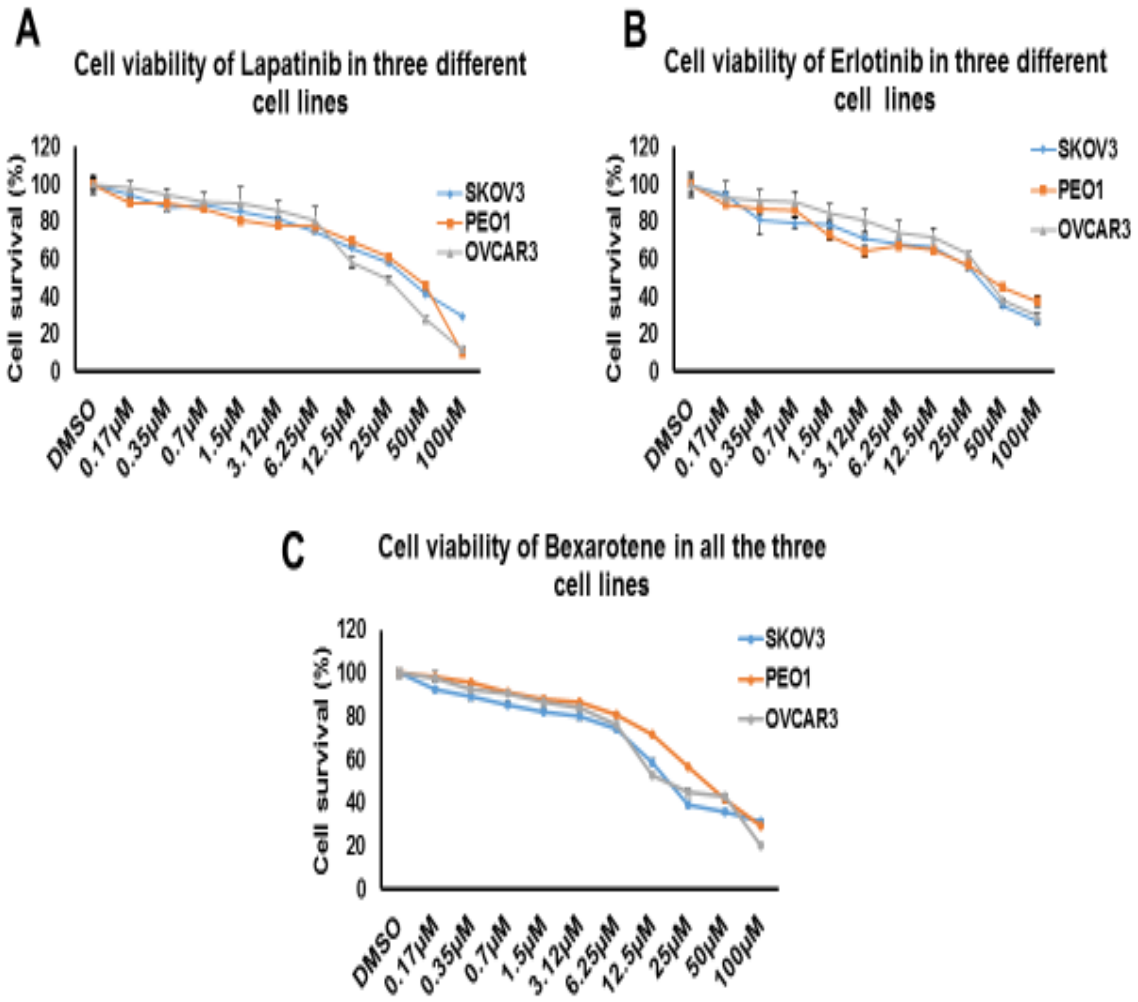

Figure 5.6: Cell viability assay: (A) lapatinib (B) erlotinib and (C) bexarotene in three ovarian cancer cells, SKOV3, PEO1 and OVCAR3. Cell viability assay was performed in order choose the less toxic consent rations for further studies. Briefly, exponentially growing cells were seeded in a 96-well plate and allowed to adhere for 18-24 h. Following 72 h treatments using different concentrations of the drugs, the plate and its contents were equilibrated to room temperature for approximately 30 min, a volume of CellTiter-Glo 2.0 reagent equal to the volume of cell culture medium present in each well the contents were then mixed for 2 min on an orbital shaker to induce cell lysis and the plate was then incubate at room temperature for 10 min to stabilize the luminescent signal and finally the luminescence was recorded using luminometer (MODULUS, Promega). The luminescent signal which is proportional to the amount of ATP in the sample, also which indicates the presence of metabolically active cells is expressed in %.

5.4.6. Treatment with lapatinib, erlotinib and bexarotene cause transcriptional downregulation of HER receptor family in ovarian cancer.

Following the determination of cytotoxicity of lapatinib, erlotinib and bexarotene in PEO1, OVCAR3 and SKOV3 cell lines, further studies were undertaken to, investigate the effects these drugs on the transcription of *HER1*, *HER2*, *HER3* and *HER4* genes. To address this, the cloned promoter regions of *HER1*, *HER2*, *HER3* and *HER4* genes as luciferase-based reporter assays (known as prHER1,

prHER2, prHER3, and prHER4 respectively) were used. These reporters were used to directly report and measure any transcriptional perturbation of the HER family receptors. The prHER1, prHER2, prHER3, prHER4 were transfected into the ovarian cancer cell lines, treated the cells with the drugs for 96 h, and then assayed for luciferase activity (Figures 5.7, 5.8 and 5.9). Untreated cells or cells treated with tBHQ were used as controls. These experiments clearly demonstrated transcriptional inhibition of *HER1*, *HER2*, *HER3* and *HER4* genes by all the three drugs tested.

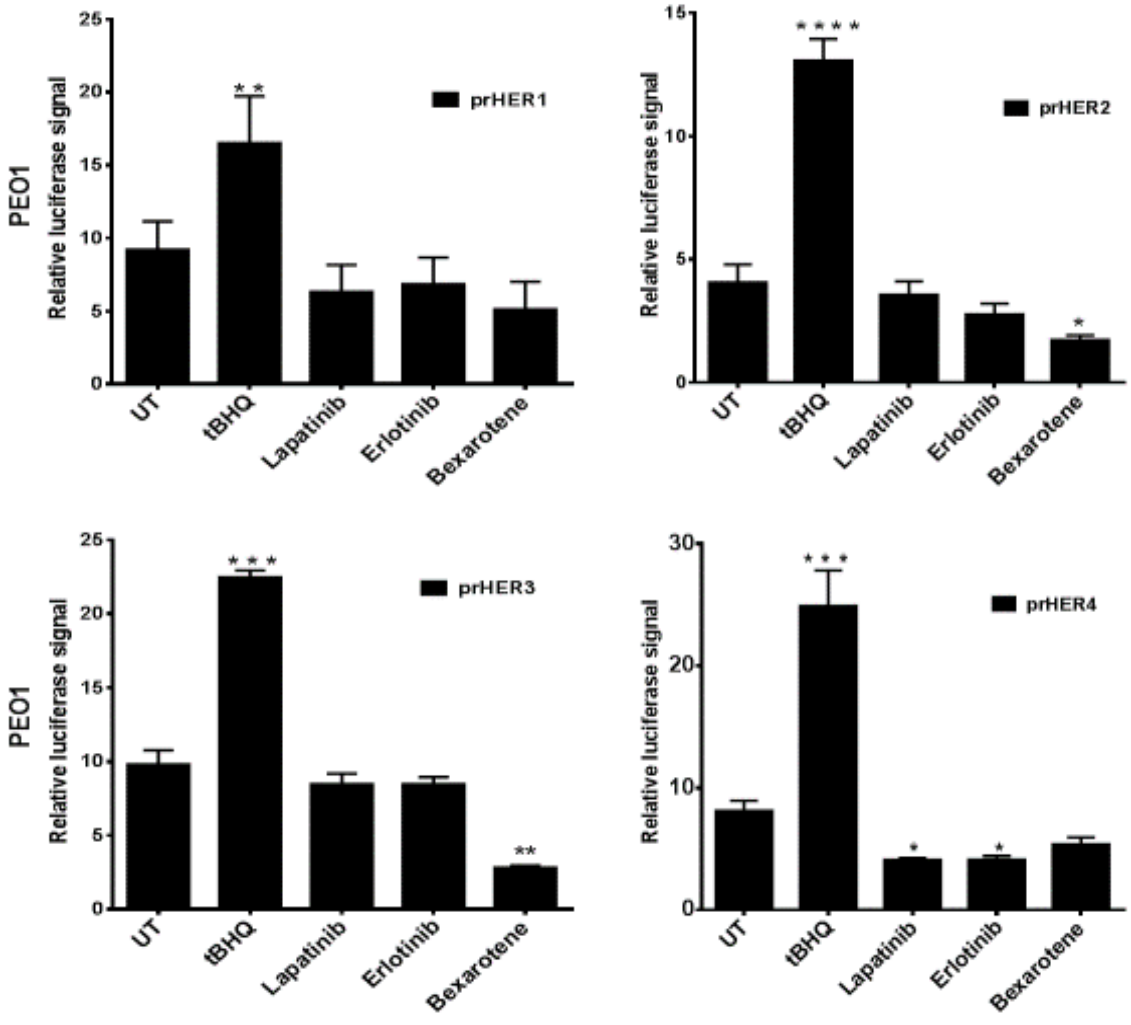

Figure 5.7: Lapatinib, erlotinib and bexarotene causes transcriptional downregulation of HER1, HER2, HER3 and HER4 in PEO1 cell line. Briefly cells were transfected in quadruplicate with either empty PGL3 basic vector or 1 μg PGL3 basic vector with cloned fragments of either HER1 (prHER1) or HER2 (prHER2) or HER3 (prHER3) or HER4 (prHER4) promoter driving the expression of luciferase gene for 24 h. Co-transfection with 0.2 μg pRL-CMV plasmid was performed as an internal transfection control. Following this, cells either left untreated or treated with 100 µM tBHQ or 5 µM each of either lapatinib or erlotinib or 2.5 µM bexarotene for 24 h. Following this, cell lysates were prepared and assayed for Luciferase activity (luciferase assay and stop and glo Luciferase system, Promega). Data presented are the means ± S.D. of n = 3; independent experiments performed in quadruplicates with statistical significance determined by ONE WAY ANOVA followed by Tukey's post hoc test according to the scale *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

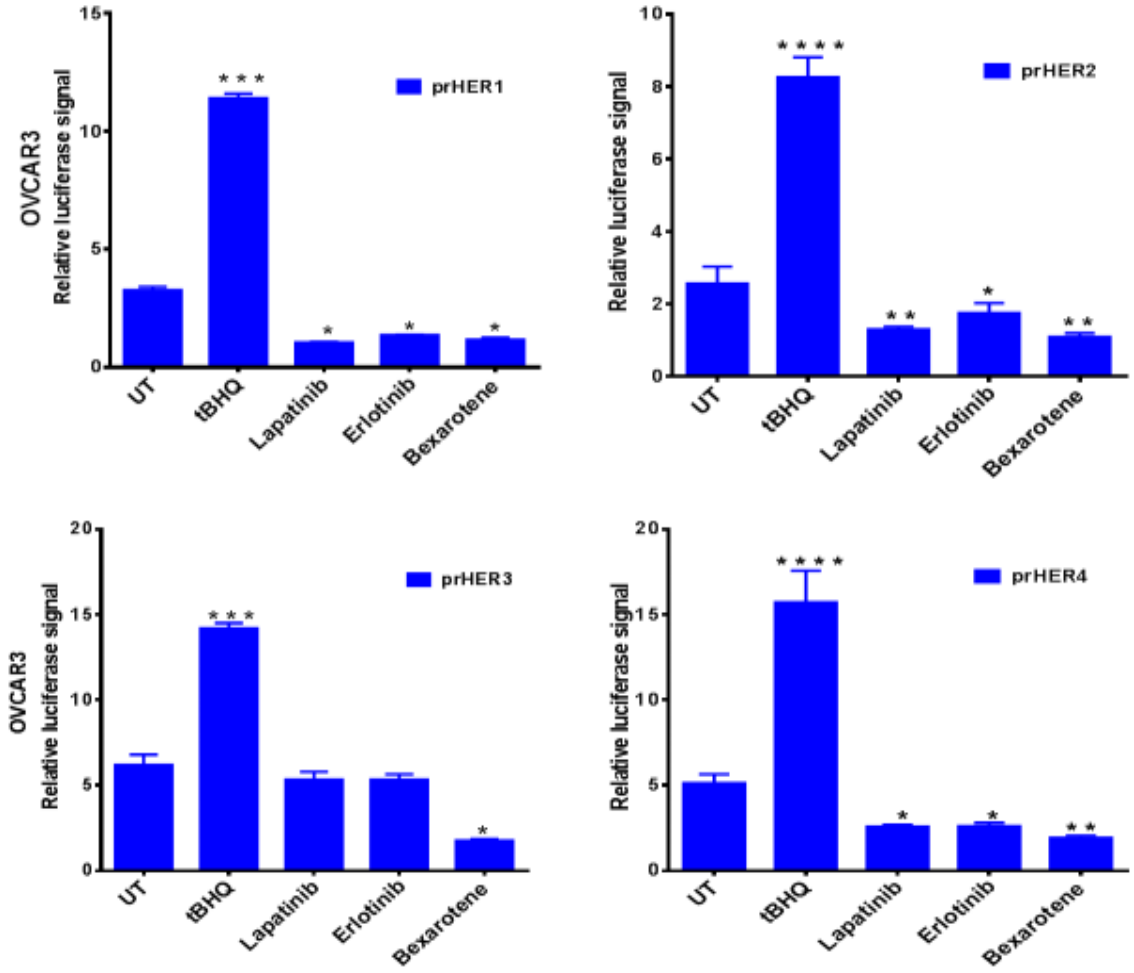

Figure 5.8: Lapatinib, erlotinib and bexarotene causes transcriptional downregulation of HER1, HER2, HER3 and HER4 in OVCAR3 cell line. Briefly cells were transfected in quadruplicate with either empty PGL3 basic vector or 1 μg PGL3 basic vector with cloned fragments of either HER1 (prHER1) or HER2 (prHER2) or HER3 (prHER3) or HER4 (prHER4) promoter driving the expression of luciferase gene for 24 h. Co-transfection with 0.2 μg pRL-CMV plasmid was performed as an internal transfection control. Following this, cells either left untreated or treated with 100 µM tBHQ or 5 µM each of either lapatinib or erlotinib or 2.5 µM bexarotene for 24 h. Following this, cell lysates were prepared and assayed for Luciferase activity (luciferase assay and stop and glo Luciferase system, Promega). Data presented are the means ± S.D. of n = 3; independent experiments performed in quadruplicates with statistical significance determined by ONE WAY ANOVA followed by Tukey's post hoc test according to the scale *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

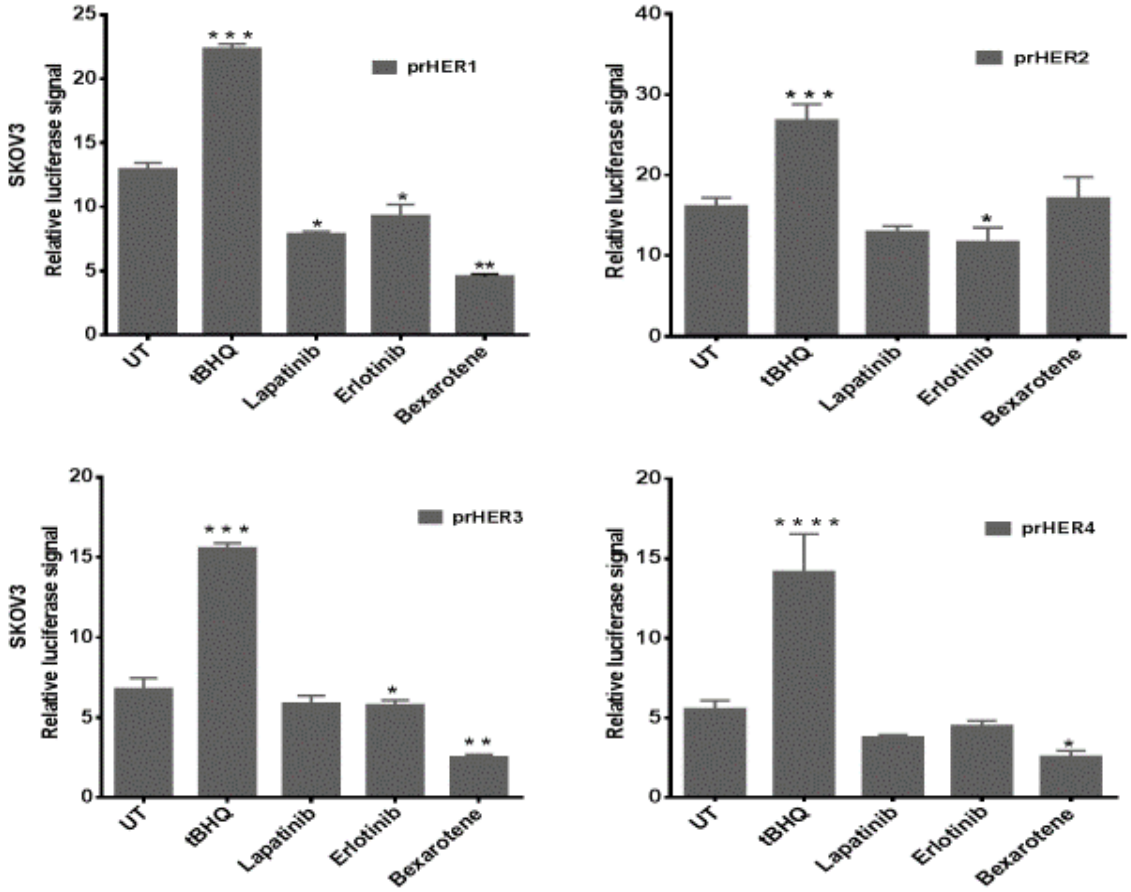

Figure 5.9: Lapatinib, erlotinib and bexarotene causes transcriptional downregulation of HER1, HER2, HER3 and HER4 in SKOV3 cell line. Briefly cells were transfected in quadruplicate with either empty PGL3 basic vector or 1 μg PGL3 basic vector with cloned fragments of either HER1 (prHER1) or HER2 (prHER2) or HER3 (prHER3) or HER4 (prHER4) promoter driving the expression of luciferase gene for 24 h. Co-transfection with 0.2 μg pRL-CMV plasmid was performed as an internal transfection control. Following this, cells either left untreated or treated with 100 µM tBHQ or 5 µM each of either lapatinib or erlotinib or 2.5 µM bexarotene for 24 h. Following this, cell lysates were prepared and assayed for Luciferase activity (luciferase assay and stop and glo Luciferase system, Promega). Data presented are the means ± S.D. of n = 3; independent experiments performed in quadruplicates with statistical significance determined by ONE WAY ANOVA followed by Tukey's post hoc test according to the scale *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

5.5. Key findings

In this section, ten compounds that alter NRF2/ARE signalling in the MCF7-AREc32 reporter cell line were investigated. The investigation included cytotoxicity screening and the ability of the compound to either cause production or sequestration of ROS. The results indicated that these compounds were able to engage and the NRF2-KEAP1 either by positive or negative feedback loop directly to stimulate or disrupt NRF2 activity and function (Figures 5.2 and 5.3). The data also indicated compounds such as RA, 9-cis RA, brusatol and

bexarotene inhibited elevated the level of ROS (Figure 5.4) and then lapatinib, erlotinib and bexarotene downregulated the transcriptional level of HER family receptors in ovarian cancer cell (Figures 5.7, 5.8 and 5.9). This work has demonstrated the feasibility of using bexarotene to treat ovarian cancers.

6. CHAPTER SIX

NRF2 co-regulates the HER family receptors to Modulate the Efficacy of Lapatinib and Erlotinib as RTK Targeting Chemotherapeutics in Ovarian Cancer

6.1 Abstract

In the previous chapters, it is observed that tBHQ and retinoid treatment led to transcriptional upregulation and downregulation of HER1 and HER4, suggesting that NRF2 is involved in regulating the receptor expression and as such may influence responses to targeted therapies involving HER RTK inhibitors, lapatinib and erlotinib. This important question was investigated by treating PEO1, OVCAR3, SKOV3 and MCF7-AREc32 cells with, either lapatinib or erlotinib alone, or by co-treatment with tBHQ and/or bexarotene to examine the consequences of NRF2 activation and/or inhibition on drug responses. It is found that pharmacological activation of NRF2 by tBHQ and/or inhibition by bexarotene alone was sufficient to enhance or reduce the proliferation of all the cell lines for up 72 h. Further, exposure of cells to lapatinib and erlotinib, inhibited the proliferation of all the cell lines for up to 72 h of treatment, with slight loss of inhibitory effect at 96 h. Interestingly however, pre-treatment of cells with 100 μM tBHQ for 5 h before the introduction of the HER family RTK inhibitors significantly protected cells from the growth inhibitory action of the subsequently added drugs. Moreover, pre-treatment of cells with 2.5 μM bexarotene for 5 h before the introduction of HER family RTK targeted drugs significantly increases their cytotoxicity. This was consistent in all the cell lines and for all the treatment days tested. The combination of tBHQ with the inhibitors did not only protect the cells but increased their survival even beyond the untreated levels on all days in all the cell lines. The combination of bexarotene with the inhibitors increases the cytotoxicity of the drugs and decreased the survival of the ovarian cancer cells far below the untreated level. This demonstrated that NRF2 activation is not only implicated in resistance to other agents as previously demonstrated but can also lead to resistance to chemotherapies involving lapatinib and erlotinib whose actions otherwise are very specific to HER family receptors with no clear relation to antioxidant response element. Thus, blockade of the cellular anti-oxidative system by NRF2 inhibition significantly augmented the lapatinib and erlotinib cytotoxicity and can potentially overcome resistance to therapies involving lapatinib and erlotinib. Taken together, it is demonstrated that bexarotene alone and its combination with either lapatinib or erlotinib exerts inhibition of the NRF2 to improve ovarian cancer treatment for a better outcome.

6.2. Introduction

There are several studies that highlight the relationship between ROS and NRF2 and the involvement of NRF2-ARE and HER receptor family pathways (Khalil et al., 2016a, Khalil et al., 2016b, Manandhar et al., 2012, Kang et al., 2014b). NRF2 is known to promote resistance to chemotherapeutic agents, in addition to its contribution to general cytoprotection, metabolic reprograming, and cell survival (Hayes and McMahon, 2009, Hayes and Ashford, 2012, Hayes et al., 2015, Wang et al., 2007, Tang et al., 2011). Moreover, targeted chemotherapy involving RTKi against HER family receptors has generated interest as a strategy to overcome chemoresistance in breast, ovarian, and other forms of cancers (Khalil et al., 2016b, Goltsov et al., 2014a, Khalil et al., 2016a, Langdon et al., 1988, Langdon et al., 2010, Langdon and Cameron, 2013, Mullen et al., 2007, Tang et al., 2011, Tu et al., 2013, Messersmith and Ahnen, 2008, Paez et al., 2004, Regales et al., 2009).

Studies have examined the crosstalk between growth promoting MAPK and PI3K pathways and NRF2 antioxidant pathways in numerous cell systems (Kang et al., 2005, He et al., 2012, Kang et al., 2000, Zipper and Mulcahy, 2000, Khalil et al., 2016a). However, in the majority of such studies, the focus was on the regulation of NRF2 activity and its functions by these kinases. While the interaction and complex formation of NRF2 with HER2 has been reported to enhance HER signalling (Kang et al., 2005, He et al., 2012, Kang et al., 2000, Zipper and Mulcahy, 2000), studies by Khalil et al., (2016a and 2016b) recently demonstrated the transcriptional regulation of HER2 and HER3 by NRF2 (Khalil et al., 2016b, Khalil et al., 2016a). Further, they demonstrated a novel relationship between NRF2 function, HER2/HER3 signalling, ROS generation and the sensitisation of ovarian cancer cells to the killing effects of the targeted therapy agents, trastuzumab, pertuzumab, or combination of the two (Khalil et al., 2016b, Khalil et al., 2016a). Interestingly, the work here (in chapters 4, 5 and 6) and (Khalil et al., 2016b) suggests that all the members of the HER family receptors are regulated by NRF2 in ovarian cancer cells. Furthermore, a mechanism of action of lapatinib and erlotinib, like trastuzumab, pertuzumab (Khalil et al., 2016b), likely involves inhibition of NRF2-ARE function, accumulation of ROS and

the subsequent killing of the cancer cells. This led us to hypothesize that inhibition of NRF2 function and concomitant cellular accumulation of ROS are possible mechanistic components and basis of action of HER2-targeted small molecule RTK inhibition. This may equally be the basis and a contributing determinant of resistance to RTK inhibitors like lapatinib and erlotinib.

6.3. Aims and objectives

6.3.1 The aim

The main aims of this chapter are:

1. To examine whether a novel mechanism of action of lapatinib and erlotinib, as RTK inhibitors, involves NRF2 inhibition and the accumulation of ROS to sensitise ovarian cancer cells to the killing effect of RTKi chemotherapeutic drugs.
2. To examine and develop novel interventions that may be more effective to treat and overcome chemoresistance to RTK inhibition in ovarian cancer.

6.3.2 The objectives are:

1. To investigate whether the mechanism of action of RTK inhibitors, lapatinib and erlotinib, involves the modulation of NRF2 function in ovarian cancer cells
2. To investigate the effectiveness and the enhancement of the cytotoxic action of lapatinib and/or erlotinib and when combined with additional NRF2 inhibitor
3. To identify the connectivity of NRF2 and HER family receptor pathways that will open up for a novel strategy of improving cancer treatment.

6.4. Results

6.4.1. Pharmacological activation of NRF2 by tBHQ enhances cellular survival and attenuates the cytotoxicity caused by HER family targeting RTK inhibitory chemotherapeutic agents in ovarian cancer

NRF2 is shown to promote resistance to chemotherapeutic agents and to increase the proliferation and survival of cancer (Hayes and McMahon, 2009, Hayes and Ashford, 2012, Hayes et al., 2015, Wang et al., 2007, Tang et al., 2011). However, several chemotherapeutic drugs including lapatinib and erlotinib are used to treat HER family overexpressing cancers including ovarian cancer (Kuang et al., 2010, Roskoski Jr, 2014, Geyer et al., 2006, Ryan et al., 2008). In this study, cell lines, PEO1, SKOV3 and OVCAR3 which are known to have different expressions of HER family and NRF2 (Khalil et al., 2015, Khalil et al., 2016a, Mullen et al., 2007) were used. Firstly, it is examined whether pharmacological preactivation of NRF2 would change the cytotoxic responses of ovarian cells to lapatinib (5 μM) or erlotinib (5 μM). In order to achieve this, cells were grown in media containing 5% charcoal-stripped FBS and 1 nmol/L Heregulin (HRG) and then all the relevant treatments were done. Pharmacological activation of NRF2 by tBHQ alone was sufficient to enhance the proliferation of all the cell lines at all the time points (24 h, 48 h, 72 h and 96 h) (Figure 6.1). However, and as expected, exposure of these cells to the RTK inhibitors, lapatinib or erlotinib, inhibited the proliferation of all the cell lines for up to 72 h of treatment, while each drug slightly loses its inhibitory effect after 96 h. Interestingly, pretreatment of cells with 100 μM tBHQ for 5 h before the introduction of either lapatinib or erlotinib significantly protected cells from the growth inhibitory action of the subsequently added RTK inhibitor. These were consistent in all the 3 cell lines and for all the treatments time points tested (Figure 6.1). Thus, combining tBHQ with the RTK inhibitors did not only protect the cells but also enhanced their proliferation and survival significantly in all cell lines (Figure 6.1). Therefore, this indicated that NRF2 and its activation is modulating the action and effectiveness of the RTK inhibitors, lapatinib and erlotinib. Thus, NRF2 is not only implicated in resistance to agents as previously demonstrated (Hayes and McMahon, 2009, Hayes and Ashford, 2012, Hayes et

al., 2015, Wang et al., 2007, Tang et al., 2011) but can also may be mediating the resistance to chemotherapies involving lapatinib and erlotinib whose actions are more specific to HER receptors than NRF2-KEAP-1 or ARE pathway.

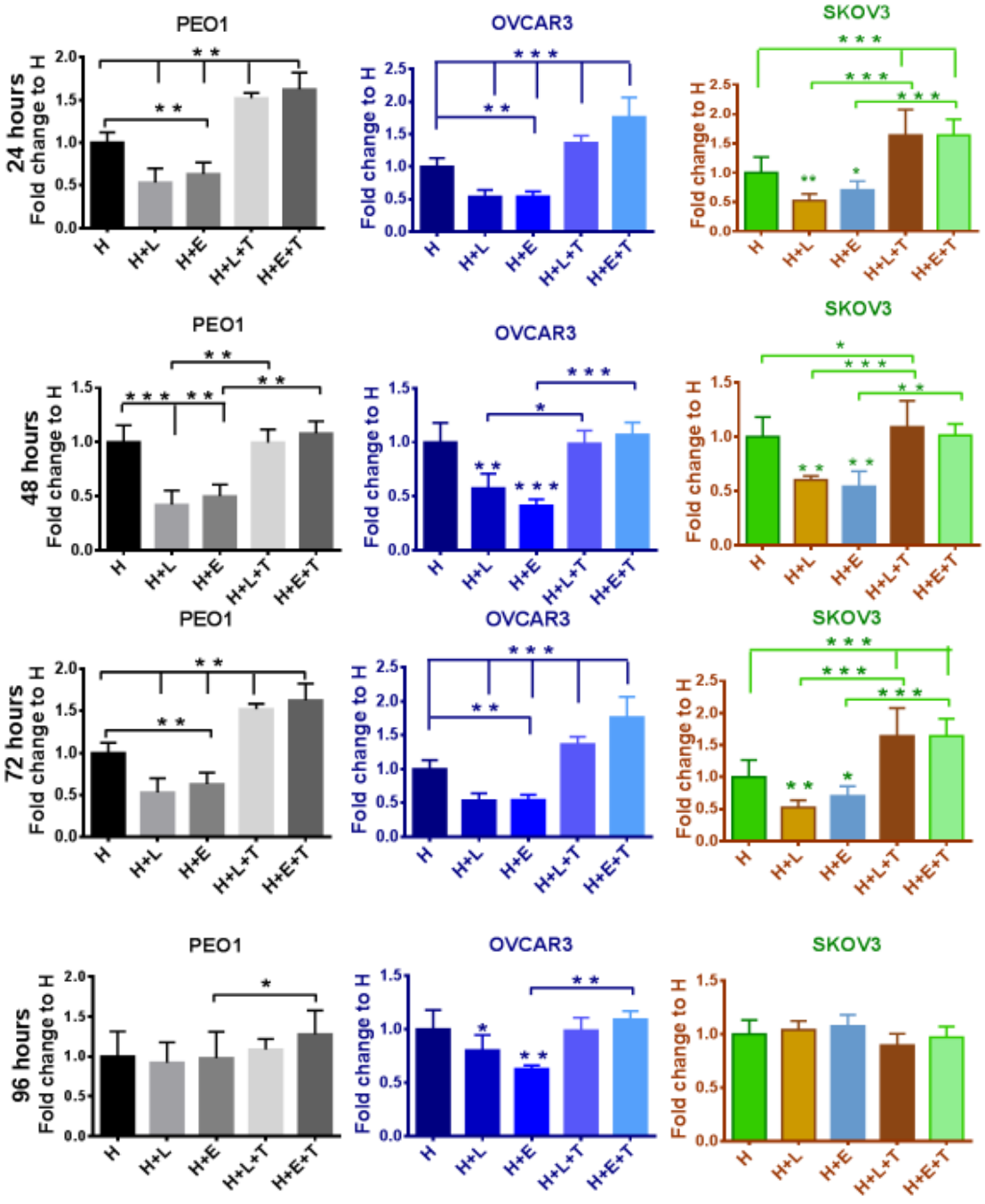

Figure 6.1: NRF2 activation causes cytoprotection from HER family chemotherapeutic agents, lapatinib and erlotinib in ovarian cancer cells. Briefly, exponentially growing cells were seeded in triplicates in 96-well plates for 24 h. Following this, cells were either left untreated in media containing 1nM Heregulin (H) or treated with same media containing in the presence of 1 nM HRG with 5 μM each of lapatinib (H+L) or erlotinib (H+E) or treated with combination 5μM lapatinib and 100 μM tBHQ (H+L+T) or combination of 5 μM erlotinib and 100 μM tBHQ (H+E+T) at different time points. Cell number was assessed by use of the cell titre glo assay. Data shown are means ± S.D. of n= 3 independent experiments performed in triplicates, normalised to (H), expressed in fold change with statistical significance was calculated by ONE WAY ANOVA followed by Tukey's post hoc test according to the scale :(*: p<0.05, **: p<0.01, ***:p<0.001, and ****:p<0.0001).

6.4.2. Pharmacological inhibition of NRF2 by bexarotene reduces the proliferation and survival of Ovarian Cancer Cells by increasing the Cytotoxicity Caused by HER Family RTK Targeted Chemotherapeutic Agents

The transcriptional control of HER family by NRF2 and the way that NRF2 hindrance prompted to the transcriptional constraint of these kinases family gives a vital way to sensitisation towards drugs that will thus be more lethal to the ovarian tumour (Khalil et al., 2016b, Khalil et al., 2016a).

To address this and to further confirm that NRF2 is modulating the cytotoxic action and effectiveness of lapatinib and erlotinib, cells (PEO1, OVCAR3 and SKOV3) were grown in media containing 5% charcoal-stripped FBS and 1 nmol/L HRG and then exposed to lapatinib (5 μM) or/and erlotinib (5 μM) alone or their combination with bexarotene (2.5 μM) (Figure 6.2). First, it was found that treatment with lapatinib alone or erlotinib alone causes cytotoxicity compared to the untreated in all the cell lines. Importantly, bexarotene co-treatment greatly enhanced both lapatinib and erlotinib cytotoxicity in all the cell lines resulting in lower cell survival in comparison with their corresponding single treatments alone at all the time points (24 h, 48 h, 72 h, 96 h) with a regain of survival at 96 h. These important results demonstrated that bexarotene treatment, which was shown to repress NRF2 activity and disrupt the antioxidant response pathway reduces ovarian cancer cell proliferation and survival by increasing the cytotoxicity caused by lapatinib and erlotinib. This confirms a role for NRF2 in modulating the action and effectiveness of RTK inhibitors, lapatinib and erlotinib, and makes this a strategy representing a novel finding by which ovarian cancer cell proliferation and survival could be diminished and eradicated.

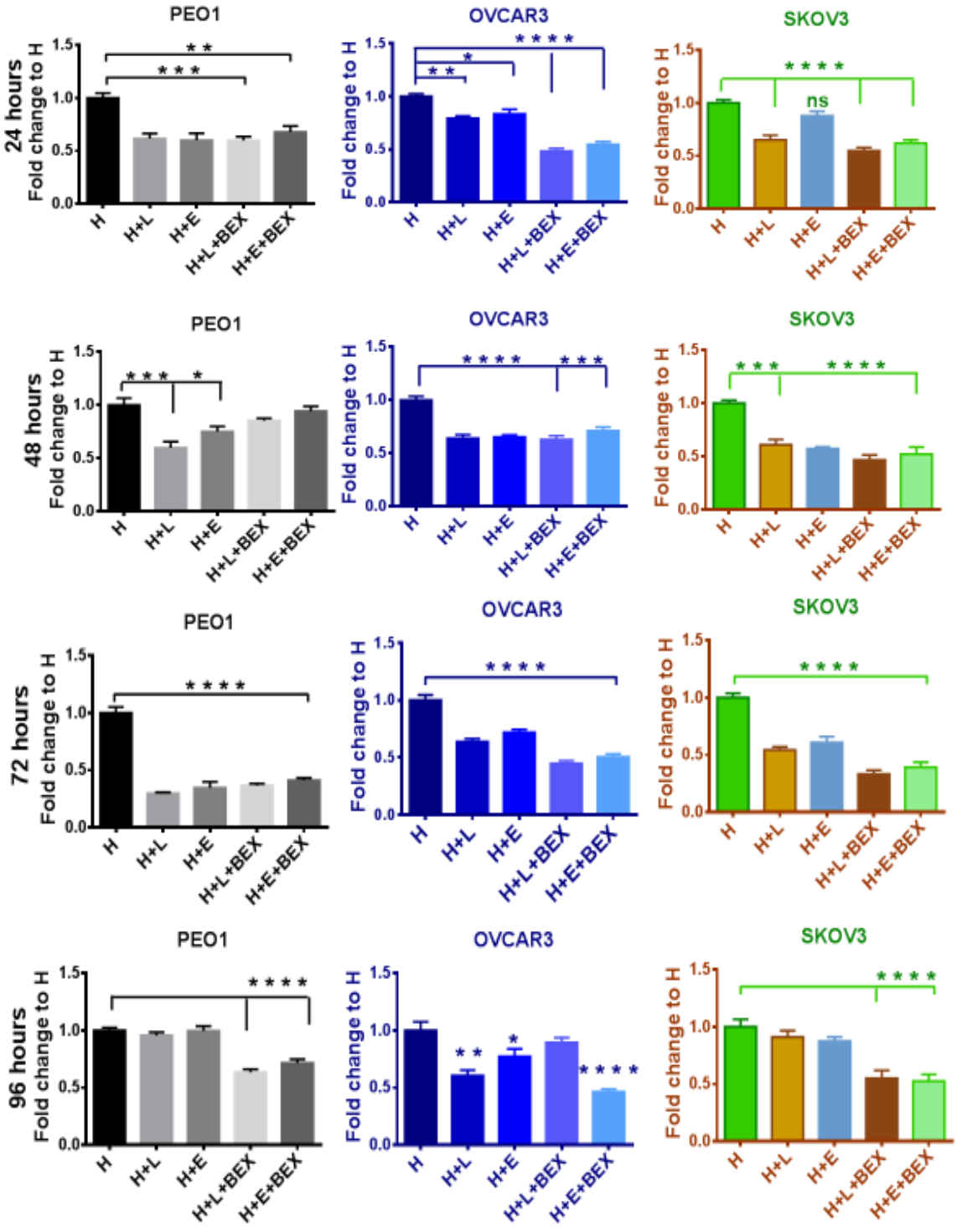

Figure 6.2: NRF2 inhibition increases the chance of cytotoxicity of HER family-targeted agents, lapatinib and erlotinib in ovarian cancer cells. Briefly, exponentially growing cells were seeded in triplicates in 96-well plates for 24 h. Following this, cells were either left untreated in media containing 1 nM Heregulin (H) or treated with same media containing in the presence of 1 nM HRG with 5 μM each of lapatinib (H+L) or erlotinib (H+E) or treated with combination 5 μM lapatinib and 2.5 μM Bexarotene (H+L+BEX) or combination of 5 μM erlotinib and 2.5 μM Bexarotene (H+E+BEX) at different time points. Cell number was assessed by the use of the cell titre glo assay. Data shown are means ± S.D. of n=3 independent experiments performed in triplicates, normalised to (H), expressed in fold change with statistical significance was calculated by ONE WAY ANOVA followed by Tukey's post hoc test according to the scale :(*: p<0.05, **: p<0.01, ***: p<0.001, and ****:p<0.0001).

6.4.3 Chemotherapeutic action of lapatinib and erlotinib involves generation of ROS in ovarian cancer

The biochemical and physiological oxidative processes in the body lead to the production of ROS which plays a major role in the initiation and progress of various human diseases which are associated with numerous physiological and pathophysiological processes. ROS has a vital effect when it is in low amount in the cell; a role that involves regulating intracellular signalling and homeostasis, processes that favour cell growth, cell division and survival (Prasad et al., 2017). Moreover, studies have illustrated the co-modulatory role and interaction of ROS with HER family receptors and other growth promoting pathways (Khalil et al., 2016a). Based on the observation in (Figures 6.1 and 6.2) and the previous work reported by Khalil et al. (2016a and 2016b) it is further hypothesised that the cytotoxic action of RTK inhibition targeting HER receptor (lapatinib and erlotinib) involves cellular accumulation of ROS concomitant to the disruption of NRF2 and its function.

To address this hypothesis using lapatinib and erlotinib, firstly total ROS levels in basal, HRG stimulated and drug-inhibited states in all three cell lines were studied. Here as well, HRG which is known to be a potent ligand for HER receptors, was used. The data in Figure 6.3A illustrates that HRG stimulation alone led to a significant increase in ROS levels in all three cell lines as compared to basal levels in unstimulated cells. Moreover, it is seen that treatments with lapatinib, erlotinib, their combination or their combination with bexarotene led to ROS generation in all the ovarian cancer cell line models. ROS elevation was seen at all the time points (24 h, 48 h, 72 h and 96 h) tested with an observed elevation of ROS being differential in cell- and time-dependent fashion (Figure 6.3B).

Investigation of the single drug treatment (lapatinib or erlotinib), in all the cell lines (Figure 6.3B), showed that lapatinib often generated more ROS than erlotinib, while their combination failed to generate significant higher levels of ROS than their singular administration at all the time points investigated. However, the fact that administration of these drugs led to the generation of ROS (Figure 6.3B), suggests ROS could be a contributing factor in cellular cytotoxicity of lapatinib

and erlotinib and implicates the engagement of AR pathway and inhibition of NRF2 function during drug action. Thus, it is next sought to investigate the status of the NRF2-ARE antioxidant response of cells following lapatinib and erlotinib treatments.

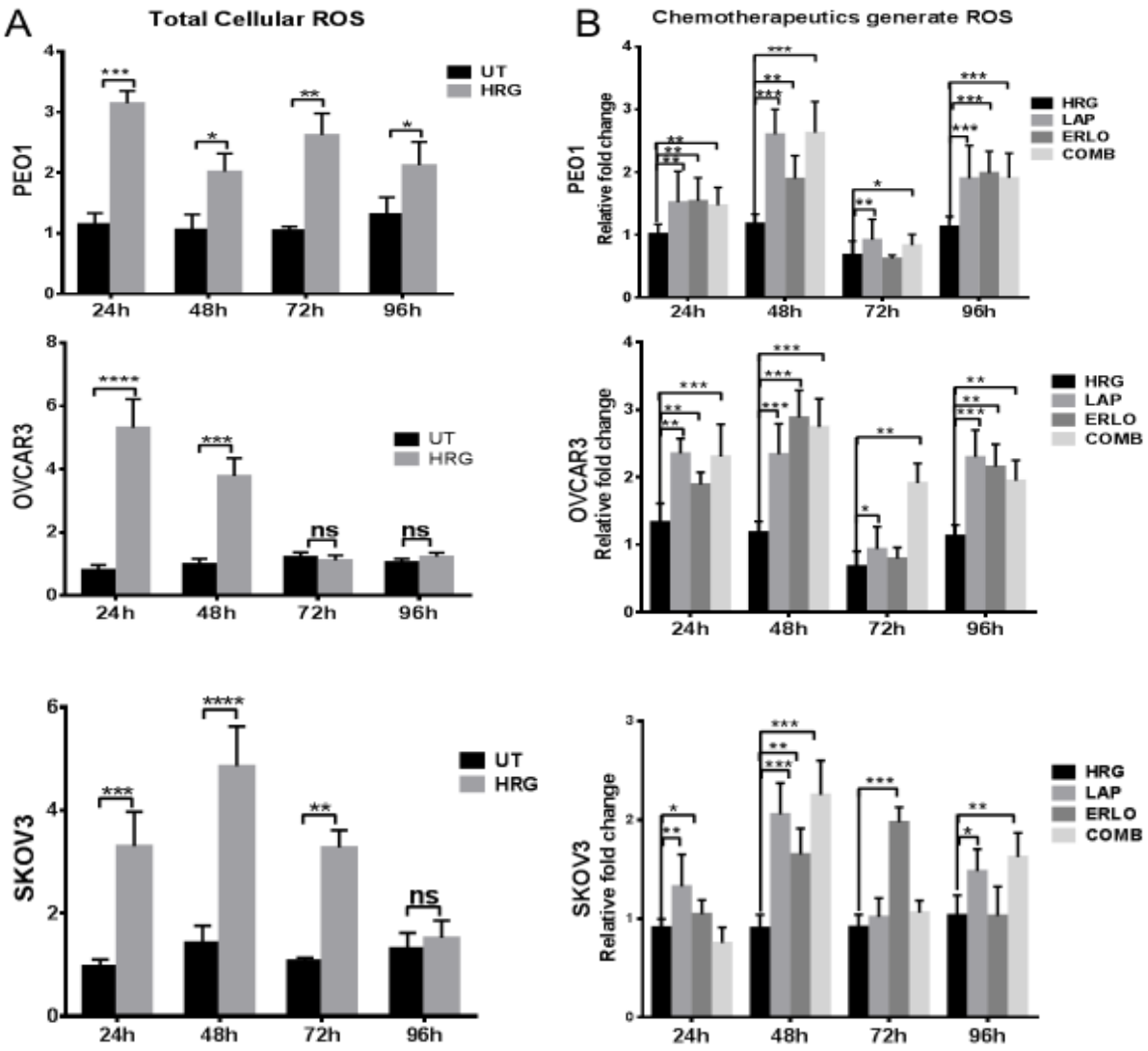

Figure 6.3: Chemotherapeutic treatment with lapatinib and erlotinib generates reactive ROS in ovarian cancer cells: **(A)** Heregulin treatment causes persistent elevation of ROS in ovarian cancer cells. Exponentially growing cells were seeded in triplicates in opaque flat bottom black walled 96-well plates for 24 h. Following this, cells were either left untreated (UT) or treated with 1 nM Heregulin for different time points as indicated. Following incubations, cells were loaded with DCFDA fluorescent stain for 45 min and assayed for ROS as described in Materials and Methods. **(B)** Lapatinib, erlotinib and their combination cause ROS generation. Cells were seeded as in **(A)** and treated with either 1 nM HRG alone or with co-treatment of 5 µM lapatinib (LAP), erlotinib (ERLO) or their combination (COMB) for different time points as indicated and ROS assay was repeated. For both **(A)** and **(B)**, the fluorescence reading recorded from each well was normalized to total cell abundance within the same wells using Bradford assay as described in Materials and Methods. Data shown are mean values ± S.D of n=3 independent experiments performed in triplicates, normalized to UT in **(A)** or HRG in **(B)** and expressed as fold change. Statistical significance was determined between treatment groups by TWO WAY ANOVA followed by post hoc Tukey's test as appropriate and significance expressed according to the scale :(*: p<0.05, **: p<0.01, ***: p<0.001, and ****: p<0.0001).

6.4.4 Lapatinib and erlotinib followed with pharmacological inhibition of NRF2 by Bexarotene disrupts its antioxidant transcription, suppresses NRF2 and HO-1 protein levels and elevates cellular ROS

Bexarotene which on its own is reported to be an anticancer agent, has previously been shown to inhibit NRF2/ARE in an NRF2 dependent manner (Garattini et al., 2014, Saito-Hakoda et al., 2015, Qi et al., 2016, Wu et al., 2014, Heo et al., 2016). In order to extend the observations reported in the previous section, the consequences of NRF2 inhibition following exposure to the chemotherapeutic drugs was further investigated. Experiments were performed, firstly in the ovarian cancer cell line models and subsequently in MCF7-AREc32 cell line in order to validate and confirm the inhibitory action of bexarotene and also drugs (lapatinib and erlotinib) on the NRF2 dependent AR pathway. Exposure to bexarotene alone caused a decrease in total NRF2 levels in OVCAR3 and SKOV3 cells (Figure 6.4A). Interestingly the levels of NRF2 in these cell lines were further decreased following co-treatment with combined chemotherapy (lapatinib and erlotinib) in PEO1 cells. This drug-induced reduction in NRF2 levels suggests that chemotherapy is also targeting NRF2. Next, using the luciferase ARE reporter MCF7-AREc32 cell line, it was found that bexarotene treatment significantly inhibited the transcriptional activity of NRF2 at all the time points tested (Figure 6.4B). Bexarotene treatment of MCF7-AREc32 reporter cell line also elevated ROS levels (Figure 6.4C). Furthermore, bexarotene enhanced the inhibitory action of the combination of lapatinib and erlotinib on AR pathway. It is also observed that bexarotene alone and in combination with lapatinib and erlotinib reduced the level of HO-1 (Figure 6.4A). These findings suggest that while bexarotene inhibits the NRF2 dependent ARE pathway, such treatment might also elevate cellular ROS levels in the ovarian cancer cell lines. Indeed, it is found that treatment with bexarotene significantly represses total NRF2 and induced ROS in the three cell lines tested (Figures 6.4A and 6.4C).

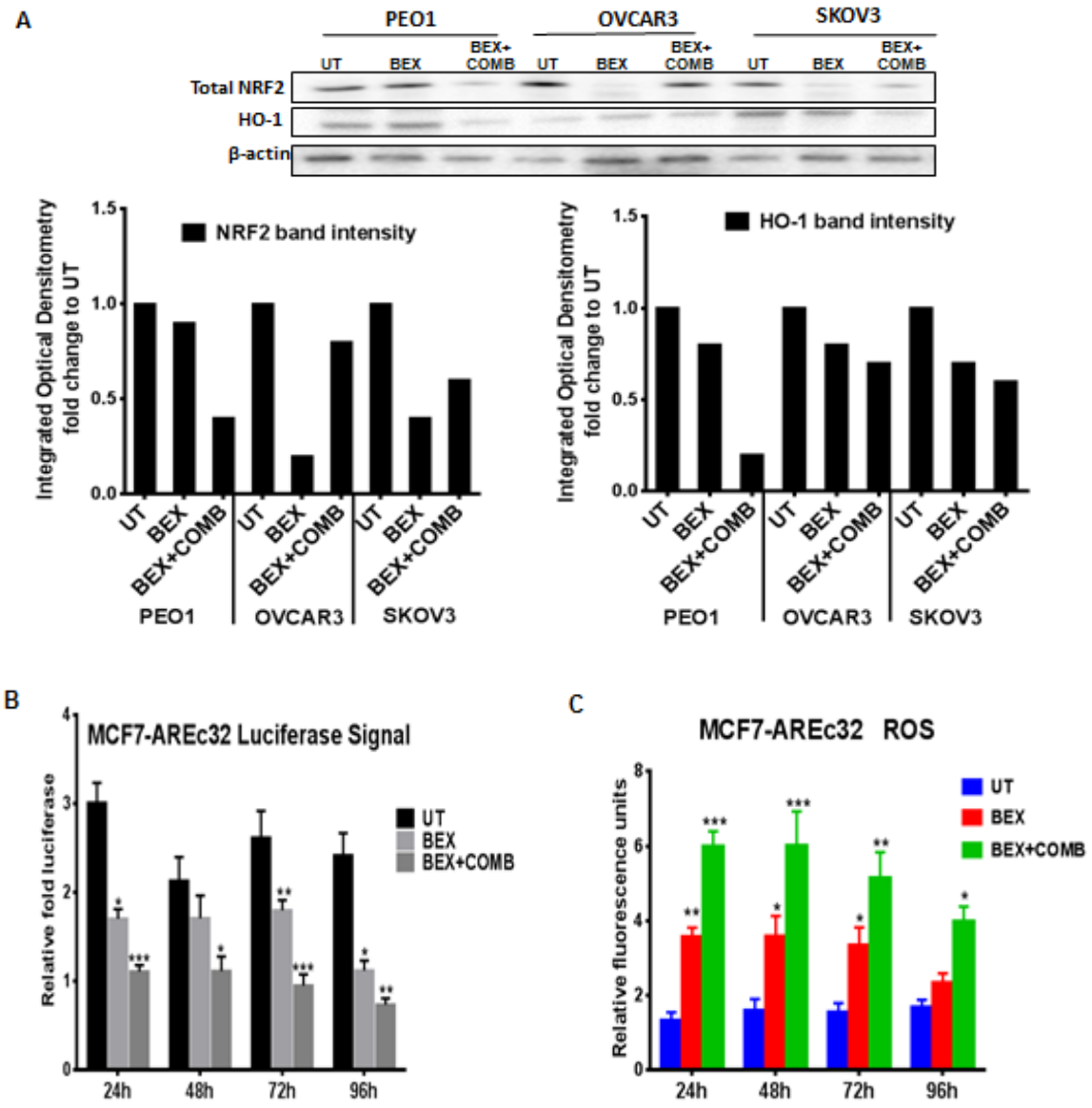

Figure 6.4: Combined treatment with bexarotene causes inhibition of NRF2 dependent antioxidant response pathway and generates ROS. **(A)** Western analysis showing repression of NRF2 and HO-1 levels following bexarotene treatment in PEO1, OVCAR3 and SKOV3 cell lines. Exponentially growing cells were either left untreated, treated with 2.5 μM bexarotene or a combination of 2.5 μM bexaroene together with 5 μM of lapatinib and erlotinib for 24 h before being harvested to prepare protein lysates and processed as described in Materials and Methods. Beta actin (β-actin) was used as loading control. The bars indicate NRF2 and HO-1 levels following quantification of immunoblot signal intensities obtained in **(A)** and normalized to the value of UT and expressed as fold change. The signal intensities of bands were quantified through integrated optical densitometry measurement. Images are representative of at least two independent experiments performed. **(B)** Bexarotene treatment causes inhibition of NRF2 dependent transcription. Exponentially growing AREc32 cell line stably expressing 8xcis-AREs driving the expression of luciferase gene in an NRF2 dependent manner were either left untreated (UT), treated with bexarotene alone, or with bexarotene and combination of lapatinib and erlotinib for different time points as indicated. Following this, cell lysates were prepared and assayed for Luciferase activity (BrightGlo Luciferase system, Promega). **(C)** Bexarotene treatment causes increase in ROS levels. Exponentially growing AREc32 cell line stably expressing 8xcis-AREs driving the expression of luciferase gene in an NRF2 dependent manner were seeded in triplicates in opaque flat bottom black walled 96-well plates for 24 h. Following this, cells were either left untreated (UT), treated with bexarotene alone or with bexarotene and combination of lapatinib or erlotinib for different time points as indicated. Following incubations, cells were loaded with DCFDA fluorescent stain for 45 min and assayed for ROS as described in Materials and Methods. Data are the mean values ± S.D of n=3 independent experiments performed in quadruplicates, with statistical significance determined by TWO WAY ANOVA followed by Tukey's post hoc test according to the scale * P <0.05, **P <0.01, ***P <0. 001.

6.4.5 Activity of lapatinib, erlotinib and bexarotene involves repression of NRF2-dependent transcription and depletion of total GSH

Based on the additionally decreased levels of NRF2 observed in MCF7-AREC32 stable cell line following combined lapatinib and erlotinib with bexarotene (Figure 6.4A), a single treatment with lapatinib or erlotinib or combination was investigated to confirm the inhibition of NRF2-dependent transcription. The MCF7-AREc32 cell line, stably expressing 8 copies of NRF2 dependent cis-regulatory AREs, as a luciferase reporter was used. The results (Figure 6.5) show that stimulation with HRG, a potent ligand for HER receptors only, a significant induction of antioxidant response pathway was observed. However, the co-treatment with the combination of lapatinib and erlotinib and with the single drug alone disrupted and suppressed the ARE-dependent induction significantly thereby inhibiting the NRF2 function. A combination of either lapatinib and bexarotene or erlotinib and bexarotene also disrupted the function of NRF2, and the greatest inhibition of NRF2/ARE activity was observed when the cells were treated with a combination of lapatinib, erlotinib and bexarotene (Figure 6.5).

Moreover, to investigate whether NRF2 repression would also lead to depletion of total cellular glutathione, the ovarian cancer cells (PEO1, OVCAR3 and SKOV3) were treated for 72 h. It is found out that the 72 h treatment with HRG induced total cellular GSH levels, while the combination of HRG with lapatinib, erlotinib or bexarotene significantly reduced the GSH levels in all the cell lines (Figure 6.6). Generally, the combination of lapatinib with erlotinib, lapatinib with bexarotene, or erlotinib with bexarotene caused more significant GSH depletion than singular treatments. These results indicated that NRF2 inhibition, ROS accumulation and GSH depletion may be contributing to the unique mechanism of cytotoxicity of lapatinib or erlotinib and that bexarotene enhances the mechanism of action and the cytotoxicity of lapatinib and/or erlotinib. Overall this provides support and strengthens the hypothesis that the cellular cytotoxicity of lapatinib and/or erlotinib involves the engagement of the AR pathway and the concomitant inhibition of NRF2 function during drug action.

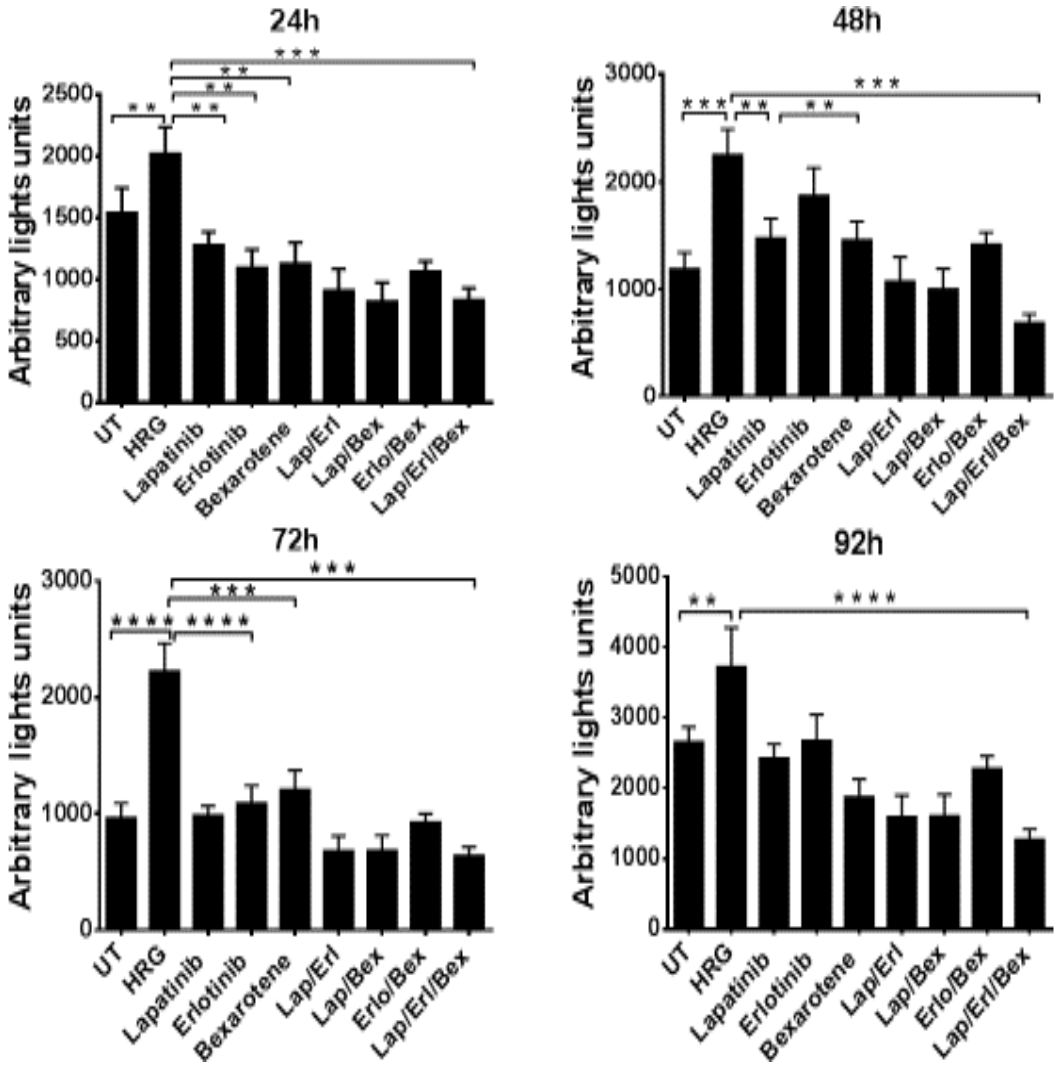

Figure 6.5: Treatments with lapatinib, erlotinib and Bexarotene causes inhibition of NRF2 dependent transcription. Single and combination of lapatinib and erlotinib cause inhibition of NRF2 dependent transcription. Exponentially growing MCF7-AREc32 cell line stably expressing *cis*-regulatory AREs driving the expression of luciferase gene in an NRF2 dependent manner were treated with 1 nM HRG alone or with co-treatment of 5 μM lapatinib and erlotinib either individually or in combination for different time points as indicated. Following this, cell lysates were prepared and assayed for Luciferase activity as described in Materials and Methods. Data shown are mean ± S.D of n=3 independent experiments performed in quadruplicates. Statistical significance is determined by ONE WAY ANOVA followed by Tukey's post hoc test. Asterisks indicate significant differences between individual groups as indicated and according to the scale *p<0.05, **p<0.01, ***p <0.001, ****p <0.0001.

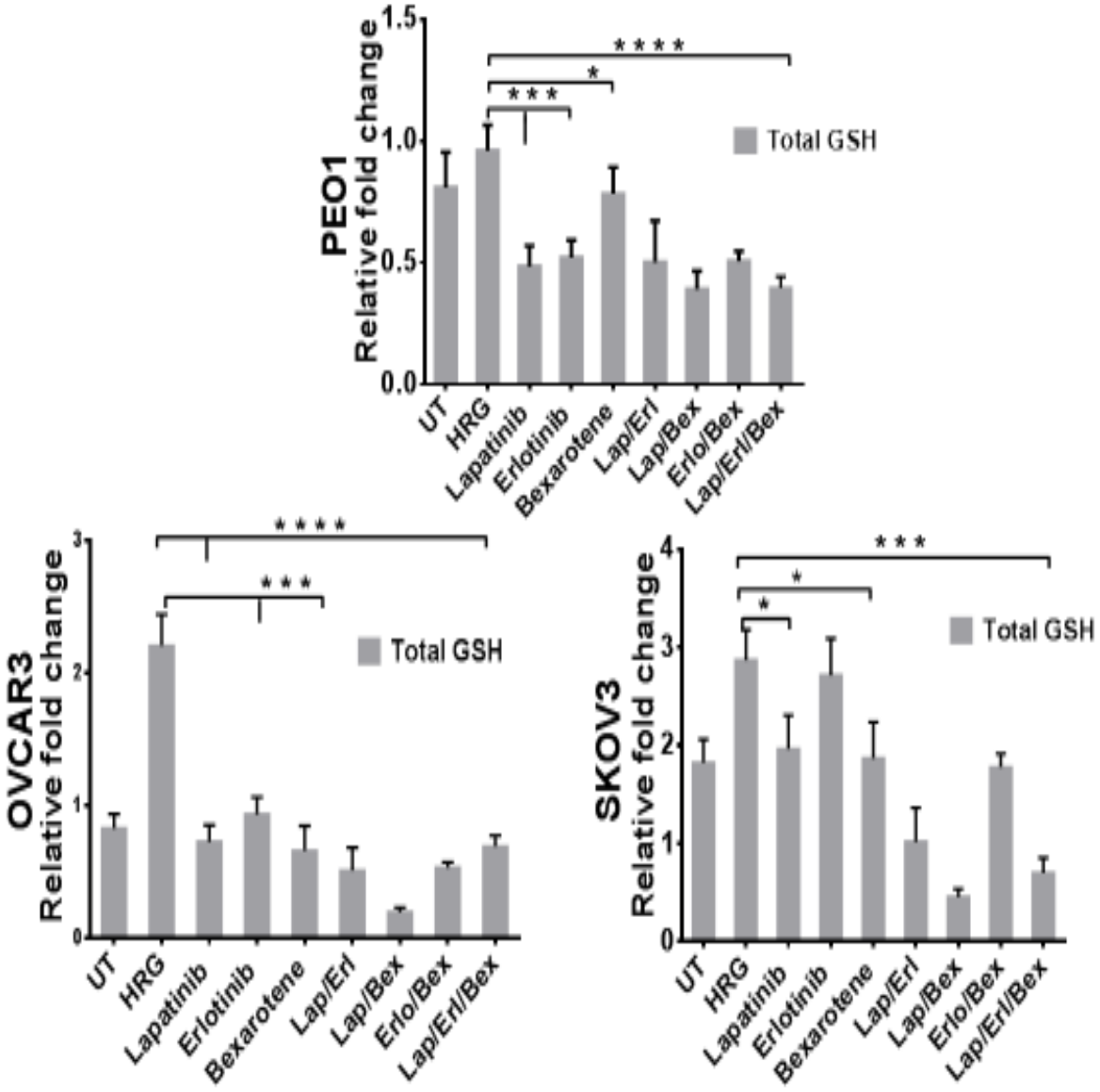

Figure 6.6: Treatments with lapatinib, erlotinib and Bexarotene causes depletion of total GSH levels. Single and combination of lapatinib and erlotinib causes decrease in glutathione level. Exponentially growing cells where seeded in luminometer-compatible 96-well plates and allow to grow overnight at 37°C in a 5% CO_2 culture incubator. The following day, the cells were either left untreated (UT) or treated with media containing 1nM Heregulin alone HRG) or with co-treatment of 5 µM lapatinib or 5 µM erlotinib or 2.5 µM bexarotene their combinations for 72 h before being processed for glutathione assay. Data are mean ± S.D of n=3 independent experiments performed in quadruplicates. Statistical significance was determined by ONE WAY ANOVA followed by Tukey's post hoc test according to the scale * p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

6.4.6 Co-treatment of lapatinib and erlotinib with bexarotene reduces the HER family and NRF2 protein levels

The observation that retinoid (bexarotene, RA) or tBHQ treatment caused downregulation or upregulation of HER receptors at transcriptional and protein levels as demonstrated in the previous chapters suggests that NRF2 may be

directly involved in regulating HER receptor expression and as such might have a role in responses to targeted chemotherapies involving HER receptor family. The cellular cytotoxicity of HER targeting lapatinib and/or erlotinib culminates with the engagement of AR pathway and the concomitant inhibition of NRF2 function during drug action.

To answer this important question, PEO1, OVCAR3 and SKOV3 cells either with lapatinib and erlotinib alone or by co-treatment with retinoid/rexinoid (bexarotene) were investigated to examine the consequences of NRF2 inhibition on drug effects and cellular responses. Treatment with lapatinib alone or erlotinib alone represses both pNRF2, pHER1 pHER2 pHER3 and pHER4 levels in the cell lines (Figure 6.7A and B). There was also similar concomitant marked repression of pAKT levels in cell lines by either drugs and in combination with bexarotene. These results are consistent with lapatinib or erlotinib inhibiting NRF2 activity and repressing HER receptor family. Moreover, co-treatment with an NRF2 inhibitor (bexarotene) can further repress the HER signalling pathway and might sensitize the ovarian cancer cells to the killing effects of lapatinib and/or erlotinib. Furthermore, results in (Figure 6.2) appears to lend support to these assertions, as significant increased cytotoxicity of lapatinib or erlotinib following the pharmacological inhibition of NRF2 with bexarotene was observed.

These findings illustrated the important role of NRF2 in influencing outcomes to targeted therapies involving HER receptor inhibition. These results indicate the effectiveness of anti-cancer therapy involving lapatinib and erlotinib could be enhanced by incorporating bexarotene to inhibit the NRF2 dependent antioxidant response pathway. As such, this represents a novel mechanism and role for NRF2 inhibition by bexarotene in sensitising cancer cells to the killing effect of lapatinib and erlotinib. It also highlights the possibility of using the combination of lapatinib and bexarotene or erlotinib with bexarotene to overcome drug resistance.

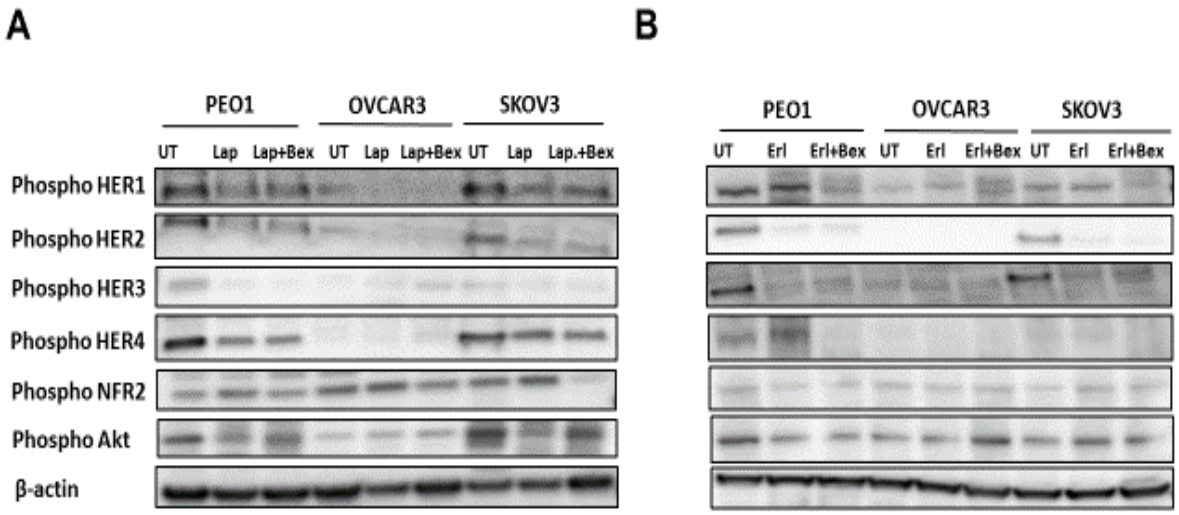

Figure 6.7: NRF2 inhibition with bexarotene sensitises HER signalling pathway to RTK inhibitors: **(A)** Lapatinib and **(B)** Erlotinib. Immunoblot analysis showing bexarotene dependent repression of HER signalling following its combination with lapatinib and erlotinib. Exponentially growing cells were either left untreated in media containing 1 nM Heregulin (UT) or treated with treated with same media containing in the presence of 1 nM Heregulin with lapatinib (Lap) alone or erlotinib (Erl) alone, each at 5 µM, or with co-treatment of 2.5 µM bexarotene (Lap+Bex) or (Erl+Bex) for 24 h before and processed for immunoblotting using relevant antibodies and β-actin was used as loading control. Images are representative of at least two independent experiments performed.

6.5. Key findings

This chapter aims to explore signalling pathways that might explain the mechanism of action and efficacy of HER family targeted chemotherapies that are critical to use or avoid in order to overcome therapeutic resistance. Specifically, it seeks to determine the action mechanism of targeted chemotherapeutic agents (lapatinib and erlotinib) and in particular, to understand enhanced cytotoxic response, ROS accumulation, NRF2 inhibition and GSH depletion triggered uniquely by a combination of either one or two chemotherapeutics, with the addition of bexarotene (NRF2 inhibitor) rather than individual agents in ovarian cancer cells. It was found that at least part of the mechanism of action of these chemotherapeutic agents involved NRF2 inhibition (Figure 6.4A) and generation of ROS (Figure 6.3), which contributed to the cytotoxic effects and killing of the ovarian cancer cells (Figure 6.2). The cell lines exhibited cytotoxicity to lapatinib and erlotinib alone, or their combination with bexarotene (Figure 6.2). However, there was inhibition of HER signalling (Figure 6.7) following lapatinib or erlotinib alone or their combination with bexarotene in ovarian cancer cells.

7. CHAPTER SEVEN

Discussion, Conclusions and Recommendations

7.1. Discussion

This research work was carried out to further the knowledge of the functional regulation of the HER receptor family and NRF2 in different conditions and cell lines. It was intended to help gain an overall understanding of how the function of NRF2 could have a role in regulating the HER receptor family in ovarian cancer cells. This, in turn, may enable the improvement of the efficacy of RTK targeting cancer chemotherapeutic agents in order to overcome chemoresistance in ovarian cancer. This was achieved by firstly cloning the proximal promoter regions *HER1* and *HER4,* and then using the already cloned (Khalil et al., 2016b) promoter regions of *HER2* and *HER3* to examine and re-examine their functionality and transcriptional regulation. The basal and inducible expression levels of the four genes were evaluated using both the NRF2 inducer (tBHQ) and inhibitors (siRNA and RA) with a panel of ovarian cancer cells. The tBHQ treatments lead to up-regulation of the transcription of all the HER receptors whilst inhibiting NRF2 function leads to their down-regulation, suggesting that NRF2 regulates the expression of HER receptors in ovarian cancer cells. Following the screening of a panel of compounds expected to be potent NRF2 modulators, bexarotene was found be one of the most potent NRF2 inhibitors as well as the most suitable to include in further studies because it is already a clinical and anticancer drug.

Next, the study looked at the protein levels of both NRF2 and HER receptors in order to examine their responses, following different treatments and perturbations of NRF2 function. This initial part of the research provided some insight into the molecular basis for regulation of the expression of these HER family receptor genes by NRF2. The research also found a novel mechanism and role for NRF2 inhibition by bexarotene in sensitising ovarian cancer cells to the killing effect of RTK inhibitors, lapatinib and erlotinib. It also highlighted the possibility of using a combination of lapatinib and bexarotene, or erlotinib with bexarotene, to sensitize and overcome drug resistance.

7.1.1 Cloning of the proximal promoter regions of human *HER1* and *HER4* genes

This section of the study reports on cloning of the promoter regions of human HER1 and HER4 genes and examines their transcriptional activity in human ovarian cancer cell lines (PEO1, OVCAR3 and SKOV3). The HER family receptors are one of the regulators of cellular proliferation and survival and are also, in part, the ones that determine the initiation and progression of cancer (Kang et al., 2014b, Schlessinger, 2000, Roskoski Jr, 2014, Yarden, 2001, Prenzel et al., 2001). Using the computer-based analysis known as bioinformatics (Sandelin et al., 2004), it was found that the promoters of *HER1* and *HER4* contained putative binding sites of NRF2 (Figure 3.2), this being a transcription factor that steers the expression of many genes including cytoprotective and detoxifying genes (Hayes, 2000, Hayes et al., 2016, Hayes and McMahon, 2009).

Recent studies have identified HER family as a target in cancer (Burgess, 2008, Downward, 2003, Khalil et al., 2016a, Khalil et al., 2016b, Goltsov et al., 2014a, Zwick et al., 2001, Gschwind et al., 2004, Mendelsohn and Baselga, 2000, Shende et al., 2017, Koustas et al., 2017, Guo et al., 2016) and showed that the expression of HER family could be regulated both at the transcriptional and translational level (Khalil et al., 2016b, Khalil et al., 2016a). However, little is known about the gene promoter and cognate transcription factors that mediate HER family gene expression. So far, only a few studies were undertaken to find potential cis-regulatory elements and investigate their influence on HER family gene transcription (Khalil et al., 2015, Khalil et al., 2016b, Khalil et al., 2016a). A study reported by Khalil et al., (2016) characterised the HER2 and HER3 promoter genes. The genes contain the putative NRF2 binding sites at the proximal promoter level. Further, it has demonstrated crosstalk between the transcription factor NRF2 and HER2/HER3 transcription (Khalil et al., 2016b).

The presence of a putative binding site of NRF2 in the promoters of these genes and other previous studies (Khalil et al., 2016b) enables the hypothesis of a possible link between HER receptors and NRF2, and is why it was decided to clone the promoter regions of *HER1* and *HER4*.

One of the aims of this study was to clone and then test basal activities HER1 and HER4 promoters. 1.5 kb and 1.3 kb DNA fragments encompassing the HER1 and HER4 gene proximal promoters were each placed upstream of the luciferase reporter gene in the pGL3 vector. Following this, the clones were confirmed via sequencing before being transiently transfected into three different panels of ovarian cell lines, namely PEO1, OVCAR3 and SKOV3. The luciferase reporter system assay indicated that both prHER1 and prHER4 showed a high and differential basal level of expressions in all the cell lines tested when compared to the control pGL3 vector (Figure 3.8). This study found that the transcriptional activity of *HER1* and *HER4* suggested the functionality of the promoter constructs, and this, therefore, provided the tools to study the regulation of HER1 and HER4 receptors.

The generation basal levels of luciferase activity suggest that the regulation of HER1 and HER4 gene expression occur at the transcriptional level. As shown in this study, the identification of the putative binding sites of NRF2 in the promoter regions of genes may enhance the promoter activity, thereby suggesting a possible role of NRF2 in HER1 and HER4 regulation. These findings suggest that the 1.5 kb and 1.3 kb fragments upstream of the transcription start site could be a core functional promoter, essential for the study of the transcriptional regulation of HER1 and HER4 in cancer cells. Recently, a study by Khalil et al. reported the cloning of promoter regions of HER2 and HER3, and so provided the importance of generating transcriptional reporter assays for both HER2 and HER3 receptors in the context of studying transcriptional regulations in cancer therapy (Khalil et al., 2016b). Another study by Horikawa et al. (1999) is similar to this study, and this provides credence and support for the importance of cloning promoter regions of a gene for the study of its regulation. Horikawa et al. (1999) reported the cloning and characterisation of human telomerase reverse transcriptase (hTERT) gene promoter providing transcriptional regulation of the hTERT gene, with this suggesting that investigation of the molecular mechanisms that regulate hTERT gene expression could lead to a better understanding of telomerase regulation, cellular senescence and immortalization, and human carcinogenesis (Horikawa et al., 1999). The expression of telomerase activity is strongly associated with human cell immortalization and carcinogenesis (Chiu and Harley,

1997, Kim et al., 1994); therefore identification of the NRF2 putative binding sites in the promoter sequence of HER1 and HER4 is essential in understanding their regulation.

Studies have reported the roles of several other transcription factors other than NRF2, such as specificity protein 1 (sp1), EGFR-specific transcription factor (ETF) and activator protein 1 (AP-1) (Kitadai et al., 1992, Johnson, 1996, Kageyama et al., 1988) in the transcriptional initiation. These transcription factors might thus be one of the critical factors promoting the initiation of HER receptor transcription. Thus, the interaction between the relevant transcription factors and their possible roles in the transcription of HER1 and HER4 could be investigated further.

7.1.2 Regulation of HER1 and HER4 by NRF2 in ovarian cancer cells

Previous evidence has implicated NRF2, ROS and HER receptors in cell growth, cell survival, cancer initiation and progression and therapeutic resistance in cancer cells (Khalil et al., 2016a, Khalil et al., 2016b), therefore highlighting the possibility of direct linkage between ROS, HER family and NRF2 pathways.

This section demonstrates that NRF2 may regulate cancer cell proliferation, susceptibility and resistance to chemotherapeutic drugs via transcriptional regulation of HER family receptors. In order to demonstrate the role of NRF2 in regulating the HER family receptors and subsequently determining the responses to targeted therapies, ovarian cancer cell lines, PEO1, OVCAR3, and SKOV3 with different HER family and NRF2 expressions were used (Langdon et al., 1988, Mullen et al., 2007, Khalil et al., 2016b), and pharmacological activation and pharmacological inhibition and genetic inhibition of NRF2-ARE pathway were employed. The mechanisms by which tBHQ induce NRF2 and RA inhibits NRF2 and its function are known (Khalil et al., 2016a, Khalil et al., 2016b, Wang et al., 2007), and in this research the basis for using RA was to modulate cellular NRF2 status and ARE-dependent transcriptional power, as well as to implicate NRF2 in regulating cellular susceptibility to RTK targeted drugs.

First, pharmacological activation of NRF2 with tBHQ up-regulated the transcriptional levels of all the HER receptors with a concomitant induction of

HER1, HER4, NFR2 and pAkt proteins (Figures 4.1 and 4.2). This is consistent with current emerging concepts of transcriptional control and gene expression (Khalil et al., 2016b). It is clear that tBHQ treatment leads to induction of NRF2, its associated antioxidant transcriptional programme and transcriptional and signalling activation of HER1 and HER4, and that this tBHQ response was evidently dependent on NRF2.

Second, to further investigate and confirm the involvement of NRF2 in the regulation of HER1 and HER4, both pharmacological and genetic approaches to deplete NRF2 level and function using RA and siRNA were employed. This strategic approach caused increased transcriptional repression of *HER1* and *HER4* (Figures 4.3 and 4.5) leading to repression of HER1, HER4, total and pNRF2 and HO-1 levels (Figures 4.4 and 4.6), while tBHQ dependent induction of ARE disrupted the knocking down effect of siRNA (Figures 4.7 and 4.8). Moreover, NRF2 depletion by RA and siRNA caused an increase in ROS (Figure 4.9) and depletion of total GSH (Figure 4.10) in all the ovarian cell lines tested.

tBHQ is widely known as an NRF2 activator and can upregulate ARE response driven genes (Arlt et al., 2013, Wang and Jaiswal, 2006, Li et al., 2014). On the other hand, retinoids like RA are chemopreventive and chemotherapeutic agents. One source of RA is vitamin A, derived from dietary β-carotene. RA regulates cell proliferation, differentiation, and morphogenesis. It inhibits tumorigenesis through suppression of cell growth and stimulation of cellular differentiation. Also, RA promotes apoptosis, and this property may contribute to its antitumor properties (Wang et al., 2007, Tan et al., 2008). siRNA, however, is a class of double-stranded RNA molecules, 20-25 base pairs in length. siRNA is similar to miRNA and operates within the RNA interference (RNAi) pathway, where it interferes with the expression of specific genes with complementary nucleotide sequences by degrading mRNA after transcription, resulting in no translation (Persengiev et al., 2004, Elbashir et al., 2001, Tiscornia et al., 2003, Caplen et al., 2001, Xia et al., 2002, Hannon, 2002). Previous studies have shown the inhibitory nature of siRNA and RA in on ARE (Khalil and Deeni, 2015, Wang et al., 2007, McMahon et al., 2014, Khalil et al., 2016b, Khalil et al., 2016a). For example, the paper published earlier in 2016 by Khalil et al (2016b) has reported that NRF2 regulates HER2 and HER3 following the use of widely used NRF2 activator (tBHQ) and

repressor (siRNA) which in turn upregulated and downregulated HER2 and HER3 respectively. Here, the study demonstrates that NRF2 regulates HER1 and HER4 following the same approach used in the case of HER2 and HER3. Pharmacological activation, pharmacological inhibition and genetic inhibition of both NRF2-ARE and HER1 and HER4 were studied.

Several possible NRF2 binding sites were identified in the HER1and HER4 promoter regions (Figure 3.1). The regulatory role of NRF2 on HER1 and HER4 expressions is supported by the observed higher induction levels of HER1 and HER4 following NRF2 activation and inhibition/knockdown in all the cell lines (Figures 4.1, 4.2, 4.3, 4.4, 4.5 and 4.6). The NRF2 activation and inhibition-dependent regulation of the HER1 and HER4 receptors and their signalling pathway were governed and executed by NRF2 at both the transcriptional and translational levels. The results from both the receptor gene reporter assays and western blotting of HER1 and HER4 expressions indicated that activation or inhibition of NRF2 also upregulated or downregulated HER1 and HER4, respectively. It is clear that tBHQ treatment led to activation of NRF2, its associated antioxidant signalling and transcriptional program, which led to the transcriptional upregulation of HER1 and HER4. Moreover, this tBHQ response was evidently dependent on NRF2. Also, RA treatment and knockdown of NRF2 with siRNA led to depletion of NRF2, its associated antioxidant signalling and transcriptional program, with subsequent transcriptional and translational downregulation of HER1 and HER4, RA or siRNA dependent responses were evidently dependent on NRF2.

In support of the finding that NRF2 depletion by siRNA causes transcriptional down-regulation of HER1 and HER4 leading to repression of HER1, HER4 and pAkt proteins, a study by Khalil et al (2016a) showed a similar approach and outcome. Moreover, it demonstrated that while inhibition of NRF2 significantly sensitised ovarian cancer cells to targeted immunotherapy, the parallel approach of knockdown of KEAP1 reversed this sensitisation (Khalil et al 2016a). These results support and confirm the earlier hypothesis of the regulatory role of NRF2 in the transcription of HER1 and HER4 receptors, as well as its possible involvement of alteration of HER1 and HER4 protein levels. Another study by (Kang et al., 2014a), reported a similar role for NRF2 in regulating the expression

of one of the members of HER family receptors, but did not show the evidence of direct transcriptional regulation as shown in (Khalil et al., 2016b) and this study.

The direct interaction of NRF2 and HER2 in regulating the expression of NRF2 target genes, including HO-1 through binding of the complex to the ARE of the target genes, has been demonstrated (Kang et al., 2014b). Moreover, the down-regulation of HER2, HER3 and pAKT as well as HO-1 and pNRF2 levels following siRNA-mediated depletion of NRF2, has been reported (Khalil et al., 2016b). This adds credence to observed down-regulation of HER1, HER4 and pAkt as well as NRF2 and HO-1 levels, following the siRNA-mediated knockdown of NRF2 in this study.

Since NRF2 overexpression has been reported in cancers (Kang et al., 2014b, Chian et al., 2014b, Chio et al., 2016, Choi and Kwak, 2016), NRF2 silencing using siRNAs as a means of therapy may be a reliable alternative approach for treating NRF2 overexpressing cancers (Kanninen et al., 2015), including ovarian. Such findings could give insight to further understanding the molecular genetic aspect of ovarian cancer and its treatment. Furthermore, understanding that there is a limitation of compounds to decrease NRF2 activity could give insight into the molecular genetic aspects of ovarian cancer and its treatment, in the context of NRF2 targeted gene therapy. To date, over 2,000 clinical trials in gene therapy have already been conducted, with cancer being the basis of the majority of these trials. Various regulated vectors have been designed so that gene transfer is specific, regulated, effective and safe in the same way that conventional drugs are. This would prevent the occurrence of unwanted side effects of long-term transgene expression, as reported by (Springer et al., 1998). Although NRF2 silencing using siRNA strategy has been shown to enhance chemo- and radiotherapy (Singh, 2006, Singh et al., 2010), there are many problems including difficulties in intracellular delivery and trafficking and clearance by the immune system, as well as the occurrence of off-target effects associated with this approach. These should be talked about further before considering this strategy in the clinic (Pecot et al., 2011). Gene therapy may well provide a more specific approach than small molecular weight inhibitor in targeting NRF2 in cancer. This is due to the fact that general systemic NRF2 inhibition is not desirable, as NRF2 serves important protective functions in healthy normal cells.

In conclusion, these results support and confirm the earlier identified role of NRF2 in the regulation of HER2 and HER3 reported by Khalil et al. (2016) (Khalil et al., 2016b). A recent study by Khalil et al. (2016) reported that NRF2 regulates HER2 and HER3, thereby adding credence to present findings on HER1 and HER4, with this further confirming the role of NRF2 as a regulator of all four members of the HER family receptors. It also gives further insight into the future possibility of using gene therapy in silencing NFR2 hyperactivation in ovarian cancer patients.

7.1.3 Screening for inhibitors of the NRF2-ARE signalling pathway and their potential applications in ovarian cancer therapy

In this section of the research, a panel of compounds anticipated to be potent NRF2-ARE modulators were screened to uncover the most potent compound. NRF2 is a transcription factor that mediates protection against electrophiles and oxidants. It regulates the expression of many genes such as cytoprotective and detoxifying genes. ROS, however, are involved in the physiological aspects of the cell, including signal transduction cascades and calcium signalling (Liou and Storz, 2010, McMahon et al., 2014). Both NRF2 activation and elevation of ROS have been detected in almost all cancers, and these promote tumour development and progression (McMahon et al., 2014, Liou and Storz, 2010).

The panels of potent NRF2 modulators and in particular NRF2 inhibitors, were investigated using the MCF7-AREc32 reporter cell line (Wang et al., 2007). Three lowest different concentrations (2.5 µM, 5.0 µM and 10 µM) of the compounds were used to evaluate their ability to inhibit NRF2 and to elevate ROS. The investigation indicated that compounds such as bexarotene, RA, 9-cis RA, luteolin and brusatol were able to inhibit the NRF2-ARE in MCF7-AREc32 cell line (Figures 5.2 and 5.3) and also elevate ROS (Figure 5.4), even at the lowest concentration of 2.5 µM. Interestingly, bexarotene, which is a clinically used anti-cancer drug, appeared to be one the most potent inhibitors of NRF2-ARE dependent activity.

The compounds that were more promising in their ability to inhibit NRF2 and elevate ROS were used at the lowest concentration (2.5 µM), to examine their effect on the level of NRF2 and its substrate gene target at a protein level in the

MCF7-AREc32 cell line. As expected, all the compounds were able to cause repression of total NRF2, pNRF2 and HO-1, with bexarotene still proving to be the most potent (Figure 5.3). The ability of bexarotene to inhibit NRF2 levels suggests that it can be used in targeting NRF2 to modulate HER receptor expression in ovarian cancer cells. The effect of bexarotene, lapatinib and erlotinib on MCF7-AREC32, was examined, the results shown that low concentrations (2.5 μM and 5 μM) of lapatinib, erlotinib and bexarotene have induced the cytotoxicity, increased ROS and inhibit NRF2 dependent ARE pathways (Figure 5.5).

Following cell viability assay to estimate the required concentrations of lapatinib, erlotinib and bexarotene for the further treatment of ovarian cancer cell lines, PEO1, OVCAR3 and SKOV3 (Figure 5.6), The effect of these drugs was examined when the ovarian cancer cells were transiently transfected with prHER1, prHER2, prHER3 and prHER4. Interestingly, the results indicated that bexarotene as well lapatinib and erlotinib, down-regulated the transcriptional level of *HER1, HER2, HER3* and *HER4* in all the ovarian cancer cell lines (Figures 5.7, 5.8 and 5.9). These findings suggest bexarotene, has the ability to inhibit NRF2 dependent ARE pathways, to elevate cellular ROS levels in MCF7-AREc32 cell lines and to down-regulate HER1 and HER4 transcriptionally in PEO1, OVCAR3 and SKOV3 ovarian cancer cell lines.

NRF2 is increasingly being recognised as a crucial transcription factor which mediates protection against electrophiles and oxidants by regulating the expression of many cytoprotective and detoxifying genes (Hayes, 2000, Hayes et al., 2016, Hayes and McMahon, 2009). Elevated rates of ROS have been detected in almost all cancers, where they promote many aspects of cellular proliferation, tumour development and progression (Khalil et al., 2016b, Khalil et al., 2016a, Khalil and Deeni, 2015, Liou and Storz, 2010, Leone et al., 2015, Park et al., 2010, Poillet-Perez et al., 2015, Trachootham et al., 2009, Prasad et al., 2017). Tumour cells also express increased levels of antioxidant proteins to detoxify ROS, suggesting that a delicate balance of intracellular ROS levels is required for cancer cell function. To effectively target cancer cells, specific ROS-sensing signalling pathways that mediate the diverse stress-regulated cellular

functions need to be identified (McMahon et al., 2014, Liou and Storz, 2010, Tebay et al., 2015).

The compounds screened in this section were able to induce NRF2-KEAP1 either by positive or negative feedback to stimulate NRF2 activity. These compounds included an RXR agonist (bexarotene), which possesses anti-cancer activity and is reported to inhibit NRF2-ARE pathway (McMahon et al., 2014). These effects may be either indirect or due to the modulation of pathways directly involved in the regulation of NRF2 function. In the case of bexarotene, an RXR agonist, for example, the findings that this compound reduced NRF2 signalling (Figure 5.3) are consistent with an earlier finding that RXR acts as a negative regulator of NRF2-ARE signalling (McMahon et al., 2014, Qi et al., 2016, Wu et al., 2014). The negative regulation occurs when a rexinoid such as bexarotene specifically binds to RXR which in turn forms a complex with NRF2 and blocks it from binding to ARE, leading to NRF2 repression (Farol and Hymes, 2004, Qi et al., 2016, Wu et al., 2014). This provides further evidence supporting the interaction of RXR with NRF2 to inhibit NRF2-ARE function that now extends to ovarian cancer cells. Thus, bexarotene could be used as a potent inhibitor of NRF2 as part of the drug development process to sensitize and overcome cancer chemoresistance.

Developments in treatment approaches together with the earlier diagnosis have considerably increased the average survival of cancer patients. Nevertheless, recent reports of the involvement of NRF2 in increasing cancer cell survival leading to chemoresistance, have made researchers focus on the screening and identification of novel inhibitors of NRF2 that would be recommended for clinical trials in treating cancer patients. Drug discovery and development often employs high-throughput screening (HTS) of libraries that may contain a huge number of compounds typically covering the commercially available chemical diversity. The study in this section has revealed a new way for researchers to consider the HTS of potent inhibitors of NRF2, so as to increase the number of those that have been discovered for improved cancer treatment (Varbanov et al., 2017).

So far, a number of studies have conducted screenings for NRF2 inhibitors and compared to the number of NRF2 activators known, the number of inhibitors is quite small (Zhu et al., 2016). One of the aims of screening for NRF2 inhibitors is

to find inhibitors with efficiency and safety profiles for application in clinical cancer therapies. Furthermore, applying these inhibitors in clinical settings would be more successful if these agents blocked the NRF2 signalling pathway in cancer cells, whilst leaving normal cells unaffected or with very low cytotoxicity to normal cells (Zhu et al., 2016). Specifically, among the inhibitors studied, ochratoxin A is reported to have nephrotoxicity and carcinogenicity (Limonciel and Jennings, 2014). Triptolide causes oxidative injuries in many cells and tissues (Liu et al., 2013) and brusatol is reported to cause loss of body weight in nude mice (Ren, 2011), with the adverse effects of other compounds remaining unexamined. More accurate and detailed preclinical studies and clinical trials need to be conducted to elucidate dose-dependent, cell-type-dependent and even stage-dependent effects (Zhu et al., 2016). In addition, as well as experiments in vitro as per this study, long-term animal model experiments are needed.

7.1.4 NRF2 co-regulates HER family receptors to modulate the efficacy of lapatinib and erlotinib as RTK targeting chemotherapeutics in ovarian cancer

The transition from cytotoxic chemotherapy to molecularly targeted cancer drugs has resulted in an increasing number of successful therapies that have impacted positively on the lives of a large number of cancer patients.

In this section, all of the HER receptor family were investigated following treatments with either bexarotene alone or in combination with lapatinib or erlotinib. A series of experiments such as cytotoxicity assays, immunoblotting, ROS level detection and total GSH were performed. The HER family receptors and NRF2 have already been implicated in numerous reports as key contributors to resistance towards anti-cancer drugs (Khalil et al., 2016a, Khalil et al., 2016b, Goltsov et al., 2014a, Kobayashi and Yamamoto, 2005, Ercan et al., 2012, Xu et al., 2013). However, the majority of these studies have reported the role of NRF2 in resistance to chemotherapeutic drugs (Namani et al., 2014, Gupta et al., 2012, Kundu and Surh, 2010, Singh et al., 2013, Ramos-Gomez et al., 2001, Wang, 2008, Singh et al., 2010, Ohta et al., 2008). One of the major challenges for cancer treatment is to identify and target key survival pathways in cancerous cells that can provide selectivity against them that will lead to improved cancer

treatment. This is a challenging task owing to the complexity of responses generated by different types of cancers, age and different settings (Khalil, 2012). Experimental and clinical studies have shown enhanced efficacy and safe tolerability of HER targeting drugs such as trastuzumab, pertuzumab, lapatinib and erlotinib (Arora and Scholar, 2005, Madhusudan and Ganesan, 2004, Haouala et al., 2009, Polli et al., 2009, Leveque, 2008, Arlt et al., 2013, Goltsov et al., 2014a, Khalil et al., 2016a, Khalil et al., 2016b, Slamon et al., 2001, Kuang et al., 2010). The small molecule cancer drugs, lapatinib and erlotinib have been recognized as promising therapeutics targeting the increased EGFR expression in triple-negative breast cancer (TNBC), specifically through their use with other chemotherapies (Hoelder et al., 2012, Kuang et al., 2010, Roskoski Jr, 2014, Haouala et al., 2009, Ryan et al., 2008). However, outcomes of treatments with HER targeting drugs either used singly or in combination, remains unpredictable. This could be due to tumour type specificity and tumour biology-dependency, especially expression levels of cell surface receptors, their dimerization preferences, recycling kinetics and ligand abundance (Khalil et al., 2015, Khalil et al., 2016a, Ryan et al., 2008, Slamon et al., 2001). Thus, the efficacy of these drugs is potentially reduced due to limited therapeutic efficacy and frequent emergence of resistance.

There are many reasons to be optimistic about cancer drug discovery and development because of the novel and improved recent scientific and technological breakthroughs. Novel concepts such as ‘non-oncogene addiction’ (Luo et al., 2009) and ‘synthetic lethality’ (Kaelin, 2005, Ashworth et al., 2011) have widened the scope beyond the exploration of oncogenic pathway addictions, and have helped guide the identification of novel targets either through hypothesis-driven research or large-scale screening campaigns. Enormous genome sequencing and molecular pathology efforts, coupled with bioinformatics and system biology approaches, are allowing continuous refinement of the understanding of how cancer cells are wired, and how they can be targeted through single agents or on several fronts through drug combinations (Hoelder et al., 2012, Heller, 2002, Loman et al., 2012, Karp et al., 2002, Weston and Hood, 2004, Voelkerding et al., 2009, Subramaniam et al., 2011, Cronin and Ross, 2011, Stratton, 2011, MacConaill and Garraway, 2010, Kitano, 2003).

This present study demonstrates that NRF2 regulates cancer cell proliferation, susceptibility and resistance to targeted therapy via transcriptional regulation and alteration of HER family receptors. This study sought to determine the mechanism of action of targeted chemotherapeutic agents, lapatinib and erlotinib, and in particular to understand their ability to inhibit NRF2, elevate ROS and enhance cytotoxic response triggered either by their combination with bexarotene or as single chemotherapeutics agents. This research used ovarian cancer cell lines PEO1, OVCAR3 and SKOV3 with different degrees of HER family and NRF2 expression status (Mullen et al., 2007, Khalil et al., 2016a, Khalil et al., 2015). The study found that the mechanism of action of lapatinib, erlotinib and retinoids used in this research involved generation of ROS and GSH depletion, which contributed to killing effects and cancer growth retardation. This is consistent with the conventional adage that depletion of GSH can cause oxidative stress and sensitise tumours to the killing effects of the therapeutic agents (Gorrini et al., 2013, Khalil et al., 2016a, Goltsov et al., 2014a). To demonstrate the role of NRF2 in HER signalling pathways and thus determine responses to targeted therapies, ovarian cancer cell lines with different expressions of both HER receptors and NRF2 (Khalil et al., 2015, Khalil et al., 2016a, Mullen et al., 2007) were grown in HER receptor ligand HRG, in association with pharmacological activation or inhibition of both NRF2-ARE and HER signalling pathways.

Firstly, pharmacological activation of NRF2 with tBHQ enhanced ovarian cancer cell growth and protected cells from cytotoxicity caused by combined RTK targeting chemotherapeutic agents, lapatinib and erlotinib took place (Figure 6.1). The pharmacological inhibition of NRF2 with bexarotene did the opposite and increased cytotoxicity caused by the agents (Figure 6.2).

It is clear that tBHQ treatment led to the induction of NRF2, its associated antioxidant transcriptional programme and transcriptional and signalling activation of HER1 and HER4, and that this tBHQ response was evidently dependent on NRF2. Thus, NRF2 activation by tBHQ desensitised the RTK signalling pathway to the inhibitory action of the HER targeting chemotherapeutic agents, lapatinib and erlotinib, whilst the inhibition of NRF2 with bexarotene did the opposite.

Furthermore, it was observed that treatments with lapatinib, erlotinib, their combination with each other or their combination with bexarotene, led to ROS generation in all the ovarian cancer cell line models (Figure 6.3). This approach also led to the repression of NRF2 and HO-1 (Figure 6.4) and disruption of the antioxidant response programme of NRF2 transcription (Figure 4.5), as well as depletion of total GSH (Figure 6.6), which confirms the crosslink between NRF2 and ROS. These results support and confirm an earlier study by Khalil et al. (2016b), which reported that treatments with pertuzumab, trastuzumab, their combination with each other or with RA, led to ROS generation in cancer cell line models, depletion of total GSH, and disruption of the antioxidant response programme as well as suppression of NRF2.

The observation that lapatinib and erlotinib alone or as co-treatment with bexarotene caused down-regulation of HER family receptor protein levels as well NRF2, suggests that NRF2 may be directly involved in regulating HER receptor expression and as such, might have a role in responses to RTK targeted chemotherapies, involving the HER receptor family. The action of lapatinib, erlotinib and bexarotene culminates with the engagement of the AR pathway and the concomitant inhibition of NRF2 function during drug action.

The fact that treatments with combinations of lapatinib with bexarotene or erlotinib with bexarotene repress pNRF2, pHER1 pHER2 pHER3, pHER4 as well as pAKT levels in the cell lines (Figure 6.7 A and B), suggests that this might sensitize ovarian cancer cells to the killing effects of lapatinib and/or erlotinib. Moreover, data in Figure 6.2 appear to support these assertions, as significantly increased cytotoxicity of lapatinib or erlotinib following the pharmacological inhibition of NRF2 with bexarotene were evident. This demonstrates that bexarotene causes repression of NRF2 dependent antioxidant pathway, which may contribute to the enhanced cytotoxicity of lapatinib and erlotinib.

The role of bexarotene as an effective anticancer drug is under active examination in various clinical trials (Qi et al., 2016, Saito-Hakoda et al., 2015, Heo et al., 2016, Farol and Hymes, 2004). The selective NRF2 inhibition and high toxicity to cancer cells and not normal cells may be the major advantage of bexarotene, which is already known to be generally safe and well tolerated (Duvic

et al., 2001, Papadavid et al., 2008, Rigas and Dragnev, 2005, Abbott et al., 2009, Whittaker et al., 2012, Yen et al., 2004b, Mehta et al., 2012, VäkEvä et al., 2012, Quéreux et al., 2013, Talpur et al., 2014). However, NRF2 inhibitory therapy combined with bexarotene will be cautiously examined in a pre-clinical setting because NRF2 has a protective effect against oxidative damage to normal tissue. This study will lead to better molecular and therapeutic understanding of NRF2 inhibition using certain compounds including bexarotene, which may lead to the sensitization and elimination of resistant cancer cells (Wu et al., 2014, Qi et al., 2016, Garattini et al., 2014, Saito-Hakoda et al., 2015). The work here has demonstrated that the effectiveness of anti-HER receptors therapies such as lapatinib and erlotinib can be reduced even in the presence of HRG, which can bind the receptors and activate alternative signalling pathways (in this case NRF2-ARE). This leads to subsequent modulation of dimerization profiles and activation of associated downstream signalling, thereby overcoming the inhibitory effects of the RTK drugs. Importantly, this research has shown an improved efficacy of lapatinib and erlotinib when combined with bexarotene against ovarian cancer cells, by repressing the HER family receptors NRF2 and AKT.

Overall, this research is in agreement with the in vitro and in vivo data reported by Khalil et al., (2016a and 2016b), which illustrate the important role of NRF2 in influencing outcomes to targeted therapies involving HER2 receptor inhibition (Khalil et al., 2016a), and on the regulation of HER2 and HER3 by NRF2 to oppose HER2 targeted immunotherapy (Khalil et al., 2016b). The study in this section opens up a new potential opportunity for improving the effectiveness of lapatinib and erlotinib via inhibition of NRF2, as well as a novel strategy of cancer cell sensitization during the course of targeted therapy for cancer employing chemotherapeutics.

7.2. Conclusions

This current PhD research study involved designing novel tools for evaluating the regulation of the HER receptor family and NRF2 pathways to ovarian cancer treatment and outcome. A number of assays involving cloning, luciferase assay, cytotoxicity assay, ROS assay, western blotting and total glutathione assay were

employed in the investigation. The research discovered regulatory mechanisms of HER family receptor function involving HER1 and HER4 receptors and NRF2/ARE-dependent transcriptional modulation through their promoter activity. The second means of regulation is via the mechanism of their NRF2 mediated modulation and their concomitant alteration at a protein level.

The novel findings in the current research work extend current knowledge regarding the mechanism of HER receptors and NRF2 expression. This research provides further insight into the molecular basis for regulation of the expression of the HER genes by NRF2, as well as the clinical potential of using siRNA via gene therapy to improve cancer treatment. It also highlights a novel mechanism and role for NRF2 inhibition by bexarotene in sensitising ovarian cancer cells to the killing effect of RTK inhibitors, lapatinib and erlotinib. Finally, it highlights the possibility of using the combination of lapatinib and bexarotene, or erlotinib with bexarotene, to overcome RTK-associated drug resistance.

7.3 Recommendations

The study presented here, albeit being generally successful, was not without limitations. In this section, the main recommendations and suggestions of how to extend the aforementioned hypothesis in future research are hereby discussed. This study has reported on the modulation of HER receptor family and NRF2 in improving ovarian cancer therapy. Further experiments such as gel shift assay and ChIP assay should be employed to investigate the interaction between other relevant transcription factors such AP-1, sp1 and ETF and their possible roles in the transcription of HER1 and HER4. The study has shown that NRF2 regulates HER1 and HER4, and further experiments are needed to confirm the role of NRF2 as a transcription factor for HER receptors. The clinical potential of appropriately designed siRNAs in various diseases including cancer has been demonstrated (Guo et al., 2013, Huang et al., 2008, Xia et al., 2002, Kanninen et al., 2015, Esmaeili, 2016). Many simple questions need to be answered to uncover the full therapeutic benefit of this mechanism. These include details of the components and function of various enzymes involved in the formation of siRNA, the role of siRNA in DNA methylation and chromatin modelling, as well as

other epigenetic mechanisms used by cells to control gene expression. Since these agents are in clinical trials, issues related to bioavailability and safety need to be critically evaluated. Furthermore, the clinical potential of using NRF2 inhibitors such as brusatol, luteolin and bexarotene has been reported (Ren, 2011, Chian et al., 2014a, Chian et al., 2014b, Wu et al., 2014). These inhibitors have been demonstrated to sensitize cancer cells to chemotherapeutic and immunotherapeutic agents, as well as to radiotherapy. Generally, the purpose of screening for these inhibitors is to find inhibitors with sufficient potency that are safer for application within clinical cancer therapies. To better recommend the use of these agents in clinical settings, there is a need for more collective research to explore whether these inhibitors can reliably be used in future either alone or as efficient sensitizing agents in combination with chemotherapy and radiotherapy to improve not only ovarian cancer treatment, but also other cancer treatments.

Most current therapies against cancer consist of empirically combined strategies as a way of improving cancer treatment and overcoming drug resistance. Drug combinations are widely used because multiple drugs affect multiple targets and cell populations, and so enhance their therapeutic effects (Bijnsdorp et al., 2011). It is important to test the potency of a combination and its synergic and/or antagonistic effects before evaluation in the clinic. A combination can be examined by combining two agents in various different ways such as simultaneous or sequential combination schedules (Bijnsdorp et al., 2011). One of the most widely used ways to evaluate whether a combination is effective is the median-drug effect analysis method (Bijnsdorp et al., 2011). Using this method, a combination index (CI) is calculated from drug cytotoxicity or growth inhibition curves (Bijnsdorp et al., 2011, Ashton, 2015). To calculate a CI, the computer software Calcusyn can be used, taking the entire shape of the growth inhibition curve into account for calculating whether a combination is synergistic, additive or antagonistic (Bijnsdorp et al., 2011, Jia et al., 2009, Ashton, 2015). It is recommended that future studies consider how combinations can be designed in vitro and then analysed using Calcusyn or Compusyn so as to identify limitations and advantages of using such combinations. Thus, future studies might expand the role of NRF2 as a key element in driving drug resistance and

opens up additional strategies of sensitising cancer cells to HER family targeted therapy, as well as overcoming the resistance of cancer cells to such drugs.

8. REFERENCES

Abbott, R. A., Whittaker, S. J., Morris, S. L., Russell-Jones, R., Hung, T., Bashir, S. J. and Scarisbrick, J. J. (2009) 'Bexarotene therapy for mycosis fungoides and Sézary syndrome', *British Journal of Dermatology,* 160(6), pp. 1299-1307.

Abdullah, A., Kitteringham, N. R., Jenkins, R. E., Goldring, C., Higgins, L., Yamamoto, M., Hayes, J. and Park, B. K. (2012) 'Analysis of the role of Nrf2 in the expression of liver proteins in mice using two-dimensional gel-based proteomics', *Pharmacological Reports,* 64(3), pp. 680-697.

Agarwal, R. and Kaye, S. B. (2003) 'Ovarian cancer: strategies for overcoming resistance to chemotherapy', *Nature Reviews Cancer,* 3(7), pp. 502-516.

Agus, V. and Janovjak, H. (2017) 'Optogenetic methods in drug screening: technologies and applications', *Current Opinion in Biotechnology,* 48, pp. 8-14.

Ahmed, S. M. U., Luo, L., Namani, A., Wang, X. J. and Tang, X. (2017) 'Nrf2 signaling pathway: Pivotal roles in inflammation', *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease,* 1863(2), pp. 585-597.

Ahn, Y.-H., Hwang, Y., Liu, H., Wang, X. J., Zhang, Y., Stephenson, K. K., Boronina, T. N., Cole, R. N., Dinkova-Kostova, A. T. and Talalay, P. (2010) 'Electrophilic tuning of the chemoprotective natural product sulforaphane', *Proceedings of the National Academy of Sciences,* 107(21), pp. 9590-9595.

Ajgal, Z., de Percin, S., Diéras, V., Pierga, J. Y., Campana, F., Fourquet, A. and Kirova, Y. M. (2017) 'Combination of radiotherapy and double blockade HER2 with pertuzumab and trastuzumab for HER2-positive metastatic or locally recurrent unresectable and/or metastatic breast cancer: Assessment of early toxicity', *Cancer/Radiothérapie,* 21(2), pp. 114-118.

Akhdar, H., Loyer, P., Rauch, C., Corlu, A., Guillouzo, A. and Morel, F. (2009) 'Involvement of Nrf2 activation in resistance to 5-fluorouracil in human colon cancer HT-29 cells', *European Journal of Cancer,* 45(12), pp. 2219-2227.

Alarcón-Aguilar, A., Luna-López, A., Ventura-Gallegos, J. L., Lazzarini, R., Galván-Arzate, S., González-Puertos, V. Y., Morán, J., Santamaría, A. and Königsberg, M. (2014) 'Primary cultured astrocytes from old rats are capable to activate the Nrf2 response against MPP+ toxicity after tBHQ pretreatment', *Neurobiology of Aging,* 35(8), pp. 1901-1912.

Alečković, M. and Kang, Y. (2015) 'Regulation of cancer metastasis by cell-free miRNAs', *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer,* 1855(1), pp. 24-42.

Allard, J. S., Perez, E. J., Fukui, K., Carpenter, P., Ingram, D. K. and Cabo, R. d. (2016) 'Prolonged metformin treatment leads to reduced transcription of Nrf2 and neurotrophic factors without cognitive impairment in older C57BL/6J mice', *Behavioural Brain Research,* 301, pp. 1-9.

Amin, D. N., Campbell, M. R. and Moasser, M. M. (2010) 'The role of HER3, the unpretentious member of the HER family, in cancer biology and cancer therapeutics', *Seminars in Cell & Developmental Biology,* 21(9), pp. 944-950.

Andrews, N. C., Erdjument-Bromage, H., Davidson, M. B., Tempst, P. and Orkin, S. H. (1993) 'Erythroid transcription factor NF-E2 is a haematopoietic-specific basic-leucine zipper protein', *Nature,* 362(6422), pp. 722.

Anuranjani and Bala, M. (2014) 'Concerted action of Nrf2-ARE pathway, MRN complex, HMGB1 and inflammatory cytokines - Implication in modification of radiation damage', *Redox Biology,* 2, pp. 832-846.

Arlt, A., Sebens, S., Krebs, S., Geismann, C., Grossmann, M., Kruse, M. L., Schreiber, S. and Schafer, H. (2013) 'Inhibition of the Nrf2 transcription factor by the alkaloid trigonelline renders pancreatic cancer cells more susceptible to apoptosis through decreased proteasomal gene expression and proteasome activity', *Oncogene,* 32(40), pp. 4825-4835.

Arora, A. and Scholar, E. M. (2005) 'Role of tyrosine kinase inhibitors in cancer therapy', *Journal of Pharmacology and Experimental Therapeutics,* 315(3), pp. 971-979.

Ashton, J. C. (2015) 'Drug Combination Studies and Their Synergy Quantification Using the Chou–Talalay Method', *Cancer research*.

Ashworth, A., Lord, C. J. and Reis-Filho, J. S. (2011) 'Genetic interactions in cancer progression and treatment', *Cell,* 145(1), pp. 30-38.

Avraham, R. and Yarden, Y. (2011) 'Feedback regulation of EGFR signalling: decision making by early and delayed loops', *Nature reviews Molecular cell biology,* 12(2), pp. 104-117.

Balogun, E., Hoque, M., Pengfei, G., Killeen, E., Green, C. J., Foresti, R., Jawed, A. and Motterlini, R. (2003) 'Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element', *Biochemical Journal,* 371(3), pp. 887-895.

Barber, H. R. K. (1986) 'Ovarian cancer', *CA: a cancer journal for clinicians,* 36(3), pp. 149-184.

Barber, H. R. K. (2012) *Ovarian carcinoma: etiology, diagnosis, and treatment.* Springer Science & Business Media.

Barbosa, M. L. d. C., Lima, L. M., Tesch, R., Sant'Anna, C. M. R., Totzke, F., Kubbutat, M. H. G., Schächtele, C., Laufer, S. A. and Barreiro, E. J. (2014) 'Novel 2-chloro-4-anilino-quinazoline derivatives as EGFR and VEGFR-2 dual inhibitors', *European Journal of Medicinal Chemistry,* 71(0), pp. 1-14.

Barrera, L. N., Cassidy, A., Wang, W., Wei, T., Belshaw, N. J., Johnson, I. T., Brigelius-Flohé, R. and Bao, Y. (2012) 'TrxR1 and GPx2 are potently induced by isothiocyanates and selenium, and mutually cooperate to protect Caco-2 cells against free radical-mediated cell death', *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research,* 1823(10), pp. 1914-1924.

BarthÉLÉMy, P., Leblanc, J., Goldbarg, V., Wendling, F. and Kurtz, J.-E. (2014) 'Pertuzumab: Development Beyond Breast Cancer', *Anticancer Research,* 34(4), pp. 1483-1491.

Baselga, J., Cortés, J., Kim, S.-B., Im, S.-A., Hegg, R., Im, Y.-H., Roman, L., Pedrini, J. L., Pienkowski, T. and Knott, A. (2012) 'Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer', *New England Journal of Medicine,* 366(2), pp. 109-119.

Berchuck, A., Kamel, A., Whitaker, R., Kerns, B., Olt, G., Kinney, R., Soper, J. T., Dodge, R., Clarke-Pearson, D. L. and Marks, P. (1990) 'Overexpression of HER-2/neu is associated with poor survival in advanced epithelial ovarian cancer', *Cancer research,* 50(13), pp. 4087-4091.

Bianco, R., Melisi, D., Ciardiello, F. and Tortora, G. (2006) 'Key cancer cell signal transduction pathways as therapeutic targets', *European journal of cancer,* 42(3), pp. 290-294.

Bijnsdorp, I. V., Giovannetti, E. and Peters, G. J. (2011) 'Analysis of drug interactions', *Cancer Cell Culture: Methods and Protocols*, pp. 421-434.

Blackwell, K. L., Burstein, H. J., Storniolo, A. M., Rugo, H., Sledge, G., Koehler, M., Ellis, C., Casey, M., Vukelja, S. and Bischoff, J. (2010) 'Randomized study of Lapatinib alone or in combination with trastuzumab in women with ErbB2-positive, trastuzumab-refractory metastatic breast cancer', *Journal of Clinical Oncology,* 28(7), pp. 1124-1130.

Bonner, J. A., Harari, P. M., Giralt, J., Cohen, R. B., Jones, C. U., Sur, R. K., Raben, D., Baselga, J., Spencer, S. A. and Zhu, J. (2010) 'Radiotherapy plus cetuximab for locoregionally advanced head and neck cancer: 5-year survival data from a phase 3 randomised trial, and relation between cetuximab-induced rash and survival', *The lancet oncology,* 11(1), pp. 21-28.

Bookman, M. A., Darcy, K. M., Clarke-Pearson, D., Boothby, R. A. and Horowitz, I. R. (2003) 'Evaluation of monoclonal humanized anti-HER2 antibody, trastuzumab, in patients with recurrent or refractory ovarian or primary peritoneal carcinoma with overexpression of HER2: a phase II trial of the Gynecologic Oncology Group', *Journal of Clinical Oncology,* 21(2), pp. 283-290.

Bowerman, B., Eaton, B. A. and Priess, J. R. (1992) 'skn-1, a maternally expressed gene required to specify the fate of ventral blastomeres in the early C. elegans embryo', *Cell,* 68(6), pp. 1061-1075.

Buchdunger, E., Zimmermann, J., Mett, H., Meyer, T., Müller, M., Druker, B. J. and Lydon, N. B. (1996) 'Inhibition of the Abl protein-tyrosine kinase in vitro and in vivo by a 2-phenylaminopyrimidine derivative', *Cancer research,* 56(1), pp. 100-104.

Burgess, A. W. (2008) 'EGFR family: structure physiology signalling and therapeutic targets', *Growth Factors,* 26(5), pp. 263-274.

Burris Iii, H. A., Hurwitz, H. I., Dees, E. C., Dowlati, A., Blackwell, K. L., O'Neil, B., Marcom, P. K., Ellis, M. J., Overmoyer, B. and Jones, S. F. (2005) 'Phase I safety, pharmacokinetics, and clinical activity study of lapatinib (GW572016), a reversible dual inhibitor of epidermal growth factor receptor tyrosine kinases, in heavily pretreated patients with metastatic carcinomas', *Journal of Clinical Oncology,* 23(23), pp. 5305-5313.

Calderwood, S. K., Khaleque, M. A., Sawyer, D. B. and Ciocca, D. R. (2006) 'Heat shock proteins in cancer: chaperones of tumorigenesis', *Trends in biochemical sciences,* 31(3), pp. 164-172.

Campbell, M. R., Amin, D. and Moasser, M. M. (2010) 'HER3 comes of age: new insights into its functions and role in signaling, tumor biology, and cancer therapy', *Clinical Cancer Research,* 16(5), pp. 1373-1383.

Cao, C., Lu, S., Sowa, A., Kivlin, R., Amaral, A., Chu, W., Yang, H., Di, W. and Wan, Y. (2008) 'Priming with EGFR tyrosine kinase inhibitor and EGF sensitizes ovarian cancer cells to respond to chemotherapeutical drugs', *Cancer Letters,* 266(2), pp. 249-262.

Cao, L., Zhou, Y., Huang, L., Dong, S. and Ma, Y. (2017) 'Development of a dual-expression vector facilitated with selection-free PCR recombination cloning strategy', *AMB Express,* 7, pp. 98.

Caplen, N. J., Parrish, S., Imani, F., Fire, A. and Morgan, R. A. (2001) 'Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems', *Proceedings of the National Academy of Sciences,* 98(17), pp. 9742-9747.

Cell, S. (2016) *ErbB / HER Signaling Pathway* (Accessed: 11/02/2017 2017).

Chan, J. Y., Han, X.-L. and Kan, Y. W. (1993a) 'Cloning of Nrf1, an NF-E2-related transcription factor, by genetic selection in yeast', *Proceedings of the National Academy of Sciences,* 90(23), pp. 11371-11375.

Chan, J. Y., Han, X.-L. and Kan, Y. W. (1993b) 'Isolation of cDNA encoding the human NF-E2 protein', *Proceedings of the National Academy of Sciences,* 90(23), pp. 11366-11370.

Chandarlapaty, S., Sawai, A., Scaltriti, M., Rodrik-Outmezguine, V., Grbovic-Huezo, O., Serra, V., Majumder, P. K., Baselga, J. and Rosen, N. (2011) 'AKT inhibition relieves feedback suppression of receptor tyrosine kinase expression and activity', *Cancer cell,* 19(1), pp. 58-71.

Chen, C.-C., Chu, C.-B., Liu, K.-J., Huang, C.-Y. F., Chang, J.-Y., Pan, W.-Y., Chen, H.-H., Cheng, Y.-H., Lee, K.-D., Chen, M.-F., Kuo, C.-C. and Chen, L.-T. (2013) 'Gene expression profiling for analysis acquired oxaliplatin resistant factors in human gastric carcinoma TSGH-S3 cells: The role of IL-6 signaling and Nrf2/AKR1C axis identification', *Biochemical Pharmacology,* 86(7), pp. 872-887.

Chen, Q., Peng, H., Dong, L., Chen, L., Ma, X., Peng, Y., Dai, S. and Liu, Q. (2016) 'Activation of the NRF2-ARE signalling pathway by the Lentinula edodes polysaccharose LNT alleviates ROS-mediated cisplatin nephrotoxicity', *International Immunopharmacology,* 36, pp. 1-8.

Cheng, W., Liu, J., Yoshida, H., Rosen, D. and Naora, H. (2005) 'Lineage infidelity of epithelial ovarian cancers is controlled by HOX genes that specify regional identity in the reproductive tract', *Nature medicine,* 11(5), pp. 531.

Chian, S., Li, Y.-Y., Wang, X.-J. and Tang, X.-W. (2014a) 'Luteolin sensitizes two oxaliplatin-resistant colorectal cancer cell lines to chemotherapeutic drugs via inhibition of the Nrf2 pathway', *Asian Pacific journal of cancer prevention: APJCP,* 15(6), pp. 2911-2916.

Chian, S., Thapa, R., Chi, Z., Wang, X. J. and Tang, X. (2014b) 'Luteolin inhibits the Nrf2 signaling pathway and tumor growth in vivo', *Biochemical and Biophysical Research Communications,* 447(4), pp. 602-608.

Chio, Iok In C., Jafarnejad, Seyed M., Ponz-Sarvise, M., Park, Y., Rivera, K., Palm, W., Wilson, J., Sangar, V., Hao, Y., Öhlund, D., Wright, K., Filippini, D., Lee, Eun J., Da Silva, B., Schoepfer, C., Wilkinson, John E., Buscaglia, Jonathan M., DeNicola, Gina M., Tiriac, H., Hammell, M., Crawford, Howard C., Schmidt, Edward E., Thompson, Craig B., Pappin, Darryl J., Sonenberg, N. and Tuveson, David A. (2016) 'NRF2 Promotes Tumor Maintenance by Modulating mRNA Translation in Pancreatic Cancer', *Cell,* 166(4), pp. 963-976.

Chiu, C.-P. and Harley, C. B. (1997) 'Replicative senescence and cell immortality: the role of telomeres and telomerase', *Proceedings of the Society for Experimental Biology and Medicine,* 214(2), pp. 99-106.

Cho, J.-M., Manandhar, S., Lee, H.-R., Park, H.-M. and Kwak, M.-K. (2008) 'Role of the Nrf2-antioxidant system in cytotoxicity mediated by anticancer cisplatin: Implication to cancer cell resistance', *Cancer Letters,* 260(1–2), pp. 96-108.

Choi, B.-h. and Kwak, M.-K. (2016) 'Shadows of NRF2 in cancer: Resistance to chemotherapy', *Current Opinion in Toxicology,* 1, pp. 20-28.

Chorley, B. N., Campbell, M. R., Wang, X., Karaca, M., Sambandan, D., Bangura, F., Xue, P., Pi, J., Kleeberger, S. R. and Bell, D. A. (2012) 'Identification of novel NRF2-regulated genes by ChIP-Seq: influence on retinoid X receptor alpha', *Nucleic acids research*, pp. gks409.

Ciardiello, F., Caputo, R., Bianco, R., Damiano, V., Pomatico, G., De Placido, S., Bianco, A. R. and Tortora, G. (2000) 'Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor', *Clinical Cancer Research,* 6(5), pp. 2053-2063.

Clarke, J. D., Dashwood, R. H. and Ho, E. (2008) 'Multi-targeted prevention of cancer by sulforaphane', *Cancer letters,* 269(2), pp. 291-304.

Clayton, A. J., Danson, S., Jolly, S., Ryder, W. D. J., Burt, P. A., Stewart, A. L., Wilkinson, P. M., Welch, R. S., Magee, B., Wilson, G., Howell, A. and Wardley, A. M. (2004) 'Incidence of cerebral metastases in patients treated with trastuzumab for metastatic breast cancer', *Br J Cancer,* 91(4), pp. 639-643.

Cohen, S. N., Chang, A. C. Y., Boyer, H. W. and Helling, R. B. (1973) 'Construction of biologically functional bacterial plasmids in vitro', *Proceedings of the National Academy of Sciences,* 70(11), pp. 3240-3244.

Colombo, N., Van Gorp, T., Parma, G., Amant, F., Gatta, G., Sessa, C. and Vergote, I. (2006) 'Ovarian cancer', *Critical reviews in oncology/hematology,* 60(2), pp. 159-179.

Cook, N., Frese, K. K. and Moore, M. (2014) 'Assessing the role of the EGF receptor in the development and progression of pancreatic cancer', *Gastrointest Cancer: Targets and Therapy,* 4, pp. 23-37.

Cragg, M. S., French, R. R. and Glennie, M. J. (1999) 'Signaling antibodies in cancer therapy', *Current opinion in immunology,* 11(5), pp. 541-547.

Cronin, M. and Ross, J. S. (2011) 'Comprehensive next-generation cancer genome sequencing in the era of targeted therapy and personalized oncology', *Biomarkers,* 5(3), pp. 293-305.

Cuello, M., Ettenberg, S. A., Clark, A. S., Keane, M. M., Posner, R. H., Nau, M. M., Dennis, P. A. and Lipkowitz, S. (2001) 'Down-regulation of the erbB-2 receptor by trastuzumab (herceptin) enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in breast and ovarian cancer cell lines that overexpress erbB-2', *Cancer research,* 61(12), pp. 4892-4900.

Cullinan, S. B. and Diehl, J. A. (2004) 'PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress', *Journal of Biological Chemistry,* 279(19), pp. 20108-20117.

Cunningham, D., Humblet, Y., Siena, S., Khayat, D., Bleiberg, H., Santoro, A., Bets, D., Mueser, M., Harstrick, A., Verslype, C., Chau, I. and Van Cutsem, E. (2004) 'Cetuximab Monotherapy and Cetuximab plus Irinotecan in Irinotecan-Refractory Metastatic Colorectal Cancer', *New England Journal of Medicine,* 351(4), pp. 337-345.

Dassonville, O., Bozec, A., Fischel, J. L. and Milano, G. (2017) 'EGFR targeting therapies: Monoclonal antibodies versus tyrosine kinase inhibitors', *Critical Reviews in Oncology / Hematology,* 62(1), pp. 53-61.

de Bono, J. S. and Rowinsky, E. K. (2002) 'The ErbB receptor family: a therapeutic target for cancer', *Trends in molecular medicine,* 8(4), pp. S19-S26.

Derjuga, A., Gourley, T. S., Holm, T. M., Heng, H. H. Q., Shivdasani, R. A., Ahmed, R., Andrews, N. C. and Blank, V. (2004) 'Complexity of CNC transcription factors as revealed by gene targeting of the Nrf3 locus', *Molecular and cellular biology,* 24(8), pp. 3286-3294.

Dietz, B. M., Kang, Y.-H., Liu, G., Eggler, A. L., Yao, P., Chadwick, L. R., Pauli, G. F., Farnsworth, N. R., Mesecar, A. D. and Van Breemen, R. B. (2005) 'Xanthohumol isolated from Humulus lupulus inhibits menadione-induced DNA damage through induction of quinone reductase', *Chemical research in toxicology,* 18(8), pp. 1296-1305.

Downward, J. (2003) 'Targeting RAS signalling pathways in cancer therapy', *Nature Reviews Cancer,* 3(1), pp. 11-22.

Druker, B. J., Sawyers, C. L., Kantarjian, H., Resta, D. J., Reese, S. F., Ford, J. M., Capdeville, R. and Talpaz, M. (2001) 'Activity of a Specific Inhibitor of the BCR-ABL Tyrosine Kinase in the Blast Crisis of Chronic Myeloid Leukemia and Acute Lymphoblastic Leukemia with the Philadelphia Chromosome', *New England Journal of Medicine,* 344(14), pp. 1038-1042.

Dry, J. R., Yang, M. and Saez-Rodriguez, J. (2016) 'Looking beyond the cancer cell for effective drug combinations', *Genome medicine,* 8(1), pp. 125.

Duong, H.-Q., You, K. S., Oh, S., Kwak, S.-J. and Seong, Y.-S. (2017) 'Silencing of NRF2 Reduces the Expression of ALDH1A1 and ALDH3A1 and Sensitizes to 5-FU in Pancreatic Cancer Cells', *Antioxidants,* 6(3), pp. 52.

Duvic, M., Hymes, K., Heald, P., Breneman, D., Martin, A. G., Myskowski, P., Crowley, C. and Yocum, R. C. (2001) 'Bexarotene is effective and safe for treatment of refractory advanced-stage cutaneous T-cell lymphoma: multinational phase II-III trial results', *Journal of clinical oncology,* 19(9), pp. 2456-2471.

Edwards, B. K., Howe, H. L., Ries, L. A. G., Thun, M. J., Rosenberg, H. M., Yancik, R., Wingo, P. A., Jemal, A. and Feigal, E. G. (2002) 'Annual report to the nation on the status of cancer, 1973–1999, featuring implications of age and aging on US cancer burden', *Cancer,* 94(10), pp. 2766-2792.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) 'Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells', *nature,* 411(6836), pp. 494-498.

Emily R. Penick, M., Chad A. Hamilton, MD, G. Larry Maxwell, MD, and Charlotte S. Marcus, M. (2018) *12- Germ Cell, Stromal, and Other Ovarian Tumors.*

Engelman, J. A. and Jänne, P. A. (2008) 'Mechanisms of acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non–small cell lung cancer', *Clinical Cancer Research,* 14(10), pp. 2895-2899.

Ercan, D., Xu, C., Yanagita, M., Monast, C. S., Pratilas, C. A., Montero, J., Butaney, M., Shimamura, T., Sholl, L. and Ivanova, E. V. (2012) 'Reactivation of ERK signaling causes resistance to EGFR kinase inhibitors', *Cancer discovery,* 2(10), pp. 934-947.

Esmaeili, M. A. (2016) 'Combination of siRNA-directed gene silencing with epigallocatechin-3-gallate (EGCG) reverses drug resistance in human breast cancer cells', *Journal of chemical biology,* 9(1), pp. 41-52.

Fabbro, D., Ruetz, S., Buchdunger, E., Cowan-Jacob, S. W., Fendrich, G., Liebetanz, J., Mestan, J., O'Reilly, T., Traxler, P., Chaudhuri, B., Fretz, H., Zimmermann, J., Meyer, T., Caravatti, G., Furet, P. and Manley, P. W. (2002) 'Protein kinases as targets for anticancer agents: from inhibitors to useful drugs', *Pharmacology & Therapeutics,* 93(2–3), pp. 79-98.

Fabian, M. A., Biggs, W. H., Treiber, D. K., Atteridge, C. E., Azimioara, M. D., Benedetti, M. G., Carter, T. A., Ciceri, P., Edeen, P. T. and Floyd, M. (2005) 'A small molecule–kinase interaction map for clinical kinase inhibitors', *Nature biotechnology,* 23(3), pp. 329-336.

Faratian, D., Zweemer, A. J. M., Nagumo, Y., Sims, A. H., Muir, M., Dodds, M., Mullen, P., Um, I., Kay, C. and Hasmann, M. (2011) 'Trastuzumab and pertuzumab produce changes in morphology and estrogen receptor signaling in ovarian cancer xenografts revealing new treatment strategies', *Clinical Cancer Research,* 17(13), pp. 4451-4461.

Farhoosh, R. and Tavassoli-Kafrani, M. H. (2010) 'Polar compounds distribution of sunflower oil as affected by unsaponifiable matters of Bene hull oil (BHO) and tertiary-butylhydroquinone (TBHQ) during deep-frying', *Food Chemistry,* 122(1), pp. 381-385.

Farol, L. T. and Hymes, K. B. (2004) 'Bexarotene: a clinical review', *Expert Review of Anticancer Therapy,* 4(2), pp. 180-188.

Farombi, E. O., Shrotriya, S., Na, H.-K., Kim, S.-H. and Surh, Y.-J. (2008) 'Curcumin attenuates dimethylnitrosamine-induced liver injury in rats through Nrf2-mediated induction of heme oxygenase-1', *Food and Chemical Toxicology,* 46(4), pp. 1279-1287.

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D. M., Forman, D. and Bray, F. (2015) 'Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012', *International journal of cancer,* 136(5), pp. E359-E386.

Ferlay, J., Steliarova-Foucher, E., Lortet-Tieulent, J., Rosso, S., Coebergh, J. W. W., Comber, H., Forman, D. and Bray, F. (2013) 'Cancer incidence and mortality patterns in Europe: Estimates for 40 countries in 2012', *European Journal of Cancer,* 49(6), pp. 1374-1403.

Friedlander, M. (1998) 'Prognostic factors', *Ovarian cancer,* 5, pp. 143-152.

Gajria, D. and Chandarlapaty, S. (2011) 'HER2-amplified breast cancer: mechanisms of trastuzumab resistance and novel targeted therapies', *Expert review of anticancer therapy,* 11(2), pp. 263-275.

Gala, K. and Chandarlapaty, S. (2014) 'Molecular pathways: HER3 targeted therapy', *Clinical cancer research,* 20(6), pp. 1410-1416.

Gao, A.-M., Ke, Z.-P., Shi, F., Sun, G.-C. and Chen, H. (2013a) 'Chrysin enhances sensitivity of BEL-7402/ADM cells to doxorubicin by suppressing PI3K/Akt/Nrf2 and ERK/Nrf2 pathway', *Chemico-Biological Interactions,* 206(1), pp. 100-108.

Gao, A.-M., Ke, Z.-P., Wang, J.-N., Yang, J.-Y., Chen, S.-Y. and Chen, H. (2013b) 'Apigenin sensitizes doxorubicin-resistant hepatocellular carcinoma BEL-7402/ADM cells to doxorubicin via inhibiting PI3K/Akt/Nrf2 pathway', *Carcinogenesis,* 34(8), pp. 1806-1814.

Garattini, E., Bolis, M., Garattini, S. K., Fratelli, M., Centritto, F., Paroni, G., Gianni', M., Zanetti, A., Pagani, A., Fisher, J. N., Zambelli, A. and Terao, M. (2014) 'Retinoids and breast cancer: From basic studies to the clinic and back again', *Cancer Treatment Reviews,* 40(6), pp. 739-749.

Gerber, H.-P. and Ferrara, N. (2005) 'Pharmacology and pharmacodynamics of bevacizumab as monotherapy or in combination with cytotoxic therapy in preclinical studies', *Cancer research,* 65(3), pp. 671-680.

Gershenson, D. M. (1994) 'Management of early ovarian cancer: germ cell and sex cord-stromal tumors', *Gynecologic oncology,* 55(3), pp. S562-S572.

Geyer, C. E., Forster, J., Lindquist, D., Chan, S., Romieu, C. G., Pienkowski, T., Jagiello-Gruszfeld, A., Crown, J., Chan, A. and Kaufman, B. (2006) 'Lapatinib plus capecitabine for HER2-positive advanced breast cancer', *New England Journal of Medicine,* 355(26), pp. 2733-2743.

Gharwan, H. and Groninger, H. (2016) 'Kinase inhibitors and monoclonal antibodies in oncology: clinical implications', *Nat Rev Clin Oncol,* 13(4), pp. 209-227.

Giantonio, B. J., Catalano, P. J., Meropol, N. J., O'Dwyer, P. J., Mitchell, E. P., Alberts, S. R., Schwartz, M. A. and Benson Iii, A. B. (2007) 'Bevacizumab in combination with oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) for previously treated metastatic colorectal cancer: results from the Eastern Cooperative Oncology Group Study E3200', *Journal of Clinical Oncology,* 25(12), pp. 1539-1544.

Gilmour, L. M. R., Macleod, K. G., McCaig, A., Gullick, W. J., Smyth, J. F. and Langdon, S. P. (2001) 'Expression of erbB-4/HER-4 growth factor receptor isoforms in ovarian cancer', *Cancer research,* 61(5), pp. 2169-2176.

Gilmour, L. M. R., Macleod, K. G., McCaig, A., Sewell, J. M., Gullick, W. J., Smyth, J. F. and Langdon, S. P. (2002) 'Neuregulin Expression, Function, and Signaling in Human Ovarian Cancer Cells', *Clinical Cancer Research,* 8(12), pp. 3933.

Goldstein, Leonard D., Lee, J., Gnad, F., Klijn, C., Schaub, A., Reeder, J., Daemen, A., Bakalarski, Corey E., Holcomb, T., Shames, David S., Hartmaier, Ryan J., Chmielecki, J., Seshagiri, S., Gentleman, R. and Stokoe, D. (2016) 'Recurrent Loss of NFE2L2 Exon 2 Is a Mechanism for Nrf2 Pathway Activation in Human Cancers', *Cell Reports,* 16(10), pp. 2605-2617.

Goltsov, A., Deeni, Y., Khalil, H. S., Soininen, T., Kyriakidis, S., Hu, H., Langdon, S. P., Harrison, D. J. and Bown, J. (2014a) 'Systems analysis of drug-induced receptor tyrosine kinase reprogramming following targeted mono- and combination anti-cancer therapy', *Cells,* 3(2), pp. 563-91.

Goltsov, A., Langdon, S. P., Goltsov, G., Harrison, D. J. and Bown, J. (2014b) 'Customizing the therapeutic response of signaling networks to promote antitumor responses by drug combinations', *Frontiers in oncology,* 4.

Gomperts, B. D., Kramer, I. M. and Tatham, P. E. R. (2009) 'Chapter 12 - Signalling Pathways Operated by Receptor Protein Tyrosine Kinases', in Tatham, B.D.G.M.K.E.R. (ed.) *Signal Transduction (Second Edition)*. San Diego: Academic Press, pp. 315-374.

Gonzales, C. B., De La Chapa, J. J., Saikumar, P., Singha, P. K., Dybdal-Hargreaves, N. F., Chavez, J., Horning, A. M., Parra, J. and Kirma, N. B. (2016) 'Co-targeting ALK and EGFR parallel signaling in oral squamous cell carcinoma', *Oral Oncology,* 59, pp. 12-19.

Gordon, A. N., Finkler, N., Edwards, R. P., Garcia, A. A., Crozier, M., Irwin, D. H. and Barrett, E. (2005) 'Efficacy and safety of erlotinib HCl, an epidermal growth factor receptor (HER1/EGFR) tyrosine kinase inhibitor, in patients with advanced ovarian carcinoma: results from a phase II multicenter study', *International Journal of Gynecological Cancer,* 15(5), pp. 785-792.

Gordon, M. S., Matei, D., Aghajanian, C., Matulonis, U. A., Brewer, M., Fleming, G. F., Hainsworth, J. D., Garcia, A. A., Pegram, M. D. and Schilder, R. J. (2006) 'Clinical activity of pertuzumab (rhuMAb 2C4), a HER dimerization inhibitor, in advanced ovarian cancer: potential predictive relationship with tumor HER2 activation status', *Journal of Clinical Oncology,* 24(26), pp. 4324-4332.

Gorrini, C., Harris, I. S. and Mak, T. W. (2013) 'Modulation of oxidative stress as an anticancer strategy', *Nature reviews Drug discovery,* 12(12), pp. 931-947.

Grossman, R. and Ram, Z. (2013) 'The Dark Side of Nrf2', *World Neurosurgery,* 80(3–4), pp. 284-286.

Gschwind, A., Fischer, O. M. and Ullrich, A. (2004) 'The discovery of receptor tyrosine kinases: targets for cancer therapy', *Nature Reviews Cancer,* 4(5), pp. 361-370.

Gui, T. and Shen, K. (2012) 'The epidermal growth factor receptor as a therapeutic target in epithelial ovarian cancer', *Cancer Epidemiology,* 36(5), pp. 490-496.

Guo, T., Zhao, L., Zhang, Y., Liu, G., Yao, Y. and Li, H. (2016) 'A monoclonal antibody targeting the dimer interface of epidermal growth factor receptor (EGFR)', *Immunology Letters,* 180, pp. 39-45.

Guo, W., Chen, W., Yu, W., Huang, W. and Deng, W. (2013) 'Small interfering RNA-based molecular therapy of cancers', *Chinese journal of cancer,* 32(9), pp. 488.

Gupta, S. C., Hevia, D., Patchva, S., Park, B., Koh, W. and Aggarwal, B. B. (2012) 'Upsides and downsides of reactive oxygen species for cancer: the roles of reactive oxygen species in tumorigenesis, prevention, and therapy', *Antioxidants & redox signaling,* 16(11), pp. 1295-1322.

Hagiwara, H., Ohwada, N., Aoki, T., Suzuki, T. and Takata, K. (2008) 'The primary cilia of secretory cells in the human oviduct mucosa', *Medical molecular morphology,* 41(4), pp. 193-198.

Hanahan, D. and Weinberg, Robert A. (2011) 'Hallmarks of Cancer: The Next Generation', *Cell,* 144(5), pp. 646-674.

Hannon, G. J. (2002) 'RNA interference', *Nature,* 418(6894), pp. 244-251.

Haouala, A., Zanolari, B., Rochat, B., Montemurro, M., Zaman, K., Duchosal, M. A., Ris, H. B., Leyvraz, S., Widmer, N. and Decosterd, L. A. (2009) 'Therapeutic drug monitoring of the new targeted anticancer agents imatinib, nilotinib, dasatinib, sunitinib, sorafenib and lapatinib by LC tandem mass spectrometry', *Journal of Chromatography B,* 877(22), pp. 1982-1996.

Harrington, E. A., Bebbington, D., Moore, J., Rasmussen, R. K., Ajose-Adeogun, A. O., Nakayama, T., Graham, J. A., Demur, C., Hercend, T. and Diu-Hercend, A. (2004) 'VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth in vivo', *Nature medicine,* 10(3), pp. 262-267.

Harrison, M. L., Hoskins, P., Du Bois, A., Quinn, M., Rustin, G. J. S., Ledermann, J. A., Baron-Hay, S. and Friedlander, M. L. (2006) 'Small cell of the ovary, hypercalcemic type—analysis of combined experience and recommendation for management. A GCIG study', *Gynecologic oncology,* 100(2), pp. 233-238.

Hatton, J. L. and Yee, L. D. (2008) 'Clinical use of PPAR Ligands in Cancer', *PPAR research,* 2008.

Hayashi, M., Inokuchi, M., Takagi, Y., Yamada, H., Kojima, K., Kumagai, J., Kawano, T. and Sugihara, K. (2008) 'High expression of HER3 is associated with a decreased survival in gastric cancer', *Clinical Cancer Research,* 14(23), pp. 7843-7849.

Hayes, A. J., Skouras, C., Haugk, B. and Charnley, R. M. (2015) 'Keap1–Nrf2 signalling in pancreatic cancer', *The International Journal of Biochemistry & Cell Biology,* 65, pp. 288-299.

Hayes, J. D. (2000) 'The Nrf2 transcription factor contributes both to the basal expression of glutathione S-transferases in mouse liver and to their induction by the chemopreventive synthetic antioxidants, butylated hydroxyanisole and ethoxyquin', *Biochem. Soc. Trans.,* 28, pp. 33-41.

Hayes, John D. and Ashford, Michael L. J. (2012) 'Nrf2 Orchestrates Fuel Partitioning for Cell Proliferation', *Cell Metabolism,* 16(2), pp. 139-141.

Hayes, J. D. and Dinkova-Kostova, A. T. (2014) 'The Nrf2 regulatory network provides an interface between redox and intermediary metabolism', *Trends in Biochemical Sciences,* 39(4), pp. 199-218.

Hayes, J. D., Ebisine, K., Sharma, R. S., Chowdhry, S., Dinkova-Kostova, A. T. and Sutherland, C. (2016) 'Regulation of the CNC-bZIP transcription factor Nrf2 by Keap1 and the axis between GSK-3 and β-TrCP', *Current Opinion in Toxicology,* 1, pp. 92-103.

Hayes, J. D. and McMahon, M. (2009) 'NRF2 and KEAP1 mutations: permanent activation of an adaptive response in cancer', *Trends in Biochemical Sciences,* 34(4), pp. 176-188.

Hayes, J. D., McMahon, M., Chowdhry, S. and Dinkova-Kostova, A. T. (2010) 'Cancer chemoprevention mechanisms mediated through the Keap1–Nrf2 pathway', *Antioxid. Redox Signal.,* 13, pp. 1713-1748.

He, J., Xu, Q., Jing, Y., Agani, F., Qian, X., Carpenter, R., Li, Q., Wang, X. R., Peiper, S. S. and Lu, Z. (2012) 'Reactive oxygen species regulate ERBB2 and ERBB3 expression via miR-199a/125b and DNA methylation', *EMBO reports,* 13(12), pp. 1116-1122.

Heller, M. J. (2002) 'DNA microarray technology: devices, systems, and applications', *Annual review of biomedical engineering,* 4(1), pp. 129-153.

Heo, J. C., Jung, T. H., Lee, S., Kim, H. Y., Choi, G., Jung, M., Jung, D., Lee, H. K., Lee, J. O., Park, J. H., Hwang, D., Seol, H. J. and Cho, H. (2016) 'Effect of bexarotene on differentiation of glioblastoma multiforme compared with ATRA', *Clinical & Experimental Metastasis,* 33(5), pp. 417-429.

Herbst, R. S., Johnson, D. H., Mininberg, E., Carbone, D. P., Henderson, T., Kim, E. S., Blumenschein, G., Lee, J. J., Liu, D. D., Truong, M. T., Hong, W. K., Tran, H., Tsao, A., Xie, D., Ramies, D. A., Mass, R., Seshagiri, S., Eberhard, D. A., Kelley, S. K. and Sandler, A. (2005) 'Phase I/II Trial Evaluating the Anti-Vascular Endothelial Growth Factor Monoclonal Antibody Bevacizumab in Combination With the HER-1/Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor Erlotinib for Patients With Recurrent Non–Small-Cell Lung Cancer', *Journal of Clinical Oncology,* 23(11), pp. 2544-2555.

Herbst, R. S., Maddox, A.-M., Rothenberg, M. L., Small, E. J., Rubin, E. H., Baselga, J., Rojo, F., Hong, W. K., Swaisland, H. and Averbuch, S. D. (2002) 'Selective oral epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 is generally well-tolerated and has activity in non–small-cell lung cancer and other solid tumors: Results of a phase I trial', *Journal of Clinical Oncology,* 20(18), pp. 3815-3825.

Higgins, L. G., Kelleher, M. O., Eggleston, I. M., Itoh, K., Yamamoto, M. and Hayes, J. D. (2009) 'Transcription factor Nrf2 mediates an adaptive response to sulforaphane that protects fibroblasts in vitro against the cytotoxic effects of electrophiles, peroxides and redox-cycling agents', *Toxicology and Applied Pharmacology,* 237(3), pp. 267-280.

Hoelder, S., Clarke, P. A. and Workman, P. (2012) 'Discovery of small molecule cancer drugs: Successes, challenges and opportunities', *Molecular Oncology,* 6(2), pp. 155-176.

Hojjat-Farsangi, M., Moshfegh, A., Daneshmanesh, A. H., Khan, A. S., Mikaelsson, E., Österborg, A. and Mellstedt, H. (2014) 'The receptor tyrosine kinase ROR1 – An oncofetal antigen for targeted cancer therapy', *Seminars in Cancer Biology,* 29(0), pp. 21-31.

Hojjat-Farsangi, M., Moshfegh, A., Daneshmanesh, A. H., Khan, A. S., Mikaelsson, E., Österborg, A. and Mellstedt, H. 'The receptor tyrosine kinase ROR1–an oncofetal antigen for targeted cancer therapy'. 2014: Elsevier, 21-31.

Holschneider, C. H. and Berek, J. S. 'Ovarian cancer: epidemiology, biology, and prognostic factors'. 2000: Wiley Online Library, 3-10.

Horikawa, I., Cable, P. L., Afshari, C. and Barrett, J. C. (1999) 'Cloning and characterization of the promoter region of human telomerase reverse transcriptase gene', *Cancer research,* 59(4), pp. 826-830.

Hough, C. D., Sherman-Baust, C. A., Pizer, E. S., Montz, F. J., Im, D. D., Rosenshein, N. B., Cho, K. R., Riggins, G. J. and Morin, P. J. (2000) 'Large-scale serial analysis of gene expression reveals genes differentially expressed in ovarian cancer', *Cancer Research,* 60(22), pp. 6281-6287.

Huang, C., Li, M., Chen, C. and Yao, Q. (2008) 'Small interfering RNA therapy in cancer: mechanism, potential targets, and clinical applications', *Expert opinion on therapeutic targets,* 12(5), pp. 637-645.

Huang, H. C., Nguyen, T. and Pickett, C. B. (2000) 'Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor 2', *Proceedings of the National Academy of Sciences,* 97(23), pp. 12475-12480.

Huang, S., Armstrong, E. A., Benavente, S., Chinnaiyan, P. and Harari, P. M. (2004) 'Dual-agent molecular targeting of the epidermal growth factor receptor (EGFR)', *Cancer Research,* 64(15), pp. 5355-5362.

Hudis, C. A. (2007) 'Trastuzumab—mechanism of action and use in clinical practice', *New England Journal of Medicine,* 357(1), pp. 39-51.

Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., Berlin, J., Baron, A., Griffing, S. and Holmgren, E. (2004) 'Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer', *New England journal of medicine,* 350(23), pp. 2335-2342.

Hynes, N. E. and Lane, H. A. (2005) 'ERBB receptors and cancer: the complexity of targeted inhibitors', *Nature Reviews Cancer,* 5(5), pp. 341-354.

Igarashi, K., Hoshino, H., Muto, A., Suwabe, N., Nishikawa, S., Nakauchi, H. and Yamamoto, M. (1998) 'Multivalent DNA binding complex generated by small Maf and Bach1 as a possible biochemical basis for β-globin locus control region complex', *Journal of Biological Chemistry,* 273(19), pp. 11783-11790.

Igarashi, K., Kataokat, K., Itoh, K., Hayashi, N., Nishizawa, M. and Yamamoto, M. (1994) 'Regulation of transcription by dimerization of erythroid factor NF-E2 p45 with small Maf proteins', *Nature,* 367(6463), pp. 568-572.

Iida, K. (2004) 'Nrf2 is essential for the chemopreventive efficacy of oltipraz against urinary bladder carcinogenesis', *Cancer Res.,* 64, pp. 6424-6431.

Ilkhani, H., Sarparast, M., Noori, A., Zahra Bathaie, S. and Mousavi, M. F. (2015) 'Electrochemical aptamer/antibody based sandwich immunosensor for the detection of EGFR, a cancer biomarker, using gold nanoparticles as a signaling probe', *Biosensors and Bioelectronics,* 74, pp. 491-497.

Imai, K. and Takaoka, A. (2006) 'Comparing antibody and small-molecule therapies for cancer', *Nature Reviews Cancer,* 6(9), pp. 714-727.

Imhoff, B. R. and Hansen, J. M. (2010) 'Tert-butylhydroquinone induces mitochondrial oxidative stress causing Nrf2 activation', *Cell biology and toxicology,* 26(6), pp. 541-551.

Itoh, K. (1997) 'An Nrf2/small Maf heterodimer mediates the induction of Phase II detoxifying enzyme genes through antioxidant response elements', *Biochem. Biophys. Res. Commun.,* 236, pp. 313-322.

Itoh, K., Igarashi, K., Hayashi, N., Nishizawa, M. and Yamamoto, M. (1995) 'Cloning and characterization of a novel erythroid cell-derived CNC family transcription factor heterodimerizing with the small Maf family proteins', *Molecular and Cellular Biology,* 15(8), pp. 4184-4193.

Ivey, J. W., Bonakdar, M., Kanitkar, A., Davalos, R. V. and Verbridge, S. S. (2016) 'Improving cancer therapies by targeting the physical and chemical hallmarks of the tumor microenvironment', *Cancer letters,* 380(1), pp. 330-339.

Jacobs, I. J., Menon, U., Ryan, A., Gentry-Maharaj, A., Burnell, M., Kalsi, J. K., Amso, N. N., Apostolidou, S., Benjamin, E., Cruickshank, D., Crump, D. N., Davies, S. K., Dawnay, A., Dobbs, S., Fletcher, G., Ford, J., Godfrey, K., Gunu, R., Habib, M., Hallett, R., Herod, J., Jenkins, H., Karpinskyj, C., Leeson, S., Lewis, S. J., Liston, W. R., Lopes, A., Mould, T., Murdoch, J., Oram, D., Rabideau, D. J., Reynolds, K., Scott, I., Seif, M. W., Sharma, A., Singh, N., Taylor, J., Warburton, F., Widschwendter, M., Williamson, K., Woolas, R., Fallowfield, L., McGuire, A. J., Campbell, S., Parmar, M. and Skates, S. J. (2016) 'Ovarian cancer screening and mortality in the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS): a randomised controlled trial', *The Lancet,* 387(10022), pp. 945-956.

Jayakumar, S., Pal, D. and Sandur, S. K. (2015) 'Nrf2 facilitates repair of radiation induced DNA damage through homologous recombination repair pathway in a ROS independent manner in cancer cells', *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 779, pp. 33-45.

Jeong, K. J., Cho, K. H., Panupinthu, N., Kim, H., Kang, J., Park, C. G., Mills, G. B. and Lee, H. Y. (2013) 'EGFR mediates LPA-induced proteolytic enzyme expression and ovarian cancer invasion: Inhibition by resveratrol', *Molecular Oncology,* 7(1), pp. 121-129.

Jia, J., Zhu, F., Ma, X., Cao, Z. W., Li, Y. X. and Chen, Y. Z. (2009) 'Mechanisms of drug combinations: interaction and network perspectives', *Nature reviews. Drug discovery,* 8(2), pp. 111.

Jiang, T. (2010) 'High levels of Nrf2 determine chemoresistance in type II endometrial cancer', *Cancer Res.,* 70, pp. 5486-5496.

Johnson, A. C. (1996) 'Activation of epidermal growth factor receptor gene transcription by phorbol 12-myristate 13-acetate is mediated by activator protein 2', *Journal of Biological Chemistry,* 271(6), pp. 3033-3038.

Johnston, S. R. and Leary, A. (2006) 'Lapatinib: a novel EGFR/HER2 tyrosine kinase inhibitor for cancer', *Drugs Today (Barc),* 42(7), pp. 441-453.

Jones, F. E. (2008) 'HER4 intracellular domain (4ICD) activity in the developing mammary gland and breast cancer', *Journal of mammary gland biology and neoplasia,* 13(2), pp. 247-258.

Jorissen, R. N., Walker, F., Pouliot, N., Garrett, T. P. J., Ward, C. W. and Burgess, A. W. (2003) 'Epidermal growth factor receptor: mechanisms of activation and signalling', *Experimental Cell Research,* 284(1), pp. 31-53.

Kaelin, W. G. (2005) 'The concept of synthetic lethality in the context of anticancer therapy', *Nature reviews cancer,* 5(9), pp. 689-698.

Kageyama, R., Merlino, G. T. and Pastan, I. (1988) 'A transcription factor active on the epidermal growth factor receptor gene', *Proceedings of the National Academy of Sciences,* 85(14), pp. 5016-5020.

Kang, H. J., Yi, Y. W., Hong, Y. B., Kim, H. J., Jang, Y.-J., Seong, Y.-S. and Bae, I. (2014a) 'HER2 confers drug resistance of human breast cancer cells through activation of NRF2 by direct interaction', *Scientific reports,* 4.

Kang, H. J., Yi, Y. W., Hong, Y. B., Kim, H. J., Jang, Y.-J., Seong, Y.-S. and Bae, I. (2014b) 'HER2 confers drug resistance of human breast cancer cells through activation of NRF2 by direct interaction', *Scientific Reports,* 4, pp. 7201.

Kang, K. W., Lee, S. J. and Kim, S. G. (2005) 'Molecular mechanism of nrf2 activation by oxidative stress', *Antioxidants & redox signaling,* 7(11-12), pp. 1664-1673.

Kang, K. W., Ryu, J. H. and Kim, S. G. (2000) 'The essential role of phosphatidylinositol 3-kinase and of p38 mitogen-activated protein kinase activation in the antioxidant response element-mediated rGSTA2 induction by decreased glutathione in H4IIE hepatoma cells', *Molecular pharmacology,* 58(5), pp. 1017-1025.

Kanninen, K. M., Pomeshchik, Y., Leinonen, H., Malm, T., Koistinaho, J. and Levonen, A.-L. (2015) 'Applications of the Keap1–Nrf2 system for gene and cell therapy', *Free Radical Biology and Medicine,* 88, pp. 350-361.

Kapoun, M., Bouda, J., Presl, J., Vlasak, P. and Slunečko, R. (2015) 'Agressive small cell carcinoma of the ovary, hypercalcemic type, surgery and oncological treatment: case report', *Ceska gynekologie,* 80(3), pp. 218-221.

Karp, P. D., Paley, S. and Romero, P. (2002) 'The pathway tools software', *Bioinformatics,* 18(suppl 1), pp. S225-S232.

Kascak, P., Zamecnik, M. and Bystricky, B. (2016) 'Small Cell Carcinoma of the Ovary (Hypercalcemic Type): Malignant Rhabdoid Tumor', *Case reports in oncology,* 9(2), pp. 305-311.

Katz, M., Amit, I. and Yarden, Y. (2007) 'Regulation of MAPKs by growth factors and receptor tyrosine kinases', *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research,* 1773(8), pp. 1161-1176.

Kensler, T. W. and Wakabayashi, N. (2010) 'Nrf2: friend or foe for chemoprevention?', *Carcinogenesis,* 31, pp. 90-99.

Khalil, H. and Deeni, Y. (2015) 'NRF2 inhibition causes repression of ATM and ATR expression leading to aberrant DNA Damage Response', *BioDiscovery,* 1.

Khalil, H. S. (2012) 'Identification and characterization of novel autoregulatory mechanism controlling ataxia telangiectasia mutated gene expression, protein traffiking and function'.

Khalil, H. S., Goltsov, A., Langdon, S. P., Harrison, D. J., Bown, J. and Deeni, Y. (2015) 'Quantitative analysis of NRF2 pathway reveals key elements of the regulatory circuits underlying antioxidant response and proliferation of ovarian cancer cells', *Journal of Biotechnology,* 202, pp. 12-30.

Khalil, H. S., Langdon, S. P., Goltsov, A., Soininen, T., Harrison, D. J., Bown, J. and Deeni, Y. Y. (2016a) 'A novel mechanism of action of HER2 targeted immunotherapy is explained by inhibition of NRF2 function in ovarian cancer cells', *Oncotarget*.

Khalil, H. S., Langdon, S. P., Kankia, I. H., Bown, J. and Deeni, Y. Y. (2016b) 'NRF2 Regulates HER2 and HER3 Signaling Pathway to Modulate Sensitivity to Targeted Immunotherapies', *Oxidative Medicine and Cellular Longevity*, pp. 22.

Kim, E.-Y., Choi, Y.-J., Park, C.-W. and Kang, I.-C. (2009) 'Erkitinib, a novel EGFR tyrosine kinase inhibitor screened using a ProteoChip system from a phytochemical library', *Biochemical and Biophysical Research Communications,* 389(3), pp. 415-419.

Kim, I. and He, Y.-Y. (2014) 'Ultraviolet radiation-induced non-melanoma skin cancer: Regulation of DNA damage repair and inflammation', *Genes & Diseases,* 1(2), pp. 188-198.

Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L. C., Coviello, G. M., Wright, W. E., Weinrich, S. L. and Shay, J. W. (1994) 'Specific association of human telomerase activity with immortal cells and cancer', *Science*, pp. 2011-2015.

Kim, S.-J., Kim, J. M., Shim, S. H. and Chang, H. I. (2014) 'Anthocyanins accelerate the healing of naproxen-induced gastric ulcer in rats by activating antioxidant enzymes via modulation of Nrf2', *Journal of Functional Foods,* 7, pp. 569-579.

Kim, S. K., Yang, J. W., Kim, M. R., Roh, S. H., Kim, H. G., Lee, K. Y., Jeong, H. G. and Kang, K. W. (2008) 'Increased expression of Nrf2/ARE-dependent anti-oxidant proteins in tamoxifen-resistant breast cancer cells', *Free Radical Biology and Medicine,* 45(4), pp. 537-546.

Kim, Y. S., Lee, H. L., Lee, K. B., Park, J. H., Chung, W. Y., Lee, K. S., Sheen, S. S., Park, K. J. and Hwang, S. C. (2011) 'Nuclear factor E2-related factor 2 dependent overexpression of sulfiredoxin and peroxiredoxin III in human lung cancer', *The Korean journal of internal medicine,* 26(3), pp. 304-313.

Kimball, K. J., Numnum, T. M., Kirby, T. O., Zamboni, W. C., Estes, J. M., Barnes, M. N., Matei, D. E., Koch, K. M. and Alvarez, R. D. (2008) 'A phase I study of lapatinib in combination with carboplatin in women with platinum sensitive recurrent ovarian carcinoma', *Gynecologic oncology,* 111(1), pp. 95-101.

Kitadai, Y., Yasui, W., Yokozaki, H., Kuniyasu, H., Haruma, K., Kajiyama, G. and Tahara, E. (1992) 'The level of a transcription factor Sp1 is correlated with the expression of EGF receptor in human gastric carcinomas', *Biochemical and biophysical research communications,* 189(3), pp. 1342-1348.

Kitano, H. (2003) 'Cancer robustness: tumour tactics', *Nature,* 426(6963), pp. 125-125.

Klapper, L. N., Glathe, S., Vaisman, N., Hynes, N. E., Andrews, G. C., Sela, M. and Yarden, Y. (1999) 'The ErbB-2/HER2 oncoprotein of human carcinomas may function solely as a shared coreceptor for multiple stroma-derived growth factors', *Proceedings of the National Academy of Sciences,* 96(9), pp. 4995-5000.

Klemm, F. and Joyce, J. A. (2015) 'Microenvironmental regulation of therapeutic response in cancer', *Trends in cell biology,* 25(4), pp. 198-213.

Kobayashi, A., Ito, E., Toki, T., Kogame, K., Takahashi, S., Igarashi, K., Hayashi, N. and Yamamoto, M. (1999) 'Molecular cloning and functional characterization of a new Cap'n'collar family transcription factor Nrf3', *Journal of Biological Chemistry,* 274(10), pp. 6443-6452.

Kobayashi, M. and Yamamoto, M. (2005) 'Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation', *Antioxidants & redox signaling,* 7(3-4), pp. 385-394.

Kobayashi, M. and Yamamoto, M. (2006) 'Nrf2–Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species', *Advances in Enzyme Regulation,* 46(1), pp. 113-140.

Koustas, E., Karamouzis, M. V., Mihailidou, C., Schizas, D. and Papavassiliou, A. G. (2017) 'Co-targeting of EGFR and autophagy signaling is an emerging treatment strategy in metastatic colorectal cancer', *Cancer Letters,* 396, pp. 94-102.

Kovac, S., Angelova, P. R., Holmström, K. M., Zhang, Y., Dinkova-Kostova, A. T. and Abramov, A. Y. (2015) 'Nrf2 regulates ROS production by mitochondria and NADPH oxidase', *Biochimica et Biophysica Acta (BBA) - General Subjects,* 1850(4), pp. 794-801.

Krajka-Kuźniak, V., Paluszczak, J. and Baer-Dubowska, W. (2016) 'The Nrf2-ARE signaling pathway: an update on its regulation and possible role in cancer prevention and treatment', *Pharmacological Reports*.

Krajka-Kuźniak, V., Paluszczak, J. and Baer-Dubowska, W. (2017) 'The Nrf2-ARE signaling pathway: An update on its regulation and possible role in cancer prevention and treatment', *Pharmacological Reports,* 69(3), pp. 393-402.

Kris, M. G., Natale, R. B., Herbst, R. S., Lynch Jr, T. J., Prager, D., Belani, C. P., Schiller, J. H., Kelly, K., Spiridonidis, H. and Sandler, A. (2003) 'Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non–small cell lung cancer: a randomized trial', *Jama,* 290(16), pp. 2149-2158.

Krähn, G., Leiter, U., Kaskel, P., Udart, M., Utikal, J., Bezold, G. and Peter, R. U. (2001) 'Coexpression patterns of EGFR, HER2, HER3 and HER4 in non-melanoma skin cancer', *European Journal of Cancer,* 37(2), pp. 251-259.

Kuang, Y.-H., Shen, T., Chen, X., Sodani, K., Hopper-Borge, E., Tiwari, A. K., Lee, J. W. K. K., Fu, L.-W. and Chen, Z.-S. (2010) 'Lapatinib and erlotinib are potent reversal agents for MRP7 (ABCC10)-mediated multidrug resistance', *Biochemical pharmacology,* 79(2), pp. 154-161.

Kundu, J. K. and Surh, Y.-J. (2010) 'Nrf2-Keap1 signaling as a potential target for chemoprevention of inflammation-associated carcinogenesis', *Pharmaceutical research,* 27(6), pp. 999-1013.

Kwak, M.-K., Itoh, K., Yamamoto, M. and Kensler, T. W. (2002) 'Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the nrf2 promoter', *Molecular and cellular biology,* 22(9), pp. 2883-2892.

Kwak, M.-K., Wakabayashi, N., Itoh, K., Motohashi, H., Yamamoto, M. and Kensler, T. W. (2003) 'Modulation of gene expression by cancer chemopreventive dithiolethiones through the Keap1-Nrf2 pathway Identification of novel gene clusters for cell survival', *Journal of Biological Chemistry,* 278(10), pp. 8135-8145.

Kwak, M. K. and Kensler, T. W. (2010) 'Targeting NRF2 signaling for cancer chemoprevention', *Toxicol. Appl. Pharmacol.,* 244, pp. 66-76.

Kweon, M.-H., Adhami, V. M., Lee, J.-S. and Mukhtar, H. (2006) 'Constitutive overexpression of Nrf2-dependent heme oxygenase-1 in A549 cells contributes to resistance to apoptosis induced by epigallocatechin 3-gallate', *Journal of Biological Chemistry,* 281(44), pp. 33761-33772.

Labi, V. and Erlacher, M. (2015) 'How cell death shapes cancer', *Cell death & disease,* 6(3), pp. e1675.

Langdon, S. and Cameron, D. (2013) 'Pertuzumab for the treatment of metastatic breast cancer', *Expert Review of Anticancer Therapy,* 13(8), pp. 907-918.

Langdon, S. P., Faratian, D., Nagumo, Y., Mullen, P. and Harrison, D. J. (2010) 'Pertuzumab for the treatment of ovarian cancer', *Expert Opinion on Biological Therapy,* 10(7), pp. 1113-1120.

Langdon, S. P., Lawrie, S. S., Hay, F. G., Hawkes, M. M., McDonald, A., Hayward, I. P., Schol, D. J., Hilgers, J., Leonard, R. C. F. and Smyth, J. F. (1988) 'Characterization and Properties of Nine Human Ovarian Adenocarcinoma Cell Lines', *Cancer Research,* 48(21), pp. 6166.

Laughner, E., Taghavi, P., Chiles, K., Mahon, P. C. and Semenza, G. L. (2001) 'HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1α (HIF-1α) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression', *Molecular and cellular biology,* 21(12), pp. 3995-4004.

Ledermann, J. A. and Raja, F. A. (2010) 'Targeted trials in ovarian cancer', *Gynecologic oncology,* 119(1), pp. 151-156.

Lee, A. W. and Templeman, C. and Stram, D. A. and Beesley, J. and Tyrer, J. and Berchuck, A. and Pharoah, P. P. and Chenevix-Trench, G. and Pearce, C. L. and Ness, R. B. and Dansonka-Mieszkowska, A. and Gentry-Maharaj, A. and Hein, A. and Whittemore, A. S. and Jensen, A. and du Bois, A. and Brooks-Wilson, A. and Rudolph, A. and Jakubowska, A. and Wu, A. H. and Ziogas, A. and Ekici, A. B. and Leminen, A. and Rosen, B. and Spiewankiewicz, B. and Karlan, B. Y. and Trabert, B. and Fridley, B. L. and Gilks, C. B. and Krakstad, C. and Phelan, C. M. and Cybulski, C. and Walsh, C. and Hogdall, C. and Cramer, D. W. and Huntsman, D. G. and Eccles, D. and Lambrechts, D. and Liang, D. and Levine, D. A. and Iversen, E. S. and Bandera, E. V. and Poole, E. M. and Goode, E. L. and Van Nieuwenhuysen, E. and Hogdall, E. and Bruinsma, F. and Heitz, F. and Modugno, F. and Giles, G. G. and Risch, H. A. and Baker, H. and Salvesen, H. B. and Nevanlinna, H. and Anton-Culver, H. and Song, H. and McNeish, I. and Campbell, I. G. and Vergote, I. and Runnebaum, I. B. and Tangen, I. L. and Schwaab, I. and Gronwald, J. and Paul, J. and Lubinski, J. and Doherty, J. A. and Chang-Claude, J. and Lester, J. and Schildkraut, J. M. and McLaughlin, J. R. and Lissowska, J. and Kupryjanczyk, J. and Kelley, J. L. and Rothstein, J. H. and Cunningham, J. M. and Lu, K. and Carty, K. and Terry, K. L. and Aben, K. K. H. and Moysich, K. B. and Wicklund, K. G. and Odunsi, K. and Kiemeney, L. A. and Sucheston-Campbell, L. and Lundvall, L. and Massuger, L. F. A. G. and Pelttari, L. M. and Kelemen, L. E. and Cook, L. S. and Bjorge, L. and Nedergaard, L. and Brinton, L. A. and Wilkens, L. R. and Pike, M. C. and Goodman, M. T. and Bisogna, M. and Rossing, M. A. and Beckmann, M. W. and Dürst, M. and Southey, M. C. and Kellar, M. and Hildebrandt, M. A. T. and Siddiqui, N. and Antonenkova, N. and Bogdanova, N. and Le, N. D. and Wentzensen, N. and Thompson, P. J. and Harrington, P. and Webb, P. M. and Fasching, P. A. and Hillemanns, P. and Harter, P. and Sobiczewski, P. and Weber, R. P. and Butzow, R. and Edwards, R. P. and Vierkant, R. A. and Glasspool, R. and Orsulic, S. and Lambrechts, S. and Olson, S. H. and Wang-Gohrke, S. and Lele, S. and Tworoger, S. S. and Gayther, S. A. and Missmer, S. A. and Narod, S. A. and Ramus, S. J. and Kjaer, S. K. and Pejovic, T. and Dörk, T. and Eilber, U. and Menon, U. and McGuire, V. and Sieh, W. and Wu, X. and Bean, Y. and Shvetsov, Y. B. (2016a) 'Evidence of a genetic link between

endometriosis and ovarian cancer', *Fertility and Sterility,* 105(1), pp. 35-43.e10.

Lee, C.-H., Huntsman, D. G., Cheang, M. C. U., Parker, R. L., Brown, L., Hoskins, P., Miller, D. and Gilks, C. B. (2005) 'Assessment of Her-1, Her-2, And Her-3 expression and Her-2 amplification in advanced stage ovarian carcinoma', *International Journal of Gynecological Pathology,* 24(2), pp. 147-152.

Lee, H. and Maihle, N. J. (1998) 'Isolation and characterization of four alternate c-erbB3 transcripts expressed in ovarian carcinoma-derived cell lines and normal human tissues', *Oncogene,* 16(25), pp. 3243-3252.

Lee, I.-S., Lim, J., Gal, J., Kang, J. C., Kim, H. J., Kang, B. Y. and Choi, H. J. (2011) 'Anti-inflammatory activity of xanthohumol involves heme oxygenase-1 induction via NRF2-ARE signaling in microglial BV2 cells', *Neurochemistry International,* 58(2), pp. 153-160.

Lee, J., Kang, U., Seo, E. K. and Kim, Y. S. (2016b) 'Heme oxygenase-1-mediated anti-inflammatory effects of tussilagonone on macrophages and 12-O-tetradecanoylphorbol-13-acetate-induced skin inflammation in mice', *International Immunopharmacology,* 34, pp. 155-164.

Lee, J.-S. and Surh, Y.-J. (2005) 'Nrf2 as a novel molecular target for chemoprevention', *Cancer letters,* 224(2), pp. 171-184.

Lee-Jones, L. (2003) 'Ovary: germ cell tumors'.

Leone, A., Roca, M. S., Ciardiello, C., Terranova-Barberio, M., Vitagliano, C., Ciliberto, G., Mancini, R., Di Gennaro, E., Bruzzese, F. and Budillon, A. (2015) 'Vorinostat synergizes with EGFR inhibitors in NSCLC cells by increasing ROS via up-regulation of the major mitochondrial porin VDAC1 and modulation of the c-Myc-NRF2-KEAP1 pathway', *Free Radical Biology and Medicine,* 89, pp. 287-299.

Leveque, D. (2008) 'Off-label use of anticancer drugs', *The lancet oncology,* 9(11), pp. 1102-1107.

Levitzki, A. (1999) 'Protein tyrosine kinase inhibitors as novel therapeutic agents', *Pharmacology & therapeutics,* 82(2), pp. 231-239.

Levitzki, A. (2000) 'Protein tyrosine kinase inhibitors as novel therapeutic agents', *CANCER DRUG DISCOVERY AND DEVELOPMENT,* 5, pp. 453-466.

Levitzki, A. and Mishani, E. (2006) 'Tyrphostins and other tyrosine kinase inhibitors', *Annu. Rev. Biochem.,* 75, pp. 93-109.

Lheureux, S., Krieger, S., Weber, B., Pautier, P., Fabbro, M., Selle, F., Bourgeois, H., Petit, T., Lortholary, A. and Plantade, A. (2012) 'Expected benefits of topotecan combined with lapatinib in recurrent ovarian cancer according to biological profile: a phase 2 trial', *International Journal of Gynecological Cancer,* 22(9), pp. 1483-1488.

Li, C., Xu, X., Tao, Z., Wang, X. J. and Pan, Y. (2015) 'Resveratrol dimers, nutritional components in grape wine, are selective ROS scavengers and weak Nrf2 activators', *Food Chemistry,* 173, pp. 218-223.

Li, Q., Jiang, H., Li, H., Xu, R., Shen, L., Yu, Y., Wang, Y., Cui, Y., Li, W., Yu, S. and Liu, T. (2016) 'Efficacy of trastuzumab beyond progression in HER2 positive advanced gastric cancer: a multicenter prospective observational cohort study', *Oncotarget,* 7(31), pp. 50656-50665.

Li, S., Li, J., Shen, C., Zhang, X., Sun, S., Cho, M., Sun, C. and Song, Z. (2014) 'tert-Butylhydroquinone (tBHQ) protects hepatocytes against lipotoxicity via inducing autophagy independently of Nrf2 activation', *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids,* 1841(1), pp. 22-33.

Li, W., Huang, Y., Yang, B., Chi, X. H., Liu, L. H., Zhang, F., Yan, J. W. and Lu, X. C. (2010) 'Cloning of ID4 gene expression regulation promoter and subcloning of recombinant ID4 promoter luciferase reporter', *Zhongguo shi yan xue ye xue za zhi,* 18(2), pp. 421-426.

Li, W., Yu, S., Liu, T., Kim, J.-H., Blank, V., Li, H. and Kong, A. N. T. (2008a) 'Heterodimerization with small Maf proteins enhances nuclear retention of Nrf2 via masking the NESzip motif', *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research,* 1783(10), pp. 1847-1856.

Li, X., Lewis, M. T., Huang, J., Gutierrez, C., Osborne, C. K., Wu, M.-F., Hilsenbeck, S. G., Pavlick, A., Zhang, X. and Chamness, G. C. (2008b) 'Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy', *Journal of the National Cancer Institute,* 100(9), pp. 672-679.

Limaye, S. A., Posner, M. R., Krane, J. F., Fonfria, M., Lorch, J. H., Dillon, D. A., Shreenivas, A. V., Tishler, R. B. and Haddad, R. I. (2013) 'Trastuzumab for the treatment of salivary duct carcinoma', *The oncologist,* 18(3), pp. 294-300.

Limonciel, A. and Jennings, P. (2014) 'A review of the evidence that ochratoxin A is an Nrf2 inhibitor: implications for nephrotoxicity and renal carcinogenicity', *Toxins,* 6(1), pp. 371-379.

Lin, W.-L., Kuo, W.-H., Chen, F.-L., Lee, M.-Y., Ruan, A., Tyan, Y.-S., Hsu, J.-D., Chiang, H. and Han, C.-P. (2011) 'Identification of the coexisting HER2 gene amplification and novel mutations in the HER2 protein-overexpressed mucinous epithelial ovarian cancer', *Annals of surgical oncology,* 18(8), pp. 2388-2394.

Linggi, B. and Carpenter, G. (2006) 'ErbB receptors: new insights on mechanisms and biology', *Trends in Cell Biology,* 16(12), pp. 649-656.

Liou, G.-Y. and Storz, P. (2010) 'Reactive oxygen species in cancer', *Free radical research,* 44(5), pp. 10.3109/10715761003667554.

Lister, A., Nedjadi, T., Kitteringham, N. R., Campbell, F., Costello, E., Lloyd, B., Copple, I. M., Williams, S., Owen, A. and Neoptolemos, J. P. (2011) 'Nrf2 is overexpressed in pancreatic cancer: implications for cell proliferation and therapy', *Molecular cancer,* 10(1), pp. 37.

Liu, Y., Chen, F., Wang, S., Guo, X., Shi, P., Wang, W. and Xu, B. (2013) 'Low-dose triptolide in combination with idarubicin induces apoptosis in AML leukemic stem-like KG1a cell line by modulation of the intrinsic and extrinsic factors', *Cell death & disease,* 4(12), pp. e948.

Loman, N. J., Constantinidou, C., Chan, J. Z. M., Halachev, M., Sergeant, M., Penn, C. W., Robinson, E. R. and Pallen, M. J. (2012) 'High-throughput bacterial genome sequencing: an embarrassment of choice, a world of opportunity', *Nature Reviews Microbiology,* 10(9), pp. 599-606.

Lu, C., Zou, Y., Liu, Y. and Niu, Y. (2017) 'Rosmarinic acid counteracts activation of hepatic stellate cells via inhibiting the ROS-dependent MMP-2 activity: Involvement of Nrf2 antioxidant system', *Toxicology and Applied Pharmacology,* 318, pp. 69-78.

Luen, S. J., Salgado, R., Fox, S., Savas, P., Eng-Wong, J., Clark, E., Kiermaier, A., Swain, S. M., Baselga, J., Michiels, S. and Loi, S. (2017) 'Tumour-infiltrating lymphocytes in advanced HER2-positive breast cancer treated with pertuzumab or placebo in addition to trastuzumab and docetaxel: a retrospective analysis of the CLEOPATRA study', *The Lancet Oncology,* 18(1), pp. 52-62.

Luo, J., Solimini, N. L. and Elledge, S. J. (2009) 'Principles of Cancer Therapy: Oncogene and Non-oncogene Addiction', *Cell,* 136(5), pp. 823-837.

Ma, J., Lyu, H., Huang, J. and Liu, B. (2014) 'Targeting of erbB3 receptor to overcome resistance in cancer treatment', *Molecular cancer,* 13(1), pp. 105.

Ma, Q. (2013) 'Role of nrf2 in oxidative stress and toxicity', *Annual review of pharmacology and toxicology,* 53, pp. 401-426.

MacConaill, L. E. and Garraway, L. A. (2010) 'Clinical implications of the cancer genome', *Journal of Clinical Oncology,* 28(35), pp. 5219-5228.

Madhusudan, S. and Ganesan, T. S. (2004) 'Tyrosine kinase inhibitors in cancer therapy', *Clinical Biochemistry,* 37(7), pp. 618-635.

Maihle, N. J., Baron, A. T., Barrette, B. A., Boardman, C. H., Christensen, T. A., Cora, E. M., Faupel-Badger, J. M., Greenwood, T., Juneja, S. C. and Lafky, J. M. (2002) 'EGF/ErbB receptor family in ovarian cancer', *Ovarian Cancer*: Springer, pp. 247-258.

Makhija, S., Amler, L. C., Glenn, D., Ueland, F. R., Gold, M. A., Dizon, D. S., Paton, V., Lin, C.-Y., Januario, T., Ng, K., Strauss, A., Kelsey, S., Sliwkowski, M. X. and Matulonis, U. (2010) 'Clinical Activity of Gemcitabine Plus Pertuzumab in Platinum-Resistant Ovarian Cancer, Fallopian Tube Cancer, or Primary Peritoneal Cancer', *Journal of Clinical Oncology,* 28(7), pp. 1215-1223.

Malik, V., Dhanjal, J. K., Kumari, A., Radhakrishnan, N., Singh, K. and Sundar, D. (2017) 'Function and structure-based screening of compounds, peptides and proteins to identify drug candidates', *Methods*.

Maly, J. J. and Macrae, E. R. (2014) 'Pertuzumab in Combination with Trastuzumab and Chemotherapy in the Treatment of HER2-Positive Metastatic Breast Cancer: Safety, Efficacy, and Progression Free Survival', *Breast Cancer : Basic and Clinical Research,* 8, pp. 81-88.

Manandhar, S., Choi, B.-h., Jung, K.-A., Ryoo, I.-g., Song, M., Kang, S. J., Choi, H.-G., Kim, J.-A., Park, P.-H. and Kwak, M.-K. (2012) 'NRF2 inhibition represses ErbB2 signaling in ovarian carcinoma cells: Implications for tumor growth retardation and docetaxel sensitivity', *Free Radical Biology and Medicine,* 52(9), pp. 1773-1785.

Manley, P. W., Cowan-Jacob, S. W., Buchdunger, E., Fabbro, D., Fendrich, G., Furet, P., Meyer, T. and Zimmermann, J. (2002) 'Imatinib: a selective tyrosine kinase inhibitor', *European Journal of Cancer,* 38, Supplement 5(0), pp. S19-S27.

Mansoori, B., Shotorbani, S. S. and Baradaran, B. (2014) 'RNA interference and its role in cancer therapy', *Advanced pharmaceutical bulletin,* 4(4), pp. 313.

Maps, I. I. V. 'pGEMŪ-T and pGEMŪ-T Easy Vector Systems'.

Marmor, M. D., Skaria, K. B. and Yarden, Y. (2004) 'Signal transduction and oncogenesis by ErbB/HER receptors', *International Journal of Radiation Oncology* Biology* Physics,* 58(3), pp. 903-913.

Mass, R. D. (2004) 'The HER receptor family: a rich target for therapeutic development', *International Journal of Radiation Oncology*Biology*Physics,* 58(3), pp. 932-940.

Matar, P., Rojo, F., Cassia, R., Moreno-Bueno, G., Di Cosimo, S., Tabernero, J., Guzmán, M., Rodriguez, S., Arribas, J. and Palacios, J. (2004) 'Combined epidermal growth factor receptor targeting with the tyrosine kinase inhibitor gefitinib (ZD1839) and the monoclonal antibody cetuximab (IMC-C225)', *Clinical Cancer Research,* 10(19), pp. 6487-6501.

McAlpine, J. N., Wiegand, K. C., Vang, R., Ronnett, B. M., Adamiak, A., Köbel, M., Kalloger, S. E., Swenerton, K. D., Huntsman, D. G. and Gilks, C. B. (2009) 'HER2 overexpression and amplification is present in a subset of ovarian mucinous carcinomas and can be targeted with trastuzumab therapy', *BMC cancer,* 9(1), pp. 433.

McCubrey, J. A., Abrams, S. L., Fitzgerald, T. L., Cocco, L., Martelli, A. M., Montalto, G., Cervello, M., Scalisi, A., Candido, S., Libra, M. and Steelman, L. S. (2015) 'Roles of signaling pathways in drug resistance, cancer initiating cells and cancer progression and metastasis', *Advances in Biological Regulation,* 57, pp. 75-101.

McMahon, M., Campbell, K. H., MacLeod, A. K., McLaughlin, L. A., Henderson, C. J. and Wolf, C. R. (2014) 'HDAC Inhibitors Increase NRF2-Signaling in Tumour Cells and Blunt the Efficacy of Co-Adminstered Cytotoxic Agents', *PLoS ONE,* 9(11), pp. e114055.

Meden, H. and Kuhn, W. (1997) 'Overexpression of the oncogene c-erbB-2 (HER2/neu) in ovarian cancer: a new prognostic factor', *European Journal of Obstetrics & Gynecology and reproductive biology,* 71(2), pp. 173-179.

Mehta, N., Wayne, A. S., Kim, Y. H., Hale, G. A., Alvarado, C. S., Myskowski, P., Jaffe, E. S., Busam, K. J., Pulitzer, M. and Zwerner, J. (2012) 'Bexarotene is active against subcutaneous panniculitis-like T-cell lymphoma in adult and pediatric populations', *Clinical Lymphoma Myeloma and Leukemia,* 12(1), pp. 20-25.

Mendelsohn, J. and Baselga, J. (2000) 'The EGF receptor family as targets for cancer therapy', *Oncogene,* 19(56), pp. 6550.

Merino, M. J. and Jaffe, G. (1993) 'Age contrast in ovarian pathology', *Cancer,* 71(S2), pp. 537-544.

Messersmith, W. A. and Ahnen, D. J. (2008) 'Targeting EGFR in colorectal cancer', *N engl j Med,* 359(17), pp. 1834-1836.

Mester, J. and Redeuilh, G. (2008) 'Proliferation of breast cancer cells: regulation, mediators, targets for therapy', *Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents),* 8(8), pp. 872-885.

Mohler, J., Mahaffey, J. W., Deutsch, E. and Vani, K. (1995) 'Control of Drosophila head segment identity by the bZIP homeotic gene cnc', *Development,* 121(1), pp. 237-247.

Mohler, J., Vani, K., Leung, S. and Epstein, A. (1991) 'Segmentally restricted, cephalic expression of a leucine zipper gene during Drosophila embryogenesis', *Mechanisms of development,* 34(1), pp. 3-9.

Moi, P., Chan, K., Asunis, I., Cao, A. and Kan, Y. W. (1994) 'Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region', *Proceedings of the National Academy of Sciences of the United States of America,* 91(21), pp. 9926-9930.

Molina, M. A., Codony-Servat, J., Albanell, J., Rojo, F., Arribas, J. and Baselga, J. (2001) 'Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells', *Cancer research,* 61(12), pp. 4744-4749.

Montemurro, F., Valabrega, G. and Aglietta, M. (2007) 'Lapatinib: a dual inhibitor of EGFR and HER2 tyrosine kinase activity', *Expert opinion on biological therapy,* 7(2), pp. 257-268.

Montero, J. C., García-Alonso, S., Ocaña, A. and Pandiella, A. (2015) 'Identification of therapeutic targets in ovarian cancer through active tyrosine kinase profiling', *Oncotarget,* 6(30), pp. 30057-30071.

Moulder, S. L., Yakes, F. M., Muthuswamy, S. K., Bianco, R., Simpson, J. F. and Arteaga, C. L. (2001) 'Epidermal growth factor receptor (HER1) tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2/neu (erbB2)-overexpressing breast cancer cells in vitro and in vivo', *Cancer research,* 61(24), pp. 8887-8895.

Mullen, P., Cameron, D. A., Hasmann, M., Smyth, J. F. and Langdon, S. P. (2007) 'Sensitivity to pertuzumab (2C4) in ovarian cancer models: cross-talk with estrogen receptor signaling', *Molecular Cancer Therapeutics,* 6(1), pp. 93.

Murphy, M. and Stordal, B. (2011) 'Erlotinib or gefitinib for the treatment of relapsed platinum pretreated non-small cell lung cancer and ovarian cancer: A systematic review', *Drug Resistance Updates,* 14(3), pp. 177-190.

Muto, A., Hoshino, H., Madisen, L., Yanai, N., Obinata, M., Karasuyama, H., Hayashi, N., Nakauchi, H., Yamamoto, M. and Groudine, M. (1998) 'Identification of Bach2 as a B-cell-specific partner for small Maf proteins that negatively regulate the immunoglobulin heavy chain gene 3′ enhancer', *The EMBO journal,* 17(19), pp. 5734-5743.

Na, H.-K., Kim, E.-H., Jung, J.-H., Lee, H.-H., Hyun, J.-W. and Surh, Y.-J. (2008) '(−)-Epigallocatechin gallate induces Nrf2-mediated antioxidant enzyme expression via activation of PI3K and ERK in human mammary epithelial cells', *Archives of Biochemistry and Biophysics,* 476(2), pp. 171-177.

Nagumo, Y., Faratian, D., Mullen, P., Harrison, D. J., Hasmann, M. and Langdon, S. P. (2009a) 'Modulation of HER3 is a marker of dynamic cell signaling in ovarian cancer: implications for pertuzumab sensitivity', *Molecular Cancer Research,* 7(9), pp. 1563-1571.

Nagumo, Y., Faratian, D., Mullen, P., Harrison, D. J., Hasmann, M. and Langdon, S. P. (2009b) 'Modulation of HER3 Is a Marker of Dynamic Cell Signaling in Ovarian Cancer: Implications for Pertuzumab Sensitivity', *Molecular Cancer Research,* 7(9), pp. 1563.

Nahta, R., Hung, M.-C. and Esteva, F. J. (2004) 'The HER-2-targeting antibodies trastuzumab and pertuzumab synergistically inhibit the survival of breast cancer cells', *Cancer research,* 64(7), pp. 2343-2346.

Namani, A., Li, Y., Wang, X. J. and Tang, X. (2014) 'Modulation of NRF2 signaling pathway by nuclear receptors: Implications for cancer', *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research,* 1843(9), pp. 1875-1885.

Nguyen, T., Sherratt, P. J., Huang, H. C., Yang, C. S. and Pickett, C. B. (2003) 'Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element Degradation of Nrf2 by the 26 S proteasome', *Journal of Biological Chemistry,* 278(7), pp. 4536-4541.

Nicholas, M. K., Lukas, R. V., Jafri, N. F., Faoro, L. and Salgia, R. (2006) 'Epidermal growth factor receptor–mediated signal transduction in the development and therapy of gliomas', *Clinical Cancer Research,* 12(24), pp. 7261-7270.

No, J. H., Kim, Y.-B. and Song, Y. S. (2014) 'Targeting Nrf2 Signaling to Combat Chemoresistance', *Journal of Cancer Prevention,* 19(2), pp. 111-117.

Noonberg, S. B. and Benz, C. C. (2000) 'Tyrosine kinase inhibitors targeted to the epidermal growth factor receptor subfamily', *Drugs,* 59(4), pp. 753-767.

Normanno, N., Bianco, C., De Luca, A., Maiello, M. R. and Salomon, D. S. (2003) 'Target-based agents against ErbB receptors and their ligands: a novel approach to cancer treatment', *Endocrine-related cancer,* 10(1), pp. 1-21.

Normanno, N., De Luca, A., Bianco, C., Strizzi, L., Mancino, M., Maiello, M. R., Carotenuto, A., De Feo, G., Caponigro, F. and Salomon, D. S. (2006) 'Epidermal growth factor receptor (EGFR) signaling in cancer', *Gene,* 366(1), pp. 2-16.

Nwabo Kamdje, A. H., Seke Etet, P. F., Vecchio, L., Muller, J. M., Krampera, M. and Lukong, K. E. (2014) 'Signaling pathways in breast cancer: Therapeutic targeting of the microenvironment', *Cellular Signalling,* 26(12), pp. 2843-2856.

O'Mealey, G. B., Berry, W. L. and Plafker, S. M. (2017) 'Sulforaphane is a Nrf2-independent inhibitor of mitochondrial fission', *Redox Biology,* 11, pp. 103-110.

O'Sullivan, C. C. and Connolly, R. M. (2014) 'Pertuzumab and its accelerated approval: evolving treatment paradigms and new challenges in the management of HER2-positive breast cancer', *Oncology,* 28(3), pp. 186-186.

Oberaigner, W., Minicozzi, P., Bielska-Lasota, M., Allemani, C., De Angelis, R., Mangone, L., Sant, M. and Eurocare Working, G. (2012) 'Survival for ovarian cancer in Europe: the across-country variation did not shrink in the past decade', *Acta oncologica,* 51(4), pp. 441-453.

Ohta, T., Iijima, K., Miyamoto, M., Nakahara, I., Tanaka, H., Ohtsuji, M., Suzuki, T., Kobayashi, A., Yokota, J. and Sakiyama, T. (2008) 'Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth', *Cancer research,* 68(5), pp. 1303-1309.

Okazaki, S., Nakatani, F., Masuko, K., Tsuchihashi, K., Ueda, S., Masuko, T., Saya, H. and Nagano, O. (2016) 'Development of an ErbB4 monoclonal antibody that blocks neuregulin-1-induced ErbB4 activation in cancer cells', *Biochemical and Biophysical Research Communications,* 470(1), pp. 239-244.

Olayanju, A., Copple, I. M., Bryan, H. K., Edge, G. T., Sison, R. L., Wong, M. W., Lai, Z.-Q., Lin, Z.-X., Dunn, K., Sanderson, C. M., Alghanem, A. F., Cross, M. J., Ellis, E. C., Ingelman-Sundberg, M., Malik, H. Z., Kitteringham, N. R., Goldring, C. E. and Park, B. K. (2015) 'Brusatol provokes a rapid and transient inhibition of Nrf2 signaling and sensitizes mammalian cells to chemical toxicity—implications for therapeutic targeting of Nrf2', *Free Radical Biology and Medicine,* 78, pp. 202-212.

Omar, N., Yan, B. and Salto-Tellez, M. (2015) 'HER2: An emerging biomarker in non-breast and non-gastric cancers', *Pathogenesis,* 2(3), pp. 1-9.

Oyake, T., Itoh, K., Motohashi, H., Hayashi, N., Hoshino, H., Nishizawa, M., Yamamoto, M. and Igarashi, K. (1996) 'Bach proteins belong to a novel family of BTB-basic leucine zipper transcription factors that interact with MafK and regulate transcription through the NF-E2 site', *Molecular and cellular biology,* 16(11), pp. 6083-6095.

Padmanabhan, B., Tong, K. I., Ohta, T., Nakamura, Y., Scharlock, M., Ohtsuji, M., Kang, M.-I., Kobayashi, A., Yokoyama, S. and Yamamoto, M. (2006) 'Structural Basis for Defects of Keap1 Activity Provoked by Its Point Mutations in Lung Cancer', *Molecular Cell,* 21(5), pp. 689-700.

Paez, J. G., Jänne, P. A., Lee, J. C., Tracy, S., Greulich, H., Gabriel, S., Herman, P., Kaye, F. J., Lindeman, N. and Boggon, T. J. (2004) 'EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy', *Science,* 304(5676), pp. 1497-1500.

Pandey, P., Singh, A. K., Singh, M., Tewari, M., Shukla, H. S. and Gambhir, I. S. (2017) 'The see-saw of Keap1-Nrf2 pathway in Cancer', *Critical Reviews in Oncology/Hematology*.

Papadavid, E., Antoniou, C., Nikolaou, V., Siakantaris, M., Vassilakopoulos, T. P., Stratigos, A., Stavrianeas, N. and Katsambas, A. (2008) 'Safety and efficacy of low-dose bexarotene and PUVA in the treatment of patients with mycosis fungoides', *American journal of clinical dermatology,* 9(3), pp. 169.

Park, E. J., Lim, J. H., Nam, S. I., Park, J. W. and Kwon, T. K. (2010) 'Rottlerin induces heme oxygenase-1 (HO-1) up-regulation through reactive oxygen species (ROS) dependent and PKC δ-independent pathway in human colon cancer HT29 cells', *Biochimie,* 92(1), pp. 110-115.

Pautier, P., Ribrag, V., Duvillard, P., Rey, A., Elghissassi, I., Sillet-Bach, I., Kerbrat, P., Mayer, F., Lesoin, A. and Brun, B. (2007) 'Results of a prospective dose-intensive regimen in 27 patients with small cell carcinoma of the ovary of the hypercalcemic type', *Annals of oncology,* 18(12), pp. 1985-1989.

Pearson, K. J., Lewis, K. N., Price, N. L., Chang, J. W., Perez, E., Cascajo, M. V., Tamashiro, K. L., Poosala, S., Csiszar, A., Ungvari, Z., Kensler, T. W., Yamamoto, M., Egan, J. M., Longo, D. L., Ingram, D. K., Navas, P. and de Cabo, R. (2008) 'Nrf2 mediates cancer protection but not prolongevity induced by caloric restriction', *Proceedings of the National Academy of Sciences of the United States of America,* 105(7), pp. 2325-2330.

Pecot, C. V., Calin, G. A., Coleman, R. L., Lopez-Berestein, G. and Sood, A. K. (2011) 'RNA interference in the clinic: challenges and future directions', *Nature reviews. Cancer,* 11(1), pp. 59.

Permuth-Wey, J. and Sellers, T. A. (2009) 'Epidemiology of ovarian cancer', *Cancer Epidemiology: Modifiable Factors*, pp. 413-437.

Persengiev, S. P., Zhu, X. and Green, M. R. (2004) 'Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs)', *Rna,* 10(1), pp. 12-18.

Phelps, S. L. B., Schorge, J. O., Peyton, M. J., Shigematsu, H., Xiang, L.-L., Miller, D. S. and Lea, J. S. (2008) 'Implications of EGFR inhibition in ovarian cancer cell proliferation', *Gynecologic oncology,* 109(3), pp. 411-417.

Pirker, R., Pereira, J. R., Szczesna, A., Von Pawel, J., Krzakowski, M., Ramlau, R., Vynnychenko, I., Park, K., Yu, C.-T. and Ganul, V. (2009) 'Cetuximab plus chemotherapy in patients with advanced non-small-cell lung cancer (FLEX): an open-label randomised phase III trial', *The Lancet,* 373(9674), pp. 1525-1531.

Poillet-Perez, L., Despouy, G., Delage-Mourroux, R. and Boyer-Guittaut, M. (2015) 'Interplay between ROS and autophagy in cancer cells, from tumor initiation to cancer therapy', *Redox Biology,* 4, pp. 184-192.

Polli, J. W., Olson, K. L., Chism, J. P., John-Williams, L. S., Yeager, R. L., Woodard, S. M., Otto, V., Castellino, S. and Demby, V. E. (2009) 'An unexpected synergist role of P-glycoprotein and breast cancer resistance protein on the central nervous system penetration of the tyrosine kinase inhibitor lapatinib (N-{3-chloro-4-[(3-fluorobenzyl) oxy] phenyl}-6-[5-({[2-(methylsulfonyl) ethyl] amino} methyl)-2-furyl]-4-quinazolinamine; GW572016)', *Drug Metabolism and Disposition,* 37(2), pp. 439-442.

Powell, J. L., McAfee, R. D., McCoy, R. C. and Shiro, B. S. (1998) 'Uterine and ovarian conservation in advanced small cell carcinoma of the ovary', *Obstetrics & Gynecology,* 91(5), pp. 846-848.

Prasad, S., Gupta, S. C. and Tyagi, A. K. (2017) 'Reactive oxygen species (ROS) and cancer: Role of antioxidative nutraceuticals', *Cancer Letters,* 387, pp. 95-105.

Praslicka, B. J., Kerins, M. J. and Ooi, A. (2016) 'The complex role of NRF2 in cancer: A genomic view', *Current Opinion in Toxicology,* 1, pp. 37-45.

Prenzel, N., Fischer, O. M., Streit, S., Hart, S. and Ullrich, A. (2001) 'The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification', *Endocrine-related cancer,* 8(1), pp. 11-31.

Præstegaard, C., Kjaer, S. K., Nielsen, T. S. S., Jensen, S. M., Webb, P. M., Nagle, C. M., Høgdall, E., Risch, H. A., Rossing, M. A., Doherty, J. A., Wicklund, K. G., Goodman, M. T., Modugno, F., Moysich, K., Ness, R. B., Edwards, R. P., Goode, E. L., Winham, S. J., Fridley, B. L., Cramer, D. W., Terry, K. L., Schildkraut, J. M., Berchuck, A., Bandera, E. V., Paddock, L., Kiemeney, L. A., Massuger, L. F., Wentzensen, N., Pharoah, P., Song, H., Whittemore, A. S., McGuire, V., Sieh, W., Rothstein, J., Anton-Culver, H., Ziogas, A., Menon, U., Gayther, S. A., Ramus, S. J., Gentry-Maharaj, A., Wu, A. H., Pearce, C. L., Pike, M. C., Lee, A. W., Chang-Claude, J. and Jensen, A. (2016) 'The association between socioeconomic status and tumour stage at diagnosis of ovarian cancer: A pooled analysis of 18 case-control studies', *Cancer Epidemiology,* 41, pp. 71-79.

Psyrri, A., Kassar, M., Yu, Z., Bamias, A., Weinberger, P. M., Markakis, S., Kowalski, D., Camp, R. L., Rimm, D. L. and Dimopoulos, M. A. (2005) 'Effect of epidermal growth factor receptor expression level on survival in patients with epithelial ovarian cancer', *Clinical Cancer Research,* 11(24), pp. 8637-8643.

Qi, L. S., Guo, Y. Y., Zhang, P., Cao, X. R. and Luan, Y. X. (2016) 'Preventive and Therapeutic Effects of the Retinoid X Receptor Agonist Bexarotene on Tumors', *Current Drug Metabolism,* 17(2), pp. 118-128.

Qiu, C., Tarrant, M. K., Choi, S. H., Sathyamurthy, A., Bose, R., Banjade, S., Pal, A., Bornmann, W. G., Lemmon, M. A., Cole, P. A. and Leahy, D. J. (2008) 'Mechanism of Activation and Inhibition of the HER4/ErbB4 Kinase', *Structure,* 16(3), pp. 460-467.

Quéreux, G., Saint-Jean, M., Peuvrel, L., Brocard, A., Knol, A.-C. and Dréno, B. (2013) 'Bexarotene in cutaneous T-cell lymphoma: third retrospective study of long-term cohort and review of the literature', *Expert Opinion on Pharmacotherapy,* 14(13), pp. 1711-1721.

Ramos-Gomez, M., Kwak, M.-K., Dolan, P. M., Itoh, K., Yamamoto, M., Talalay, P. and Kensler, T. W. (2001) 'Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice', *Proceedings of the National Academy of Sciences,* 98(6), pp. 3410-3415.

Reczek, C. R. and Chandel, N. S. (2017) 'The Two Faces of Reactive Oxygen Species in Cancer'.

Reese, D. M. and Slamon, D. J. (1997) 'HER-2/neu Signal Transduction in Human Breast and Ovarian Cancer', *Stem cells,* 15(1), pp. 1-8.

Regales, L., Gong, Y., Shen, R., de Stanchina, E., Vivanco, I., Goel, A., Koutcher, J. A., Spassova, M., Ouerfelli, O. and Mellinghoff, I. K. (2009) 'Dual targeting of EGFR can overcome a major drug resistance mutation in mouse models of EGFR mutant lung cancer', *The Journal of clinical investigation,* 119(10), pp. 3000-3010.

Reid, A., Vidal, L., Shaw, H. and de Bono, J. (2007) 'Dual inhibition of ErbB1 (EGFR/HER1) and ErbB2 (HER2/neu)', *European journal of cancer,* 43(3), pp. 481-489.

Ren, D. (2011) 'Brusatol enhances the efficacy of chemotherapy by inhibiting the Nrf2-mediated defense mechanism', *Proc. Natl Acad. Sci. USA,* 108, pp. 1433-1438.

Ren, D., Villeneuve, N. F., Jiang, T., Wu, T., Lau, A., Toppin, H. A. and Zhang, D. D. (2011) 'Brusatol enhances the efficacy of chemotherapy by inhibiting the Nrf2-mediated defense mechanism', *Proceedings of the National Academy of Sciences of the United States of America,* 108(4), pp. 1433-1438.

Reschke, M., Mihic-Probst, D., Van Der Horst, E. H., Knyazev, P., Wild, P. J., Hutterer, M., Meyer, S., Dummer, R., Moch, H. and Ullrich, A. (2008) 'HER3 is a determinant for poor prognosis in melanoma', *Clinical Cancer Research,* 14(16), pp. 5188-5197.

Richard Dickersin, G., Kline, I. W. and Scully, R. E. (1982) 'Small cell carcinoma of the ovary with hypercalcemia: a report of eleven cases', *Cancer,* 49(1), pp. 188-197.

Rigas, J. R. and Dragnev, K. H. (2005) 'Emerging role of rexinoids in non-small cell lung cancer: focus on bexarotene', *The Oncologist,* 10(1), pp. 22-33.

Ritter, C. A. and Arteaga, C. L. 'The epidermal growth factor receptor–tyrosine kinase: A promising therapeutic target in solid tumors'. Elsevier, 3-11.

Ritter, C. A., Perez-Torres, M., Rinehart, C., Guix, M., Dugger, T., Engelman, J. A. and Arteaga, C. L. (2007) 'Human breast cancer cells selected for resistance to trastuzumab in vivo overexpress epidermal growth factor receptor and ErbB ligands and remain dependent on the ErbB receptor network', *Clinical Cancer Research,* 13(16), pp. 4909-4919.

Rohozinski, J., Diaz-Arrastia, C. and Edwards, C. L. (2017) 'Do some epithelial ovarian cancers originate from a fallopian tube ciliate cell lineage?', *Medical Hypotheses*.

Rolfo, C., Giovannetti, E., Hong, D. S., Bivona, T., Raez, L. E., Bronte, G., Buffoni, L., Reguart, N., Santos, E. S., Germonpre, P., Taron, M., Passiglia, F., Van Meerbeeck, J. P., Russo, A., Peeters, M., Gil-Bazo, I., Pauwels, P. and Rosell, R. (2014) 'Novel therapeutic strategies for patients with NSCLC that do not respond to treatment with EGFR inhibitors', *Cancer Treatment Reviews,* 40(8), pp. 990-1004.

Roskoski Jr, R. (2014) 'The ErbB/HER family of protein-tyrosine kinases and cancer', *Pharmacological Research,* 79, pp. 34-74.

Roy, R. V., Pratheeshkumar, P., Son, Y.-O., Wang, L., Hitron, J. A., Divya, S. P., Zhang, Z. and Shi, X. (2016) 'Different roles of ROS and Nrf2 in Cr(VI)-induced inflammatory responses in normal and Cr(VI)-transformed cells', *Toxicology and Applied Pharmacology,* 307, pp. 81-90.

Rubin, I. and Yarden, Y. (2001) 'The basic biology of HER2', *Annals of Oncology,* 12(suppl 1), pp. S3-S8.

Rusch, V., Klimstra, D., Venkatraman, E., Pisters, P. W., Langenfeld, J. and Dmitrovsky, E. (1997) 'Overexpression of the epidermal growth factor receptor and its ligand transforming growth factor alpha is frequent in resectable non-small cell lung cancer but does not predict tumor progression', *Clinical Cancer Research,* 3(4), pp. 515-522.

Rushworth, S. A., Ogborne, R. M., Charalambos, C. A. and O'Connell, M. A. (2006) 'Role of protein kinase C δ in curcumin-induced antioxidant response element-mediated gene expression in human monocytes', *Biochemical and biophysical research communications,* 341(4), pp. 1007-1016.

Ryan, Q., Ibrahim, A., Cohen, M. H., Johnson, J., Ko, C.-w., Sridhara, R., Justice, R. and Pazdur, R. (2008) 'FDA drug approval summary: lapatinib in combination with capecitabine for previously treated metastatic breast cancer that overexpresses HER-2', *The oncologist,* 13(10), pp. 1114-1119.

Saito-Hakoda, A., Uruno, A., Yokoyama, A., Shimizu, K., Parvin, R., Kudo, M., Saito-Ito, T., Sato, I., Kogure, N., Suzuki, D., Shimada, H., Yoshikawa, T., Fujiwara, I., Kagechika, H., Iwasaki, Y., Kure, S., Ito, S. and Sugawara, A. (2015) 'Effects of RXR Agonists on Cell Proliferation/Apoptosis and ACTH Secretion/Pomc Expression', *Plos One,* 10(12).

Saltz, L. B., Clarke, S., Díaz-Rubio, E., Scheithauer, W., Figer, A., Wong, R., Koski, S., Lichinitser, M., Yang, T.-S. and Rivera, F. (2008) 'Bevacizumab in combination with oxaliplatin-based chemotherapy as first-line therapy in metastatic colorectal cancer: a randomized phase III study', *Journal of clinical oncology,* 26(12), pp. 2013-2019.

Sandelin, A., Wasserman, W. W. and Lenhard, B. (2004) 'ConSite: web-based prediction of regulatory elements using cross-species comparison', *Nucleic acids research,* 32(suppl 2), pp. W249-W252.

Sarkar, F. H. (2011) *Nutraceuticals and Cancer.* Springer Science & Business Media.

Sartore-Bianchi, A., Martini, M., Molinari, F., Veronese, S., Nichelatti, M., Artale, S., Di Nicolantonio, F., Saletti, P., De Dosso, S. and Mazzucchelli, L. (2009) 'PIK3CA mutations in colorectal cancer are associated with clinical resistance to EGFR-targeted monoclonal antibodies', *Cancer research,* 69(5), pp. 1851-1857.

Scartozzi, M., Bearzi, I., Berardi, R., Mandolesi, A., Fabris, G. and Cascinu, S. (2004) 'Epidermal growth factor receptor (EGFR) status in primary colorectal tumors does not correlate with EGFR expression in related metastatic sites: implications for treatment with EGFR-targeted monoclonal antibodies', *Journal of clinical oncology,* 22(23), pp. 4772-4778.

Scheuer, W., Friess, T., Burtscher, H., Bossenmaier, B., Endl, J. and Hasmann, M. (2009) 'Strongly enhanced antitumor activity of trastuzumab and pertuzumab combination treatment on HER2-positive human xenograft tumor models', *Cancer research,* 69(24), pp. 9330-9336.

Schlegel, J., Stumm, G., Brändle, K., Merdes, A., Mechtersheimer, G., Hynes, N. E. and Kiessling, M. (1994) 'Amplification and differential expression of members of theerbB-gene family in human glioblastoma', *Journal of neuro-oncology,* 22(3), pp. 201-207.

Schlessinger, J. (2000) 'Cell Signaling by Receptor Tyrosine Kinases', *Cell,* 103(2), pp. 211-225.

Schneider, M. R. a. W., E (2009) 'The epidermal growth factor receptor ligands at a glance', *Journal of Cellular Physiology,* 218(3), pp. 460-466.

Schraml, P., Schwerdtfeger, G., Burkhalter, F., Raggi, A., Schmidt, D., Ruffalo, T., King, W., Wilber, K., Mihatsch, M. J. and Moch, H. (2003) 'Combined array comparative genomic hybridization and tissue microarray analysis suggest PAK1 at 11q13. 5-q14 as a critical oncogene target in ovarian carcinoma', *The American journal of pathology,* 163(3), pp. 985-992.

Scully, R. E. (1979) 'Tumors of the ovary and maldeveloped gonads', *Atlas of tumor pathology,* 16, pp. 316-317.

Seidman, J. D., Cho, K. R., Ronnett, B. M. and Kurman, R. J. (2011) 'Surface epithelial tumors of the ovary', *Blaustein's pathology of the female genital tract*: Springer, pp. 679-784.

Sequist, L. V., Martins, R. G., Spigel, D., Grunberg, S. M., Spira, A., Jänne, P. A., Joshi, V. A., McCollum, D., Evans, T. L. and Muzikansky, A. (2008) 'First-line gefitinib in patients with advanced non–small-cell lung cancer harboring somatic EGFR mutations', *Journal of Clinical Oncology,* 26(15), pp. 2442-2449.

Sergina, N. V., Rausch, M., Wang, D., Blair, J., Hann, B., Shokat, K. M. and Moasser, M. M. (2007) 'Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3', *Nature,* 445(7126), pp. 437-441.

Serrano-Olvera, A., Dueñas-González, A., Gallardo-Rincón, D., Candelaria, M. and De la Garza-Salazar, J. (2006) 'Prognostic, predictive and therapeutic implications of HER2 in invasive epithelial ovarian cancer', *Cancer treatment reviews,* 32(3), pp. 180-190.

Shattuck, D. L., Miller, J. K., Carraway, K. L. and Sweeney, C. (2008) 'Met receptor contributes to trastuzumab resistance of Her2-overexpressing breast cancer cells', *Cancer research,* 68(5), pp. 1471-1477.

Shende, P., Patil, S. and Gaud, R. S. (2017) 'A combinatorial approach of inclusion complexation and dendrimer synthesization for effective targeting EGFR-TK', *Materials Science and Engineering: C,* 76, pp. 959-965.

Sheng, Q. and Liu, J. (2011) 'The therapeutic potential of targeting the EGFR family in epithelial ovarian cancer', *British Journal of Cancer,* 104(8), pp. 1241-1245.

Sheng, Q., Liu, X., Fleming, E., Yuan, K., Piao, H., Chen, J., Moustafa, Z., Thomas, R. K., Greulich, H., Schinzel, A., Zaghlul, S., Batt, D., Ettenberg, S., Meyerson, M., Schoeberl, B., Kung, A. L., Hahn, W. C., Drapkin, R., Livingston, D. M. and Liu, J. F. (2010) 'An Activated ErbB3/NRG1 Autocrine Loop Supports In Vivo Proliferation in Ovarian Cancer Cells', *Cancer Cell,* 17(3), pp. 298-310.

Shibata, T., Ohta, T., Tong, K. I., Kokubu, A., Odogawa, R., Tsuta, K., Asamura, H., Yamamoto, M. and Hirohashi, S. (2008) 'Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy', *Proceedings of the National Academy of Sciences,* 105(36), pp. 13568-13573.

Sims, A. H., Zweemer, A. J., Nagumo, Y., Faratian, D., Muir, M., Dodds, M., Um, I., Kay, C., Hasmann, M. and Harrison, D. J. (2012a) 'Defining the molecular response to trastuzumab, pertuzumab and combination therapy in ovarian cancer', *British journal of cancer,* 106(11), pp. 1779-1789.

Sims, A. H., Zweemer, A. J., Nagumo, Y., Faratian, D., Muir, M., Dodds, M., Um, I., Kay, C., Hasmann, M., Harrison, D. J. and Langdon, S. P. (2012b) 'Defining the molecular response to trastuzumab, pertuzumab and combination therapy in ovarian cancer', *Br J Cancer,* 106(11), pp. 1779-1789.

Singh, A. (2006) 'Dysfunctional KEAP1–NRF2 interaction in non-small-cell lung cancer', *PLoS Med.,* 3, pp. e420.

Singh, A., Bodas, M., Wakabayashi, N., Bunz, F. and Biswal, S. (2010) 'Gain of Nrf2 function in non-small-cell lung cancer cells confers radioresistance', *Antioxidants & redox signaling,* 13(11), pp. 1627-1637.

Singh, A., Happel, C., Manna, S. K., Acquaah-Mensah, G., Carrerero, J., Kumar, S., Nasipuri, P., Krausz, K. W., Wakabayashi, N. and Dewi, R. (2013) 'Transcription factor NRF2 regulates miR-1 and miR-206 to drive tumorigenesis', *The Journal of clinical investigation,* 123(7), pp. 2921-2934.

Sirota, R., Gibson, D. and Kohen, R. (2015) 'The role of the catecholic and the electrophilic moieties of caffeic acid in Nrf2/Keap1 pathway activation in ovarian carcinoma cell lines', *Redox Biology,* 4, pp. 48-59.

Siwak, D. R., Carey, M., Hennessy, B. T., Nguyen, C. T., McGahren Murray, M. J., Nolden, L. and Mills, G. B. (2010) 'Targeting the Epidermal Growth Factor Receptor in Epithelial Ovarian Cancer: Current Knowledge and Future Challenges', *Journal of Oncology,* 2010, pp. 568938.

Slamon, D. J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J. and Pegram, M. (2001) 'Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2', *New England Journal of Medicine,* 344(11), pp. 783-792.

Solis, L. M., Behrens, C., Dong, W., Suraokar, M., Ozburn, N. C., Moran, C. A., Corvalan, A. H., Biswal, S., Swisher, S. G., Bekele, B. N., Minna, J. D., Stewart, D. J. and Wistuba, I. I. (2010) 'Nrf2 and Keap1 Abnormalities in Non-Small Cell Lung Carcinoma and Association with Clinicopathologic Features', *Clinical cancer research : an official journal of the American Association for Cancer Research,* 16(14), pp. 3743-3753.

Springer, M. L., Chen, A. S., Kraft, P. E., Bednarski, M. and Blau, H. M. (1998) 'VEGF gene delivery to muscle: potential role for vasculogenesis in adults', *Molecular cell,* 2(5), pp. 549-558.

Srinivasan, R., Benton, E., McCormick, F., Thomas, H. and Gullick, W. J. (1999) 'Expression of the c-erbB-3/HER-3 and c-erbB-4/HER-4 growth factor receptors and their ligands, neuregulin-1 α, neuregulin-1 β, and betacellulin, in normal endometrium and endometrial cancer', *Clinical cancer research,* 5(10), pp. 2877-2883.

Stacy, D. R., Ely, K., Massion, P. P., Yarbrough, W. G., Hallahan, D. E., Sekhar, K. R. and Freeman, M. L. (2006) 'Increased expression of nuclear factor E2 p45-related factor 2 (NRF2) in head and neck squamous cell carcinomas', *Head & neck,* 28(9), pp. 813-818.

Stratton, M. R. (2011) 'Exploring the genomes of cancer cells: progress and promise', *science,* 331(6024), pp. 1553-1558.

Subramaniam, S., Fahy, E., Gupta, S., Sud, M., Byrnes, R. W., Cotter, D., Dinasarapu, A. R. and Maurya, M. R. (2011) 'Bioinformatics and systems biology of the lipidome', *Chemical reviews,* 111(10), pp. 6452-6490.

Suganuma, K., Yamasaki, S., Molefe, N. I., Musinguzi, P. S., Davaasuren, B., Mossaad, E., Narantsatsral, S., Battur, B., Battsetseg, B. and Inoue, N. (2017) 'The establishment of in vitro culture and drug screening systems for a newly isolated strain of Trypanosoma equiperdum', *International Journal for Parasitology: Drugs and Drug Resistance,* 7(2), pp. 200-205.

Takakura, M., Kyo, S., Kanaya, T., Hirano, H., Takeda, J., Yutsudo, M. and Inoue, M. (1999) 'Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells', *Cancer research,* 59(3), pp. 551-557.

Tal, M., King, C. R., Kraus, M. H., Ullrich, A., Schlessinger, J. and Givol, D. (1987) 'Human HER2 (neu) promoter: evidence for multiple mechanisms for transcriptional initiation', *Molecular and cellular biology,* 7(7), pp. 2597-2601.

Talalay, P., Fahey, J. W., Healy, Z. R., Wehage, S. L., Benedict, A. L., Min, C. and Dinkova-Kostova, A. T. (2007) 'Sulforaphane mobilizes cellular defenses that protect skin against damage by UV radiation', *Proceedings of the National Academy of Sciences,* 104(44), pp. 17500-17505.

Talerman, A. and Vang, R. (2011a) 'Germ cell tumors of the ovary', *Blaustein's pathology of the female genital tract*: Springer, pp. 847-907.

Talerman, A. and Vang, R. (2011b) 'Nonspecific Tumors of the Ovary, Including Mesenchymal Tumors', *Blaustein's Pathology of the Female Genital Tract*: Springer, pp. 909-928.

Talpur, R., Thompson, A., Gangar, P. and Duvic, M. (2014) 'Pralatrexate alone or in combination with bexarotene: long-term tolerability in relapsed/refractory mycosis fungoides', *Clinical Lymphoma Myeloma and Leukemia,* 14(4), pp. 297-304.

Tan, K. P., Kosuge, K., Yang, M. and Ito, S. (2008) 'NRF2 as a determinant of cellular resistance in retinoic acid cytotoxicity', *Free Radical Biology and Medicine,* 45(12), pp. 1663-1673.

Tang, X., Wang, H., Fan, L., Wu, X., Xin, A., Ren, H. and Wang, X. J. (2011) 'Luteolin inhibits Nrf2 leading to negative regulation of the Nrf2/ARE pathway and sensitization of human lung carcinoma A549 cells to therapeutic drugs', *Free Radical Biology and Medicine,* 50(11), pp. 1599-1609.

Tanner, B., Hasenclever, D., Stern, K., Schormann, W., Bezler, M., Hermes, M., Brulport, M., Bauer, A., Schiffer, I. B. and Gebhard, S. (2006) 'ErbB-3 predicts survival in ovarian cancer', *Journal of clinical oncology,* 24(26), pp. 4317-4323.

Tapia, C., Glatz, K., Novotny, H., Lugli, A., Horcic, M., Seemayer, C. A., Tornillo, L., Terracciano, L., Spichtin, H. and Mirlacher, M. (2007) 'Close association between HER-2 amplification and overexpression in human tumors of non-breast origin', *Modern pathology,* 20(2), pp. 192.

Tarumoto, T., Nagai, T., Ohmine, K., Miyoshi, T., Nakamura, M., Kondo, T., Mitsugi, K., Nakano, S., Muroi, K., Komatsu, N. and Ozawa, K. (2004) 'Ascorbic acid restores sensitivity to imatinib via suppression of Nrf2-dependent gene expression in the imatinib-resistant cell line', *Experimental Hematology,* 32(4), pp. 375-381.

Tebay, L. E., Robertson, H., Durant, S. T., Vitale, S. R., Penning, T. M., Dinkova-Kostova, A. T. and Hayes, J. D. (2015) 'Mechanisms of activation of the transcription factor Nrf2 by redox stressors, nutrient cues, and energy status and the pathways through which it attenuates degenerative disease', *Free Radical Biology and Medicine,* 88, Part B, pp. 108-146.

Tew, W. P. (2016) 'Ovarian cancer in the older woman', *Journal of geriatric oncology,* 7(5), pp. 354-361.

Tew, W. P., Muss, H. B., Kimmick, G. G., Von Gruenigen, V. E. and Lichtman, S. M. (2014) 'Breast and ovarian cancer in the older woman', *Journal of Clinical Oncology,* 32(24), pp. 2553-2561.

Thimmulappa, R. K., Mai, K. H., Srisuma, S., Kensler, T. W., Yamamoto, M. and Biswal, S. (2002) 'Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray', *Cancer research,* 62(18), pp. 5196-5203.

Tiscornia, G., Singer, O., Ikawa, M. and Verma, I. M. (2003) 'A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA', *Proceedings of the National Academy of Sciences,* 100(4), pp. 1844-1848.

Torre, L. A., Bray, F., Siegel, R. L., Ferlay, J., Lortet-Tieulent, J. and Jemal, A. (2015) 'Global cancer statistics, 2012', *CA: a cancer journal for clinicians,* 65(2), pp. 87-108.

Trachootham, D., Alexandre, J. and Huang, P. (2009) 'Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach?', *Nature Rev. Drug Discov.,* 8, pp. 579-591.

Tsuchida, K., Tsujita, T., Hayashi, M., Ojima, A., Keleku-Lukwete, N., Katsuoka, F., Otsuki, A., Kikuchi, H., Oshima, Y., Suzuki, M. and Yamamoto, M. (2017) 'Halofuginone enhances the chemo-sensitivity of cancer cells by suppressing NRF2 accumulation', *Free Radical Biology and Medicine,* 103, pp. 236-247.

Tu, S.-H., Ho, C.-T., Liu, M.-F., Huang, C.-S., Chang, H.-W., Chang, C.-H., Wu, C.-H. and Ho, Y.-S. (2013) 'Luteolin sensitises drug-resistant human breast cancer cells to tamoxifen via the inhibition of cyclin E2 expression', *Food Chemistry,* 141(2), pp. 1553-1561.

Turke, A. B., Zejnullahu, K., Wu, Y.-L., Song, Y., Dias-Santagata, D., Lifshits, E., Toschi, L., Rogers, A., Mok, T. and Sequist, L. (2010) 'Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC', *Cancer cell,* 17(1), pp. 77-88.

Turley, A. E., Zagorski, J. W. and Rockwell, C. E. (2015) 'The Nrf2 activator tBHQ inhibits T cell activation of primary human CD4 T cells', *Cytokine,* 71(2), pp. 289-295.

Van Cutsem, E., Köhne, C.-H., Hitre, E., Zaluski, J., Chang Chien, C.-R., Makhson, A., D'Haens, G., Pintér, T., Lim, R. and Bodoky, G. (2009) 'Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer', *New England Journal of Medicine,* 360(14), pp. 1408-1417.

Vanneman, M. and Dranoff, G. (2012) 'Combining immunotherapy and targeted therapies in cancer treatment', *Nature Reviews Cancer,* 12(4), pp. 237-251.

Varbanov, H. P., Kuttler, F., Banfi, D., Turcatti, G. and Dyson, P. J. (2017) 'Repositioning approved drugs for the treatment of problematic cancers using a screening approach', *PloS one,* 12(2), pp. e0171052.

Verri, E., Guglielmini, P., Puntoni, M., Perdelli, L., Papadia, A., Lorenzi, P., Rubagotti, A., Ragni, N. and Boccardo, F. (2005) 'HER2/neu oncoprotein overexpression in epithelial ovarian cancer: evaluation of its prevalence and prognostic significance', *Oncology,* 68(2-3), pp. 154-161.

Voelkerding, K. V., Dames, S. A. and Durtschi, J. D. (2009) 'Next-generation sequencing: from basic research to diagnostics', *Clinical chemistry,* 55(4), pp. 641-658.

VäkEvä, L., RankI, A. and Hahtola, S. (2012) 'Ten-year experience of bexarotene therapy for cutaneous T-cell lymphoma in Finland', *Acta dermato-venereologica,* 92(3), pp. 258-263.

Wang, H., Liu, K., Geng, M., Gao, P., Wu, X., Hai, Y., Li, Y., Li, Y., Luo, L. and Hayes, J. D. (2013) 'RXRα inhibits the NRF2-ARE signaling pathway through a direct interaction with the Neh7 domain of NRF2', *Cancer research,* 73(10), pp. 3097-3108.

Wang, L., Yuan, H., Li, Y. and Han, Y. (2014) 'The role of HER3 in gastric cancer', *Biomedicine & Pharmacotherapy,* 68(6), pp. 809-812.

Wang, W. and Jaiswal, A. K. (2006) 'Nuclear factor Nrf2 and antioxidant response element regulate NRH:quinone oxidoreductase 2 (NQO2) gene expression and antioxidant induction', *Free Radical Biology and Medicine,* 40(7), pp. 1119-1130.

Wang, X.-J., Sun, Z., Villeneuve, N. F., Zhang, S., Zhao, F., Li, Y., Chen, W., Yi, X., Zheng, W. and Wondrak, G. T. (2008) 'Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of Nrf2', *Carcinogenesis,* 29(6), pp. 1235-1243.

Wang, X. J. (2008) 'Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of Nrf2', *Carcinogenesis,* 29, pp. 1235-1243.

Wang, X. J., Hayes, J. D., Henderson, C. J. and Wolf, C. R. (2007) 'Identification of retinoic acid as an inhibitor of transcription factor Nrf2 through activation of retinoic acid receptor alpha', *Proceedings of the National Academy of Sciences of the United States of America,* 104(49), pp. 19589-19594.

Weston, A. D. and Hood, L. (2004) 'Systems biology, proteomics, and the future of health care: toward predictive, preventative, and personalized medicine', *Journal of proteome research,* 3(2), pp. 179-196.

White, M., Cohen, J., Hummel, C., Burky, R., Cruz, A. and Farias-Eisner, R. (2014) 'Chapter 5 - The Role of Oxidative Stress in Ovarian Cancer: Implications for the Treatment of Patients', in Preedy, V. (ed.) *Cancer*. San Diego: Academic Press, pp. 41-50.

Whittaker, S., Ortiz, P., Dummer, R., Ranki, A., Hasan, B., Meulemans, B., Gellrich, S., Knobler, R., Stadler, R. and Karrasch, M. (2012) 'Efficacy and safety of bexarotene combined with psoralen–ultraviolet A (PUVA) compared with PUVA treatment alone in stage IB–IIA mycosis fungoides: final results from the EORTC Cutaneous Lymphoma Task Force phase III randomized clinical trial 21011 (NCT00056056)', *British Journal of Dermatology,* 167(3), pp. 678-687.

WHO (2017) *Cancer fact sheet* 2017).

Wieduwilt, M. J., Moasser, M. M. (2008) 'The epidermal growth factor receptor family: Biology driving targeted therapeutics', *Cellular and Molecular Life Sciences,* 65(10), pp. 1566–1584. .

Williams, T. I., Toups, K. L., Saggese, D. A., Kalli, K. R., Cliby, W. A. and Muddiman, D. C. (2007) 'Epithelial ovarian cancer: disease etiology, treatment, detection, and investigational gene, metabolite, and protein biomarkers', *Journal of proteome research,* 6(8), pp. 2936-2962.

Witkowski, L., Goudie, C., Foulkes, W. D. and McCluggage, W. G. (2016) 'Small-cell carcinoma of the ovary of hypercalcemic type (malignant rhabdoid tumor of the ovary): A review with recent developments on pathogenesis', *Surgical pathology clinics,* 9(2), pp. 215-226.

Witton, C. J., Reeves, J. R., Going, J. J., Cooke, T. G. and Bartlett, J. (2003) 'Expression of the HER1–4 family of receptor tyrosine kinases in breast cancer', *The Journal of pathology,* 200(3), pp. 290-297.

Wood, E. R., Truesdale, A. T., McDonald, O. B., Yuan, D., Hassell, A., Dickerson, S. H., Ellis, B., Pennisi, C., Horne, E. and Lackey, K. (2004) 'A unique structure for epidermal growth factor receptor bound to GW572016 (Lapatinib)', *Cancer research,* 64(18), pp. 6652-6659.

Workman, P. and Collins, I. (2014) 'Chapter 1 - Modern Cancer Drug Discovery: Integrating Targets, Technologies, and Treatments for Personalized Medicine', in Neidle, S. (ed.) *Cancer Drug Design and Discovery (Second Edition)*. San Diego: Academic Press, pp. 3-53.

Wu, J., Wang, H. and Tang, X. (2014) 'Rexinoid inhibits Nrf2-mediated transcription through retinoid X receptor alpha', *Biochemical and Biophysical Research Communications,* 452(3), pp. 554-559.

Wu, Q., Yao, B., Li, N., Ma, L., Deng, Y., Yang, Y., Zeng, C., Yang, Z. and Liu, B. (2017) 'Nrf2 mediates redox adaptation in NOX4-overexpressed non-small cell lung cancer cells', *Experimental Cell Research,* 352(2), pp. 245-254.

Xia, H., Mao, Q., Paulson, H. L. and Davidson, B. L. (2002) 'siRNA-mediated gene silencing in vitro and in vivo', *Nature biotechnology,* 20(10), pp. 1006-1010.

Xiang, M., Namani, A., Wu, S. and Wang, X. (2014) 'Nrf2: bane or blessing in cancer?', *Journal of cancer research and clinical oncology,* 140(8), pp. 1251-1259.

Xiong, H. Q., Rosenberg, A., LoBuglio, A., Schmidt, W., Wolff, R. A., Deutsch, J., Needle, M. and Abbruzzese, J. L. (2004) 'Cetuximab, a Monoclonal Antibody Targeting the Epidermal Growth Factor Receptor, in Combination With Gemcitabine for Advanced Pancreatic Cancer: A Multicenter Phase II Trial', *Journal of Clinical Oncology,* 22(13), pp. 2610-2616.

Xu, F., Yu, Y., Le, X.-F., Boyer, C., Mills, G. B. and Bast, R. C. (1999) 'The outcome of heregulin-induced activation of ovarian cancer cells depends on the relative levels of HER-2 and HER-3 expression', *Clinical cancer research,* 5(11), pp. 3653-3660.

Xu, Y., Duan, C., Kuang, Z., Hao, Y., Jeffries, J. L. and Lau, G. W. (2013) 'Pseudomonas aeruginosa pyocyanin activates NRF2-ARE-mediated transcriptional response via the ROS-EGFR-PI3K-AKT/MEK-ERK MAP kinase signaling in pulmonary epithelial cells', *PLoS One,* 8(8), pp. e72528.

Yap, T. A., Carden, C. P. and Kaye, S. B. (2009) 'Beyond chemotherapy: targeted therapies in ovarian cancer', *Nature Reviews Cancer,* 9(3), pp. 167-181.

Yarden, Y. (2001) 'The EGFR family and its ligands in human cancer: signalling mechanisms and therapeutic opportunities', *European Journal of Cancer,* 37, Supplement 4, pp. 3-8.

Yarden, Y. and Sliwkowski, M. X. (2001) 'Untangling the ErbB signalling network', *Nature reviews Molecular cell biology,* 2(2), pp. 127-137.

Yen, W.-C., Corpuz, M. R., Prudente, R. Y., Cooke, T. A., Bissonnette, R. P., Negro-Vilar, A. and Lamph, W. W. (2004a) 'A Selective Retinoid X Receptor Agonist Bexarotene (Targretin) Prevents and Overcomes Acquired Paclitaxel (Taxol) Resistance in Human Non–Small Cell Lung Cancer', *Clinical cancer research,* 10(24), pp. 8656-8664.

Yen, W.-c., Prudente, R. Y. and Lamph, W. W. (2004b) 'Synergistic effect of a retinoid X receptor-selective ligand bexarotene (LGD1069, Targretin) and paclitaxel (Taxol) in mammary carcinoma', *Breast cancer research and treatment,* 88(2), pp. 141-148.

Yen, W. C. and Lamph, W. W. (2006) 'A selective retinoid X receptor agonist bexarotene (LGD1069, Targretin) prevents and overcomes multidrug resistance in advanced prostate cancer', *The Prostate,* 66(3), pp. 305-316.

Yu, X. and Kensler, T. (2005) 'Nrf2 as a target for cancer chemoprevention', *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 591(1), pp. 93-102.

Zaczek, A., Bielawski, K. P. and Brandt, B. (2005) 'The diverse signaling network of EGFR, HER2, HER3 and HER4 tyrosine kinase receptors and the consequences for therapeutic approaches', *Histology and histopathology*.

Zhang, J., Wang, X., Vikash, V., Ye, Q., Wu, D., Liu, Y. and Dong, W. (2016a) 'ROS and ROS-mediated cellular signaling', *Oxidative medicine and cellular longevity,* 2016.

Zhang, L., Li, J., Zong, L., Chen, X., Chen, K., Jiang, Z., Nan, L., Li, X., Li, W. and Shan, T. (2016b) 'Reactive oxygen species and targeted therapy for pancreatic cancer', *Oxidative medicine and cellular longevity,* 2016.

Zhang, X. L., Yang, Y. S., Xu, D. P., Qu, J. H., Guo, M. Z., Gong, Y. and Huang, J. (2009) 'Comparative study on overexpression of HER2/neu and HER3 in gastric cancer', *World journal of surgery,* 33(10), pp. 2112-2118.

Zhou, J., Chen, J., Mokotoff, M. and Ball, E. D. (2004) 'Targeting gastrin-releasing peptide receptors for cancer treatment', *Anti-cancer drugs,* 15(10), pp. 921-927.

Zhu, J., Wang, H., Chen, F., Fu, J., Xu, Y., Hou, Y., Kou, H. H., Zhai, C., Nelson, M. B. and Zhang, Q. (2016) 'An overview of chemical inhibitors of the Nrf2-ARE signaling pathway and their potential applications in cancer therapy', *Free Radical Biology and Medicine,* 99, pp. 544-556.

Zipper, L. M. and Mulcahy, R. T. (2000) 'Inhibition of ERK and p38 MAP kinases inhibits binding of Nrf2 and induction of GCS genes', *Biochemical and biophysical research communications,* 278(2), pp. 484-492.

Zwick, E., Bange, J. and Ullrich, A. (2001) 'Receptor tyrosine kinase signalling as a target for cancer intervention strategies', *Endocrine-related cancer,* 8(3), pp. 161-173.

Zwick, E., Bange, J. and Ullrich, A. (2002) 'Receptor tyrosine kinases as targets for anticancer drugs', *Trends in Molecular Medicine,* 8(1), pp. 17-23.

9. APPENDICES

9.1 Appendix I

Table: 9.1.1: List of cell lines sources, nature and media used

Cell line	Type	Source	Nature	Media (Invitrogen)
MCF7-AREc32	Breast cell line	pGL-8xARE, along with the pcDNA3.1 plasmid containing the neomycin selectable marker, was stably transfected into MCF7 cells	Breast cancer	RPMI
SKVO3	Ovarian adenocarcinoma	Human	Ovarian cancer	RPMI
PEO1	Ovarian epithelial cell line	Human	Ovarian cancer	RPMI
OVCAR3	Ovarian epithelial cell line	Human	Ovarian cancer	RPMI

Table 9.1.2*: T*ype of tissue culture flasks and plates used

Flasks or Plates	Number of cells seeded	Growth media(mL)	Experiment type
96-well	2.5×10^3	0.2	Cytotoxicity assays (Cell viability), Total Gluthathione and ROS assays
24-well	5×10^4	0.5	Promoter transfection for luciferase assay
12-well	1×10^5	1	Immunofluorescence studies: For drug treatments of cells grown on Poly-L lysine coated coverslips
60mm	8×10^5	3	For liposome mediated cell transfections by harvesting protein lysates of cells treated with different drugs.
100mm	2×10^6	10	For liposome mediated cell transfections by harvesting protein lysates of cells treated with different drugs.
25cm^2 flask	7×10^5	5	For cell propagation and maintenance
75 cm^2 flask	2×10^6	12	For cell propagation and maintenance

Table 9.1.3: Antibodies used in the study.

Antibody	Isotype	Catalogue number	Company
HER1- EP38Y	Rabbit	ab52894	Abcam
pHER1	Rabbit	ab40815	Abcam
HER2	Rabbit	2165S	Cell signalling
pHER2-Try877	Rabbit	2241S	Cell signalling
HER3	Rabbit	4754S	Cell signalling
pHER3-Tyr1289	Rabbit	mAb #4791	Cell signalling
HER4	Rabbit	ab32375	Abcam
pHER4- Y1284	Rabbit	ab61059	Abcam
NRF2	Rabbit	ab89443	Abcam
pNRF2- EP1809Y	Rabbit	ab76026	Abcam
HO-1	Rabbit	5853S	Cell signalling
pAkt- Ser473	Rabbit	ab9271	Cell signalling
Akt	Rabbit	ab 9272	Cell signalling
HRP linked anti secondary antibody	Rabbit	7074	Cell signalling
β-actiin	Rabbit	1801	Abcam

9.1.4: Bradford assay

The standard protein concentration curve for Bradford assay was established by using a standard protein Bovine Serum Albumin (BSA). The stock was at a concentration of 2mg/mL and was diluted to produce varying concentrations, carrying out Bradford assay and plotting the standard graph. The dilutions were made as shown in the Table 9.1.

Table 9.1.4.1: Dilution of the standard protein.

2mg/mL BSA (µL)	**Water (µL)**	**Final concentration (mg/mL)**
0	20	0
1	19	0.1
2.5	17.5	0.25
5	15	0.5
7.5	12.5	0.75
10	10	1.0

Each of the 20µL mix was transferred to wells in 96-well plate and 180µL of 1x Bradford reagent was introduced and the contents mixed together. After 10min of incubation, absorbance from each well was measured at 595nm shown in table 9.2.

Table 9.1.4.2: Absorbance of standard protein

BSA (mg/mL)	**Absorbance @ 595nm**
0	0.214
0.1	0.401
0.25	0.780
0.5	1.371
0.75	1.834
1.0	2.309

The absorbance value of the blank (0mg/mL) was subtracted from the absorbance values of the samples and the resulting values were plotted on a graph to establish a concentration curve.

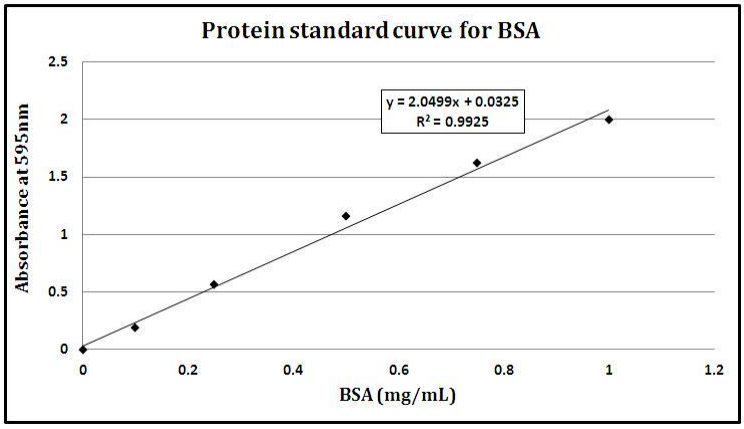

Figure 9.2: Protein standard curve for Bradford assay. To provide a protein standard curve for Bradford assay different dilutions of a known standard (2mg/mL BSA) were made as set out in table A1.6 and their absorbance measured at 595nm. The data was plotted in a graph using the x,y scatter and its trend line shown. Using the equation shown in the figure, protein concentrations of the experimental protein lysates were determined and used as mentioned.

9.2 Appendix-II:

Buffers and chemicals

9.2.1 50x TAE buffer

121gm Tris Base

28.5mL Glacial acetic acid

100mL 0.25M EDTA

Contents mixed together and water added to the final volume of 500mL. The pH of the buffer is around 8.5.

To make 0.25M EDTA stock, 46.5 gm of EDTA disodium salt was added to 400mL of water, pH adjusted to 8 with NaOH, and the contents dissolved until clear. The final volume was taken up to 500mL with water.

9.2.2 Poly L-lysine coating

For immunostaining procedures, cells were seeded on sterile cover-slips which were first coated with Poly L-lysine. All the procedure was performed in tissue culture hood under sterile condition in the following way:

Cover-slips to be coated were first autoclaved and placed in tissue culture hood. 1% Poly L-lysine solution was made in sterile water by taking 9.9mL of sterile water and 100µL of Poly
L-lysine solution. The cover-slips to be coated were then put in 50mL eppendorf tube and the solution was poured in it so that all the cover-slips are covered in the liquid. The eppendorf was capped tightly and the tube was placed on a gyro shaker and shaken gently at 30rpm for 2 hr. After that, the tube was placed back into the hood, the solution poured off and the coverslips washed three times with sterile water in the same tube. After the last wash, the water was decanted, the coated cover-slips placed flat on a sterile paper towel placed in the hood for to dry them for 30min. Cover-slips were stored in a sterile jar ready to use.

9.2.3 3% Paraformaldehyde (PFA)

3gm Paraformaldehyde

100mL Phosphate buffered saline

100µL 0.1M NaOH

Heated to 70°C and dissolved. Allowed to cool to room temperature and pH adjusted to 7.2 with concentrated HCL.

To make 0.1M NaOH, 40mg of NaOH was dissolved in 10mL of water.

9.2.4 Terrific Broth (TB)

12 gm Tryptone

24gm Yeast extract

4mL Glycerol

Distilled water added to 900mL

Next, salts for TB made in another bottle as:

1.85gm KH_2PO_4

10gm K_2HPO_4

Distilled water added to 100mL

Both bottles autoclaved and the contents of each mixed together in a sterile environment to make a litre of TB.

9.2.5 10x Phosphate Buffered Saline (PBS)

80gm NaCL

2gm KCL

14.4gm Na_2HPO_4

2.4gm KH_2PO_4

Water was added to the volume of 800mL. pH was set at 7.4 and again water was added until the final volume of 1 L. 1x PBS was made from the stock with water and usually autoclaved before using.

9.2.6 Preparation of Luciferase Assay Reagent II (LAR-II):

All the reagents used were provided in the Dual Luciferase Assay kit (Promega, UK). The lyophilized Luciferase Assay Substrate was resuspended in the 10ml of the supplied Luciferase Assay Buffer II. Once the substrates and buffer were mixed, the vial was labelled “LAR II” and stored at -80°C. At the time of use, the frozen LAR-II was thawed in water bath set at 37°C.

9.2.7 Preparation of Passive Lysis buffer:

The passive lysis buffer was provided as 5x concentrate (Dual Luciferase Assay kit, Promega, UK). 5mL of this concentrate was mixed with 20mL of distilled water to make a working stock of passive lysis buffer.

9.2.8 Preparation of Stop and Glo® reagent:

The Stop and Glo® reagent was provided as 50x concentrate (Dual Luciferase Assay kit,
Promega, UK). It was always prepared on the day of use by transferring 10µL of this reagent with 490µL of distilled water. The working stock was protected from light and stored at - 80°C until used. At the time of use, the frozen stock was thawed in water bath set at 37°C.

9.2.9: Running buffer for immunoblotting:

50mL 20x Nupage® MOPS SDS running buffer (Invitrogen, UK)

1mL Nupage® Sample reducing agent (Invitrogen, UK)

Distilled water was added to the final volume of 1 Litre. Mixed and stored at 4°C until used.

9.2.10 Transfer Buffer for immunoblotting:

50mL 20x Nupage® Transfer buffer (Invitrogen, UK)

100mL Methanol

1mL Nupage® Sample reducing agent (Invitrogen, UK)

Distilled water was added to the final volume of 1 Litre. Mixed and stored at 4°C until used.

9.2.11: Washing buffer for Immunoblotting:

A 10x concentrated washing buffer provided with WesternDOT™625 Goat anti rabbit/mouse western blot kit (Invitrogen, UK) was diluted to 1x with distilled water. Alternatively, a 0.1% solution of Tween20 in PBS was used in the same manner.

9.2.12: Blocking buffer for Immunoblotting:

For antibodies used (Table 9.1.5), BSA based blocking buffer was used as:

2.5gm BSA

50µL Tween20

TBS or PBS added to 50mL and mixed well until all contents were dissolved.

Also, milk based blocking buffer was used by replacing BSA above with 2.5gm milk keeping everything else the same.

9.2.13 SDS sample buffer for western blot:

A 4x concentrated Nupage® LDS sample buffer was used by diluting it to 1x with the RIPA extracted protein lysates.

9.2.14 Super Optimal Broth (SOB) media:

20gm Tryptone

5gm Yeast Extract

0.5gm NaCl

Dissolved in 800mL distilled water.

10mL 0.25M KCl

pH adjusted to 7. Water added to the final volume of 1 Litre. The media was autoclaved. Before use, 5mL of sterile 2M $MgCl_2$ added.0.25M KCl made by dissolving 0.93gm of KCl in water to the final volume of 50mL. 2M $MgCl_2$ made by dissolving 9.52gm of $MgCl_2$ in water to the final volume of 50mL.

9.2.15 Freezing Medium for mammalian cell storage:

Freezing medium was made in tissue culture hood using sterile reagents. 10mL of freezing medium was made in a 15mL falcon tube in the following way:

7mL Cell culture media

2mL Foetal bovine serum

1mL DMSO

These contents were mixed together and stored at -20°C. During use, the freezing medium was thawed in water bath set at 37°C. 1mL of the medium was usually used to store 100% confluent cells from a T75 flask in cryotubes.

9.2.16 Stripping Buffer for reprobing immunoblot:

20% SDS

67.5µM Tris-HCl of a pH 6.7

100µm β-Marcaptoethanol

The solution was applied to the immunoblot and incubated usually for 1 h at 37°C.

9.2.29 Drug solutions used in this study:

All solutions were filter sterilized before using.

9.2.29.1 tBHQ (Sigma-Aldrich, UK):

20mM stock solution of tBHQ was prepared by dissolving 34mg tBHQ powder in sterile DMSO to a final volume of 10mL. The vial was protected from light and stored at -20°C. The final concentration of the drug (usually 100µM) was

obtained by diluting it in the cell culture media.

9.2.29.2 9-CIS-RETINOIC ACID (Carbosynth, UK):

6.7mM stock of 9-Cis retinoic acid was prepared by dissolving 2mg 9-Cis retinoic acid powder in DMSO to a final volume of 1mL. The vial was protected from light and stored at -80°C. The final concentrations of drug (usually 2.5, 5.0, 10μM) were obtained by diluting the stock in cell culture media during treatment.

9.2.29.3 RETINOIC ACID (Carbosynth, UK):

20mM stock of retinoic acid was prepared by dissolving 60mg retinoic acid powder in sterile DMSO to a final volume of 10mL. The vial was protected from light and stored at -80°C. The final concentrations of the drug (usually 2.5, 5.0, 10μM) were obtained by diluting the stock in cell culture media during treatment.

9.2.29.4 PROCYANIDIN B_2 (Carbosynth, UK):

3.5mM stock of Procyanidin B_2 was prepared by dissolving 2mg Procyanidin B_2 powder in sterile DMSO to a final volume of 1mL. The vial was stored at -20°C. The final concentrations of the drug (usually 2.5, 5.0, 10μM) were obtained by diluting the stock in cell culture media during treatment.

9.2.29.5 BRUSATOL (Carbosynth, UK):

3.85mM stock of Brusatol was prepared by dissolving 2mg cisplatin in sterile distilled water to a final volume of 1mL. The vial was protected from light and stored at -20°C. The final concentrations of the drug (usually 2.5, 5.0, 10Mm) were obtained by diluting it in cell culture media during treatment.

9.2.29.6 NALIXIDIC ACID (Sigma-Aldrich, UK):

20mM stock of Nalixidic acid was prepared by dissolving 23.8mg of Nalixidic acid powder in sterile DMSO to a final volume of 5ml. The vial was protected from light and stored at

-20°C. The final concentrations of the drug (usually 2.5, 5.0, 10μM) were obtained by diluting it in cell culture media during drug treatment.

9.2.29.7 QUERCETIN (Carbosynth, UK):

20mM stock of Quercetin was prepared by dissolving 61mg of Quercetin powder in sterile DMSO to a final volume of 1ml. The vial was carefully wrapped and stored at

-20°C. The final concentrations of the drug (usually 2.5, 5.0,10μM) were obtained by diluting it in cell culture media during drug treatment.

9.2.29.8 BEXAROTENE (Carbosynth, UK):

20mM stock of Bexarotene acid was prepared by dissolving 35mg of Bexarotene powder in sterile DMSO to a final volume of 5ml. The vial was protected from light and stored at

-20°C. The final concentrations of the drug (usually 2.5, 5.0, 10μM) were obtained by diluting it in cell culture media during drug treatment.

9.2.29.9 LUTEOLIN (Carbosynth, UK):

35mM stock of Nalixidic acid was prepared by dissolving 100mg of Luteolin powder in sterile DMSO to a final volume of 10ml. The vial was protected from light and stored at

-20°C. The final concentrations of the drug (usually 2.5, 5.0, 10μM) were obtained by diluting it in cell culture media during drug treatment.

9.2.29.10 ASCORBIC ACID (Carbosynth, UK):

20mM stock of Ascorbic acid was prepared by dissolving 35.2mg of Ascorbic acid powder in sterile DMSO to a final volume of 10ml. The vial was protected from light and stored at

-20°C. The final concentrations of the drug (usually 2.5, 5.0, 10μM were obtained by diluting it in cell culture media during drug treatment.

9.2.29.11 GLYCETEIN (Carbosynth, UK):

35mM stock of Glycetein was prepared by dissolving 100mg of Glycetein powder in sterile DMSO to a final volume of 10ml. The vial was protected from light and stored at

-20°C. The final concentrations of the drug (usually 2.5, 5.0, 10μM) were obtained by diluting it in cell culture media during drug treatment.

9.2.29.12 XANTHOHUMOL (Carbosynth, UK):

20mM stock of Xanthohumol acid prepared by dissolving 72mg of Xanthohumol powder in sterile DMSO to a final volume of 10ml. The vial was protected from light and stored at

-20°C. The final concentrations of the drug (usually 2.5, 5.0, 10μM) were obtained by diluting it in cell culture media during drug treatment.

9.2.29.13 *p*-COUMARIC ACID (Carbosynth, UK):

20mM stock of *p*-Coumaric acid prepared by dissolving 33mg of *p*-Coumaric acid powder in sterile DMSO to a final volume of 1ml. The vial was protected from light and stored at

-20°C. The final concentrations of the drug (usually 2.5, 5.0, 10μM) were obtained by diluting it in cell culture media during drug treatment.

9.2.29.14 LAPATINIB (Cell Signaling, UK):

10mM stock of lapatinib prepared by dissolving 14gm of lapatinib powder in sterile DMSO to a final volume of 1ml. The vial was protected from light and stored at

-20°C. The final concentrations of the drug (usually 5.0Mm) was obtained by diluting it in cell culture media during drug treatment.

9.2.29.13 ERLOTINB (Cell Signaling, UK):

10mM stock of Erlotinib prepared by dissolving 14gm of Erlotinib in sterile DMSO to a final volume of 1ml. The vial was protected from light and stored at

-20°C. The final concentrations of the drug (usually 5.0Mm) was obtained by diluting it in cell culture media during drug treatment.

9.3 Appendix-III

Purchased reagents, chemicals and kits

Table 9.3. 1: Chemicals, reagents and kits used

Chemical/Reagent/Kits	Company and catalogue number
Agar	A5306-250G Sigma-Aldrich
Agarose	BIO-41025 Bioline
Bovine serum albumin	A2153-100G Sigma Aldrich
Bradford reagent	23200 Pierce Biotechnology
DMEM cell culture media	12491-015 Invitrogen
Lipofectamine™	Invitrogen
Dual luciferase assay reagent	E1910 Promega
Gel pilot DNA loading dye	239901 Qiagen
Gel pilot DNA loading ladders	239045 Qiagen
Glycerol	G5516 Sigma Aldrich
GoTaq® Flexi DNA polymerase	M8305 Promega
HaltTM Protease phosphatase Inhibitor Cocktail	78440 Pierce Biotechnology
HRP linked anti mouse antibody	7076 Cell signalling

HRP linked anti rabbit antibody	7074 Cell signalling
Hybond ECL nitrocellulose membrane	NP0322BOX Invitrogen

Cont. Chemicals, reagents and kits used

Novex®sharp prestained protein standard	LC5800 Invitrogen
Nupage® LDS sample buffer	NP0007 Invitrogen
Nupage® MOPS SDS running buffer	NP0001 Invitrogen
Nupage® Sample reducing agent	NP0009 Invitrogen
Nupage® Transfer buffer	NP0006 Invitrogen
I Blot dry blotting system	Thermofisher
Omni swab pack	WHA-WB100035 Whatman
OPTIMEM serum reduced media	31985070 Invitrogen
PBS	20012-027 Invitrogen
Poly L lysine solution	P4707-50ML Cell signalling
QIAprep spin miniprep kit	27104 Qiagen
QIAquick gel extraction kit	28704 Qiagen
plasmid plus Maxi kit	12963 Qiagen
Restriction endonucleases	R6161 Promega
RIPA buffer	89901 Pierce biotechnology
GelRed DNA stain	NBS-SV NBs biological
Sodium chloride	S3014-1KG Sigma Aldrich
T4 DNA ligase	M1801 Promega
	M1801 Promega
Trypsin	25300054 Invitrogen
Tryptone	T7293-1KG Sigma Aldrich
Shield mounting medium with DAPI	H-1200 Vectashield
	H-1200 Vectashield

Xcell Surelock® mincell system	E10007 Invitrogen
Yeast Extract	Y1625 Sigma Aldrich

Table 9.3.2: Scientific Equipment used in the current research.

Equipment	**Makers**
Ultra violet transillumation box	UVP Upland USA
Gel documentation machine	Alpha-innotech, USA
PCR amplification machine	Techgene, UK
96 well plate reader for absorption	Rosys anthos HTIII
96 well Luminometer and fluorometer	Modulus microplate, Turner biosystems, UK.
Autoclave machine	Priorclave, UK
Nanopure water	Barnsted Nanopure Diamond^{TM}, USA
Microscope	Lieca DM-IRE2 inverted fluorescence microscope
Syngene G:BOX	Chemi-XX6 Gel Documentation System (Synoptics, UK)
Spectrophotometer	Thermospectronic, USA
Thermomixer comfort	Eppendorf, UK