Protection from Endoplasmic Reticulum Stress and Apoptosis via PPARδ and Bcl-2 in Breast Cancer

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Biochemistry
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Abstract

Estrogen receptor positive breast cancer accounts for the majority of breast cancer cases. Anti-estrogens, such as tamoxifen, are used to prevent the growth and survival of these cancer cells. However, patients often develop resistance to this drug due to dysregulated apoptosis. The overexpression of either Bcl-2, an anti-apoptotic protein, or PPARδ, a nuclear receptor protein, allows cancer cells to evade endoplasmic reticulum stress and survive.

MCF-7 cells were used to investigate how dysregulated apoptosis contributes to tamoxifen resistance. Synergistic protection was observed in cells that overexpressed both PPARδ and Bcl-2, suggesting that the proteins work through different pathways. This implies that patients would require different treatments depending on the expression levels of Bcl-2 and or PPARδ in their tumours. In addition, the Bcl-2 inhibitor, ABT-199, was unable to sensitize cells that overexpressed Bcl-2 to drug treatments that elicit endoplasmic reticulum stress, revealing a novel BH3-independent mechanism of resistance through Bcl-2.
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Table of Contents

Acknowledgments........................................................................................................ iii
Table of Contents........................................................................................................v
List of Tables ................................................................................................................. ix
List of Figures ............................................................................................................... x
List of Abbreviations: ................................................................................................. xiii

1 Introduction.................................................................................................................1

1.1 Breast cancer ...........................................................................................................1
  1.1.1 Subtypes and statistics ......................................................................................1
  1.1.2 MCF-7 cells and their use in an estrogen receptor-positive breast cancer model ........................................................................................................3

1.2 Estrogen receptor signalling in breast cancer .......................................................4
  1.2.1 Estrogen and its receptors ................................................................................5
  1.2.2 Aberrant estrogen receptor signalling in cancer .............................................6
  1.2.3 Estrogen receptor signalling .............................................................................6

1.3 Current resistance mechanisms to endocrine therapy ........................................9
  1.3.1 Tamoxifen resistance ......................................................................................9
  1.3.2 Evasion of endoplasmic reticulum stress in cancer and the unfolded protein response ........................................................................................................12

1.4 The family of cadherins in cancer .....................................................................15

1.5 Cell death and the Bcl-2 family ...........................................................................16
  1.5.1 Apoptosis in cancer .......................................................................................16
  1.5.2 The Bcl-2 family ............................................................................................17
  1.5.3 Apoptotic signalling pathways ........................................................................19
  1.5.4 The pro-survival Bcl-2 and its role in breast cancer .....................................20
1.6 The PPAR family of proteins ................................................................. 20
  1.6.1 Subtypes and function ................................................................... 20
  1.6.2 PPAR ligands and activation ......................................................... 22
  1.6.3 Synthetic PPARδ agonists and their mechanisms of actions .......... 23
  1.6.4 Synthetic PPARδ antagonists and their mechanisms of action ....... 23
  1.6.5 PPARδ and its role in breast cancer ............................................. 24
1.7 Comparing Bcl-2 and PPARδ ............................................................... 25
  1.7.1 Rationale for this project .................................................................. 25
  1.7.2 Separating Bcl-2’s two pathways of protection .............................. 25
  1.7.3 MCF-7 cell lines as models for patients with resistant cells .......... 26
1.8 Background on drugs used to induce endoplasmic reticulum stress and apoptosis ................................................................. 27
  1.8.1 Mechanisms of actions for the drug panel .................................... 27
  1.8.2 Adding tamoxifen to the drug panel as a query compound .......... 29
1.9 High-Content Analysis ..................................................................... 30
  1.9.1 Classification of cell state using multiparametric, phenotypic analysis ....................................................................................... 32
  1.9.2 Random forests classifier ................................................................ 33
1.10 Background on transfection techniques ............................................ 34

2 Materials and Methods ....................................................................... 35
  2.1 Cell culture and mycoplasma testing .............................................. 35
  2.2 Bcl-2 overexpressing cell lines ....................................................... 35
  2.3 Generating PPARδ overexpressing cell lines .................................... 36
  2.4 Testing the drug panel with Bcl-2\textsuperscript{hi} cell lines ................. 39
  2.5 Staining, drug dosing, and image collection ................................. 39
  2.6 Random Forests classification ...................................................... 40
  2.7 Testing mC3-PPARδ cell lines with the drug panel ....................... 40
2.8 Sensitizing PPARδ<sup>hi</sup>/Bcl-2<sup>hi</sup> cells with Bcl-2 inhibitor and PPARδ antagonist .................................................................40

2.9 ABT-199 combination with drug panel .........................................................41

2.10 Transient transfection with mC3-Bcl-2 plasmids ........................................41

2.11 Immunofluorescence experiment with N & E-cadherin ................................42

2.12 Statistics used .................................................................................................42

3 Results and Data Interpretation ........................................................................43

3.1 Confirming Bcl-2’s protection through different pathways: endoplasmic reticulum and mitochondria .................................................................43

3.1.1 Mitochondrial hyperpolarization observed with endoplasmic reticulum stress drugs .................................................................44

3.1.2 Stains used for imaging ................................................................................45

3.1.3 Training controls for the experiment ............................................................47

3.1.4 Workflow of experiments ............................................................................48

3.1.5 Optimizing concentrations and time points for imaging experiments 49

3.1.6 Results of the 3-way classification ...............................................................50

3.2 Generating PPARδ<sup>hi</sup> cell lines to model patients with high Bcl-2 and/or PPARδ levels ............................................................................53

3.2.1 Verifying overexpression of PPARδ through fluorescent microscopy and Western blots .................................................................55

3.3 Immunofluorescence experiment with E & N-cadherin to assess cell invasiveness .........................................................................................58

3.3.1 Results for immunofluorescence experiment with the 8 cell lines .....58

3.4 Comparing PPARδ<sup>hi</sup> and Bcl-2<sup>hi</sup> cells .........................................................................................................................61

3.4.1 Media controls for the experiment ...............................................................62

3.4.2 Tamoxifen elicits endoplasmic reticulum stress that is protected through overexpression of either Bcl-2 or PPARδ ........................................63

3.4.3 Missing differences in protection with 2-way classification ....................66

3.4.4 PPARδ protects cells against endoplasmic reticulum stress drugs ......67
3.4.5 PPARδ’s protection against drugs that induce mitochondrial-dependent death .................................................................70

3.4.6 Different pathways of protection: Bcl-2 prevents cell death while PPARδ prevents mitochondrial hyperpolarization .......................75

3.5 Targeting protection through PPARδ and Bcl-2 with a PPARδ antagonist and a Bcl-2 inhibitor .................................................................76

3.5.1 ABT-199 sensitizes Bcl-2^{hi} cells to TNF-α+CHX and ActD, but not TAM ........................................................................................................77

3.5.2 ABT-199 unable to sensitize Bcl-2^{hi} cells to other endoplasmic reticulum stress drugs ................................................................78

3.5.3 ABT-199 phenomenon independent of Bcl-2 localization ..........79

3.5.4 Results with the PPARδ antagonist, NXT1511 .....................81

3.6 Titration of PPARδ agonist: optimal range for PPARδ activity? .............82

3.6.1 Culturing the cells in media containing the agonist, GW0742 ..........82

3.7 Transient transfection with mC3-Bcl-2 plasmids to exclude possibility of cell culture-selection of ABT-199 resistant cells .....................86

3.7.1 Results of the transient transfection experiments .....................86

4 Discussion and Future Directions ...................................................89

4.1.1 Using a tool compound screen to identify compounds protected solely by Bcl-2 or PPARδ .................................................................89

4.1.2 ABT-199’s inability to sensitize Bcl-2^{hi} cells to drug treatments points to a possible new target: the BH4 domain of Bcl-2 ......................90

4.1.3 Controversy surrounding PPARδ agonists: protective or tumorigenic? .......................................................................................91

4.1.4 Other assays to assess invasiveness and metastatic potential ..........93

4.1.5 Testing other estrogen receptor-positive breast cancer cell lines ......94

5 Conclusion and Summary .........................................................................................................................94

Bibliography ................................................................................................................................................97
List of Tables

Table 1. The drug panel separated into 2 classes: drugs that induced apoptosis through endoplasmic reticulum stress and drugs that induced mitochondrial dependent apoptosis. .......................................................... 27

Table 2. Primers used for amplifying PPAR and mC3 transcripts......................................... 36

Table 3. Sequencing data for mC3-PPARδ................................................................. 39
List of Figures

Figure 1. Summary of the different breast tumor subtypes and their respective prognosis. ............................................................ 3

Figure 2. Classical and non-classical genomic mechanisms of estrogen receptor signaling. .................................................. 7

Figure 3. Other signaling pathways mediated by estrogen or the ERs. ......................... 9

Figure 4. The Bcl-2 family regulates MOMP. ................................................................. 18

Figure 5. PPARs are a family of nuclear transcription factors. ................................. 22

Figure 6. Schematic of the Random Forest algorithm. .................................................. 33

Figure 7. Images of mitochondrial hyperpolarization in MCF-7 cells. ..................... 45

Figure 8. Three states of the cells defined by the stains used for imaging. ............... 46

Figure 9. Images of the control group of MCF-7 cells treated with the drug panel. ...... 47

Figure 10. Workflow of drug panel experiment. ......................................................... 49

Figure 11. Titration of the drug to find the optimal dose and time point. ................. 50

Figure 12. 3-way classification results for the experiment testing Bcl-2 protection. ...... 51

Figure 13. Plasmid map of the lentiviral expression vector, pLVX-EF1α-IRES-Puro.... 54

Figure 14. Image of unstained MCF-7 cells that overexpress the fusion construct of mCerulean3, tagged to the N terminus of PPARδ. ......................................................... 55

Figure 15. The images of the 8 cell lines using the modified GFP channel settings (433nm excitation/475nm emission) to detect mC3. ..................................................... 55

Figure 16. Histograms showing the distribution of CFP intensity for the 8 cell lines.... 56
Figure 17. Western blots for the cell lines to detect the relative levels of Bcl-2 and PPARδ. ................................................................. 57

Figure 18. Graphs showing the correlation of E and N-cadherin with respect to CFP mean. ............................................................. 59

Figure 19. Bar graph of the E/N-cadherin intensity ratio for the 8 cell lines. .......... 60

Figure 20. Images from immunofluorescence experiment with N and E-cadherin. .... 61

Figure 21. Graphs showing the 3-way classification results for the media controls in the experiment. .................................................. 62

Figure 22. 3-way classification results for cells treated with tamoxifen (14µM) for 24hrs using the three cell lines: control, Bcl2-wt, and mC3-PPARδ. ....................... 64

Figure 23. Percentage of stressed cells through TAM and TG treatment. ............... 65

Figure 24. Percentage of stressed cells via TAM and TG treatments at 48hrs. .......... 66

Figure 25. Capturing differences in protection through 3-way classification that would otherwise be missed in 2-way classification. ........................................ 67

Figure 26. 3-way classification results for the 8 cell lines treated with TG (10nM) for 48 hrs. .................................................................................................................. 68

Figure 27. 3-way classification results for the 8 cell lines treated with TN (30µM) for 48 hrs. ................................................................................................................. 69

Figure 28. 3-way classification results for the 8 cell lines treated with TAM (14µM) for 48 hrs. .................................................................................................................. 70

Figure 29. 3-way classification results for the 8 cell lines treated with TNF-α (3ng/mL) + CHX (10µg/mL) for 48 hrs. ................................................................. 72

Figure 30. 3-way classification results for the 8 cell lines treated with STS (30nM) for 48 hrs. .................................................................................................................. 73
Figure 31. 3-way classification results for the 8 cell lines treated with ActD (30nM) for 48 hrs........................................................................................................................................75

Figure 32. Summary of the 3-way classification results for the 8 cell lines treated with the whole drug panel..................................................................................................................76

Figure 33. ABT-199 sensitizes Bcl-2 hi cells to TNF-α + CHX and ActD, but not TAM. 78

Figure 34. 3-way classification results showing ABT-199 ineffectively sensitizing Bcl-2 hi cells to endoplasmic reticulum stress drugs like TG.................................................................80

Figure 35. Attempting to sensitize cells that overexpress PPARδ and Bcl-2 with a PPARδ antagonist and a Bcl-2 inhibitor........................................................................................................81

Figure 36. Increasing doses of the PPARδ agonist did not increase protection against the drug panel...........................................................................................................................................83

Figure 37. Morphology changes in the cell lines following PPARδ agonist treatment..84

Figure 38. Incubation with the PPARδ agonists did not improve the protection of cell lines overexpressing mC3-PPARδ to the drug panel.................................................................85

Figure 39. MCF-7 cells transiently transfected with the mC3-plasmids: control (“.”), Bcl2-wt (wild type Bcl-2; “WT”), Bcl2-acta (mitochondria-targeting Bcl-2; “Mito”), or Bcl2-cb5 (endoplasmic reticulum-targeting Bcl-2; “ER”) plasmids via inverse PEI transfection......................................................................................................................................88

Figure 40. 3 repeats of the transient transfection experiment........................................89
List of Abbreviations:

Bcl-2 = B-cell lymphoma-2

PPARδ = Peroxisome Proliferator-Activated Receptor δ

ABT-199 = Bcl-2 inhibitor

NXT1511 = PPARδ antagonist

ER = endoplasmic reticulum

TNF-α + CHX = Tumor Necrosis Factor alpha + Cycloheximide

ActD = Actinomycin D

STS = Staurosporine

TG = Thapsigargin

TN = Tunicamycin

TAM = Tamoxifen

mC3 = mCerulean3, cyan fluorescent protein

CMV = empty vector control

Bcl2-wt = wild type Bcl-2, expressed at both the mitochondria and ER

Bcl2-acta = fusion protein of Bcl-2 that targets the mitochondria

Bcl2-cb5= fusion protein of Bcl-2 that targets the ER

“hi” = refers to overexpression of a particular protein in a cell line

$\Delta \psi_m$ = mitochondrial hyperpolarization

BH = Bcl-2 Homology
1 Introduction

1.1 Breast cancer

1.1.1 Subtypes and statistics

Breast cancer is the leading cause of death among women in most developed countries (Spitale et al., 2008) and 30% of patients with early stage disease will go on to relapse with metastatic disease (Reinert & Barrios, 2015). It is a heterogeneous disease, which varies greatly among patients, and within the tumour itself, bearing diagnostic and therapeutic challenges (Weigelt et al., 2010; Burrell, McGranahan, Bartek, & Swanton, 2013). Intratumor heterogeneity occurs at the genomic, transcriptomic, proteomic, and morphologic levels, which warrant different therapeutic strategies (Rye et al., 2018). As such, determining an effective therapy plan requires accurate grouping of breast cancers into clinically relevant subtypes.

The study conducted by Sørlie et al. sought to classify breast carcinomas based on variations in gene expression patterns derived from cDNA microarrays that correlate tumor characteristics to clinical outcome (Sørlie et al., 2001). Variations in gene expression patterns in 40 grossly dissected human breast tumors were analyzed by cDNA microarrays and hierarchical clustering provided a “molecular signature” of each tumor. These molecular signatures were then used to classify tumours into five intrinsic subtypes with distinct clinical outcomes: luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) overexpression, basal, and normal-like tumors (Perou et al., 2000).

Luminal A tumours are estrogen receptor (ER) positive and progesterone receptor (PR) positive, but negative for HER2. They also have low levels of the protein Ki-67, a cellular marker for proliferation. In addition, luminal A cancers tend to grow slowly and have the best prognosis out of the five subtypes (Kennecke et al., 2010). These types of breast cancers are likely to benefit from hormone therapy and may benefit from chemotherapy (Diessner et al., 2016).
Luminal B tumours are ER positive, PR negative and HER2 positive. These types of cancers have high levels of Ki-67 and generally grow faster than luminal A cancers (Creighton, 2012). While their prognosis is slightly worse than luminal A breast cancers, luminal B breast cancers are likely to benefit more from the combined therapeutic strategy of chemotherapy and hormonal treatment, as well as treatment targeted to HER2 (Masoud & Pagès, 2017).

HER2 overexpression tumors are ER and PR negative, but HER2 positive. These tumors show aggressive clinical behavior and tend to grow faster than luminal cancers (Yersal & Barutca, 2014). While they have a worse prognosis, they are often successfully treated with chemotherapy and targeted therapies aimed at the HER2 protein.

Basal-like tumors, also known as triple-negative breast cancer, includes tumors that are ER, PR and HER2 negative. Basal-like breast cancers are likely to benefit from chemotherapy. This type of cancer is more common in women with breast cancer 1 (BRCA1) gene mutations; BRCA1 is a tumour suppressor and mutations in this gene leads to increased breast cancer susceptibility. Though it is not clear why, this type of cancer also is more common among younger and African-American women (Stark et al., 2010).

Finally, normal-like tumours are similar to luminal A disease in terms of their immunohistochemistry (IHC) status and are characterized by a normal breast tissue profiling (Perou et al., 2000). This subtype accounts for 7.8% of all breast cancer cases in
a lymph-node negative group (Smid et al., 2008). Still, while normal-like breast cancer has a good prognosis, its prognosis is slightly worse than luminal A types.

**Figure 1.** Summary of the different breast tumor subtypes and their respective prognosis. From “Breast cancer intrinsic subtype classification, clinical use and future trends,” by Dai et al. (2010). *American Journal of Cancer Research, 5* (10), 2929. Each of the subtypes is defined by its IHC status except for normal-like tumors. Those with positive ER and PR expression have better prognosis. The absence of ERs and PRs, along with BRCA1 mutations, is associated with worse prognosis.

Immunohistochemistry markers such as ER, PR and HER2, along with clinical variables such as tumor size, tumor grade and nodal involvement, are conventionally used for patient prognosis (Vallejos et al., 2010). Although these markers show strong association with prognosis and outcome, they cannot fully capture the diversity of clinical behaviors of breast cancer and are not sufficient to tailor patient-specific therapies. Another concern is that the assessments of the morphological features such as tumor grade have some degree of subjectivity. Fortunately, the advent of high-throughput technologies that screen thousands of genes and their products in a single assay, coupled with powerful analytical tools, has expanded our ability to classify breast cancer into distinct groups based on gene expression patterns and elucidates how intrinsic molecular characteristics affect tumor cell response to treatment (Reis-Filho et al., 2010; Güler, 2017). Improved stratification of breast cancer patients subsequently increases the robustness and accuracy of disease prognosis and therapeutic strategies (Russnes et al., 2017; Yu et al., 2018).

1.1.2 **MCF-7 cells and their use in an estrogen receptor-positive breast cancer model**

Michigan Cancer Foundation-7 (MCF-7) is an epithelial, breast cancer cell line that was first isolated in 1970 from the breast tissue of a Caucasian woman with adenocarcinoma (Soule et al., 1973). MCF-7 cells have been used for breast cancer investigations worldwide and have been propagated for many years by various groups. Over time, MCF-7 has produced more data of practical knowledge for patient care than any other breast cancer cell line (Sweeney et al., 2012). It falls under the luminal A subtype being ER and PR
positive and HER2 negative. MCF-7 is a poorly aggressive and non-invasive cell line (Gest et al., 2013), normally being considered to have low metastatic potential (Shirazi et al., 2011). Furthermore, it has been demonstrated that tamoxifen inhibited the growth of MCF-7 cells, but the inhibition could be reversed by estrogen (Comşa, Cimpean, & Raica, 2015).

MCF-7 cells are used ubiquitously in research for ER-positive breast cancer cell experiments, with the majority of these studies looking at acquired anti-estrogen drug resistance. Another factor that makes this cell line suitable for endocrine therapy resistance studies is how easily they can be cultured and retain ER expression when they are treated with these targeted therapies.

A drawback about MCF-7 cells that nevertheless limits the extent to which they model patients is that there are genetic and phenotypic heterogeneity within the cell line. This was first highlighted by Osborne et al. in 1987, where MCF-7 cells from different laboratories showed differential expression of ERs and PRs, as well as having different proliferation rates. This heterogeneity makes reproduction of experimental findings difficult among labs since differential expression of hormone receptors would alter the cell line’s response to hormone therapy. Other groups have also confirmed the heterogeneity of MCF-7 cells over the years (Coser et al., 2009; Kleensang et al., 2016). Still, this genetic and phenotypic drift is not surprising because breast cancers also evolve over time in the patient, either through the pressure of therapy or spontaneously (Lee, Oesterreich, & Davidson, 2015).

Overall, MCF-7 cells have a solid background, owing to the substantial collection of papers that have described them and will remain a staple for research despite their limitations. By standardizing protocols, limiting the number of passages, and cell line authentication, the use of MCF-7 cells allows us to conduct in vitro assays to research breast cancer pathogenesis and treatments.

1.2 Estrogen receptor signalling in breast cancer

Over 70% of the breast cancers diagnosed today are ER-positive, meaning the cells require estrogen to proliferate. Consequently, endocrine therapies use anti-estrogens to prevent the cancer cells from growing. Estrogen signalling is a complex pathway that governs cell
proliferation, apoptosis and invasion in breast cancer cells through the estrogen receptor (Manavathi, Dey, Gajulapalli, Bhatia, Bugide, & Kumar, 2012). Typically, the ER acts as a transcription factor to regulate genes involved in normal cellular function and tumor growth; binding of estrogen to this receptor consequently modulates the expression of its target genes through up or downregulation (Bourdeau, Deschênes, Laperrière, Aid, White, & Mader, 2007).

1.2.1 Estrogen and its receptors

Estrogen is the primary female sex hormone that regulates a variety of reproductive and non-reproductive processes. In premenopausal women, this hormone is mainly synthesized in the ovaries, corpus luteum, and placenta, with a small amount being produced by peripheral tissues such as the brain, liver, heart, and skin (Cui, Shen, & Li, 2013). There are three major physiological forms of estrogen in females: estrone, estradiol, and estriol. Estradiol is the predominant form of estrogen in females who are in their reproductive years. The subsequent mention of “estrogen” in this paper will refer to estradiol and its contribution to breast cancer progression.

One of endocrine therapies target estrogen production through aromatase inhibitors (AIs), which control the rate limiting step of estrogen synthesis through the enzyme, aromatase (Chumsri, Howes, Bao, Sabnis, & Brodie, 2011). This form of hormone therapy however, is usually more effective for postmenopausal women because only small amounts of estrogen are produced by the peripheral tissues; in premenopausal women, the ovaries produce most of the circulating estrogen and usually overpowers the effect of the AIs (Fabian, 2007).

Two distinct estrogen receptors, ERα and ERβ, mediate estrogen signaling. While these two nuclear receptors are highly homologous in the DNA- and ligand-binding domains, they have a distinct transcriptional activating function-1 domain (Delaunay et al., 2000). ERα mediates unregulated cell proliferation in breast cancer cells (Acconcia & Marino, 2011). However, ERβ opposes the actions of ERα by modulating the expression of ERα-regulated genes and reducing migration of cancer cells (Thomas & Gustafsson, 2011). Experimental and clinical evidence suggests that ERα subtype is the major factor involved
in the development of the majority of the breast cancers (Manavathi, Dey, Gajulapalli, Bhatia, Bugide, & Kumar, 2012).

1.2.2 Aberrant estrogen receptor signalling in cancer

When estrogen signalling becomes dysregulated, it can lead to breast carcinogenesis and progression (Saha Roy & Vadlamudi 2012). Thus far, estrogen receptor signaling is the most attractive target for clinical therapy of estrogen receptor-positive breast cancer due to their ability to regulate genes that are involved in cell proliferation, differentiation, apoptosis, and cell migration (Brisken et al., 2010). Many studies have uncovered that a cause of endocrine therapy resistance is crosstalk between estrogen receptor signaling and other oncogenic signaling pathways such as HER2 (Giuliano, Trivedi, & Schiff, 2013), epidermal growth factor receptor (EGFR), or insulin-growth factor receptor (IGFR) signaling (Skandalis, Afratis, Smirlaki, Nikitovic, Theocharis, Tzanakakis, & Karamanos, 2014). Thoroughly exploring the regulatory mechanisms of estrogen receptor signal is still a critical area for breast cancer study.

1.2.3 Estrogen receptor signalling

In the absence of estrogen, the ER is localized predominantly in the cytoplasm, as part of a complex consisting of a dimer of heat shock protein 90 (Hsp90) and a p23 monomer. Hsp90 is a chaperone protein that interacts with a variety of proteins including the ER, tumor suppressor p53 protein, angiogenesis transcription factor HIF-1α, anti-apoptotic kinases, and receptor tyrosine kinases such as HER2 (Zagouri et al., 2012). Elevated expression of Hsp90 in breast ductal carcinomas is frequently observed and contributes to the proliferative activity of breast cancer cells (Zagouri et al., 2012). p23 on the other hand, is a co-chaperone of Hsp90 that is often upregulated in several cancers and plays a role in stabilizing unliganded steroid receptors as well as regulating the catalytic activity of certain kinases (Simpson et al., 2010). Hsp90-p23 chaperone complex inactivates the ER’s ability to regulate transcription and maintains the ER in a conformation that is accessible for estrogen binding.

The ER has both non-genomic and genomic responses, owing to its ability to shuttle between the cytoplasm and the nucleus (Knoblauch & Garabedian, 1999). The classical
mechanism of the ER involves estrogen binding to receptors in the nucleus, which causes the dissociation of the ER from the chaperone complex. Following this release, the ER dimerizes and binds to specific DNA sequences known as estrogen response elements (EREs) located in the promoters of estrogen-responsive genes, where it modulates transcription of the target gene along with coregulators (Moghadam, Hanks, & Keyomarsi, 2011). However, ERs can also regulate gene expression without binding to DNA. This is a non-classical mechanism involving protein-protein interactions with other transcription factors in the nucleus, which allows for the regulation of genes that do not contain EREs (Yaşar, Ayaz, User, Güpür, & Muyan, 2017). In fact, around one third of the estrogen responsive genes lack EREs (Vrtačnik et al., 2014).

**Figure 2.** Classical and non-classical genomic mechanisms of estrogen receptor signaling. Adapted from “Breaking the cycle: An insight into the role of ERα in eukaryotic cell cycles,” by Moghadam, Hanks, & Keyomarsi (2011). *Journal of carcinogenesis*, 10. Upon the binding of estrogen to ERα, the receptor dissociates from the heat shock protein 90 (HSP90) in the cytoplasm and forms a homodimer, which translocates to the nucleus. Here,
ERα homodimers can initiate transcription from the estrogen response elements (ERE) sites of ERα target genes or through interactions with other transcription factors (TF). Some examples of genes transcribed through the classical and non-classical pathways are listed; most of these genes play a role in cell proliferation and survival.

On the other hand, some of the estrogen effects are so rapid that they cannot be associated with gene transcription and protein synthesis—these are known as non-genomic actions and are believed to be mediated through membrane-associated ERs. The non-genomic actions of the ER are frequently associated with the activation of various protein-kinase cascades, which lead to enzymatic modifications of premade signaling molecules and end products (Watson, Alyea, Jeng, & Kochukov, 2007). These cascades can also indirectly influence gene expression, through the activation of signal transduction pathways that eventually act on target transcription factors (Yaşar, Ayaz, User, Güpür, & Muyan, 2017). Overall, the indirect genomic signalling as well as the direct genomic signalling broadens the range of genes that can be regulated by the classical mechanism of ER action alone.

In addition to ligand-dependent genomic and non-genomic signalling, ERs can also be activated in the absence of estrogen. Phosphorylation of the ERs on certain residues or their associated coregulators can cause ligand-independent ER activation with the two most frequently targeted amino acids being serine and tyrosine (Murphy, Seekallu, & Watson, 2011). Moreover, there is cross talk between signal transduction pathways and estrogen receptors. For example, the epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) can regulate transcriptional activity of ERs in the absence of estrogen (Skandalis, Afratis, Smirlaki, Nikitovic, Theocharis, Tzanakakis, & Karamanos, 2014). The molecular mechanisms involved in ligand-independent activation of ER have only been partially characterized and the biological role of these processes is not clear yet. It is thought that ligand-independent pathways allow ER activation in the presence of low estrogen levels and serves to amplify growth factor pathways and consequently enhance proliferation within estrogen receptor-positive tissues.
Figure 3. Other signaling pathways mediated by estrogen or the ERs.
Adapted from “Estrogen synthesis and signaling pathways during aging: from periphery to brain,” by Cui, Shen, & Li (2013). *Trends in Molecular Medicine*, 19 (3), 197-209. Ligand-independent genomic actions involve growth factors (GF) to activate protein kinase cascades, leading to phosphorylation (P) and activation of nuclear ERs at EREs. The membrane ERs can lead to secondary messenger systems, modifications of transcription factors, and other non-transcriptional activities. Estrogen can also exert anti-oxidant effects in an ER-independent manner and inhibit oxidative stress.

1.3 Current resistance mechanisms to endocrine therapy
1.3.1 Tamoxifen resistance

Tamoxifen, a selective estrogen receptor modulator (SERM), is currently the standard adjuvant therapy for patients with estrogen receptor-positive breast cancer. SERMs can function as antagonists or agonists, depending on the tissue or target gene. Upon binding,
SERMs can induce conformational changes that affect the ER’s binding to cofactors which will ultimately determine target gene activation or repression (Martinkovich, Shah, Planey, & Arnott, 2014). In breast cancer cells, tamoxifen acts as an estrogen receptor antagonist, leading to inhibition of estrogenic effects, which are responsible for cancer cell growth or proliferation. Unfortunately, while patients may respond initially, resistance often develops; a third of women with early-stage breast cancer treated with tamoxifen may be refractory within 2-5 years or develop resistance to the drug with ongoing treatment (Fan, Chan, & Fu, 2015).

Although the molecular mechanisms underlying resistance to tamoxifen remains unclear, various mechanisms have been proposed; these include the loss of ER function or expression, crosstalk between the ER and growth factor-mediated signalling pathways, altered expression of specific miRNAs, balance of ER coregulators, and diminished metabolic activation of tamoxifen (Garcia-Becerra et al., 2013).

Since tamoxifen is dependent on the ER, a lack of ER expression or mutations of this receptor will result in intrinsic resistance to antiestrogen therapy (Zhao & Ramaswamy, 2014). Crosstalk between ER and different signalling pathways, such as growth factor receptor, stress, and cell survival signalling pathways have also been implicated in acquired and intrinsic resistance to endocrine therapy (Osborne & Schiff., 2011; Giuliano, Trivedi, & Schiff, 2013; Skandalis, Afratis, Smirlaki, Nikitovic, Theocharis, Tzanakakis, & Karamanos, 2014). The growth factor receptor signalling pathways can stimulate cancer growth together with or independent of the ER pathway. ER activity is also associated to the phosphatidylinositol-3-kinases (PI3K) pathway, which plays a role in oncogenic transformation and governs cellular proliferation through proteins such as cyclins, cyclin-dependent kinases and cyclin-dependent kinase inhibitors (Garcia-Becerra et al., 2013).

MicroRNAs (miRs) are small non-coding RNA molecules that modulate the expression of specific proteins based on complementary messenger RNA (mRNA) sequences with their target molecules. This process occurs by either degrading the target mRNA or suppressing the protein synthesis. miRs have been shown to regulate a variety of cellular processes such as differentiation, cell growth, and cell death (Hwang & Mendell, 2006). Consequently,
expression of specific miRs can influence the responses to therapeutics in breast cancer and has been implicated in tamoxifen resistance (Lyng, Lænholm, Søkilde, Gravgaard, Litman, & Ditzel, 2012).

Although administered as an ER antagonist in breast cancer, tamoxifen acts as an ER agonist in uterine, heart and bone tissue (Martinkovich, Shah, Planey, & Arnott, 2014). One of the mechanisms that explain this difference in activity involves changes in the level of expression of co-regulatory proteins that can influence regulation of ER transcriptional activity (Chang, 2012). In other words, high levels of ER coactivators may augment the estrogen-agonist activity of tamoxifen and contribute to resistance and vice versa (Osborne & Schiff., 2011). Another mechanism that explains the difference in tamoxifen activity involves the activation function domains of the ER. Transcriptional activation is mediated by two activation function (AF) domains: AF-1, which is located near the N-terminus, and AF-2, which is near the C-terminus. Typically, estrogen binds the ER in a way that allows specific amino acids in the AF-2 domain to interact with coactivators. However, when tamoxifen binds, its side chain blocks the interaction site between AF-2 and its coactivators. In other words, tamoxifen serves as an antagonist for genes that require AF-2 activation for ER-mediated transcription (Chang, 2012).

Finally, limited metabolism of tamoxifen can contribute to resistance to endocrine therapy. Tamoxifen is typically metabolized in the liver by two cytochromes: CYP3A4 and CYP2D6, which produce N-desmethy ltamoxifen and 4-hydroxytamoxifen, respectively (Cronin-Fenton, Damkier & Lash, 2010). Further oxidation of these metabolites yields the active metabolite: 4-hydroxy-N-desmethy ltamoxifen, also known as endoxifen. Both endoxifen and 4-hydroxytamoxifen have greater binding affinities for the ER and suppress cell proliferation more effectively than tamoxifen itself. In the plasma, the concentration of endoxifen is higher compared to 4-hydroxytamoxifen. Moreover, endoxifen is thought to be the responsible for the pharmacological activity of tamoxifen in vivo as its anti-estrogen activity is 100-fold higher than 4-hydroxytamoxifen (Rodriguez-Antona et al., 2006). Genetic mutations of the cytochromes mentioned previously may result in incomplete activation of tamoxifen to its more potent forms, decreasing the effectiveness of this drug (Mürdter et al., 2011).
1.3.2 Evasion of endoplasmic reticulum stress in cancer and the unfolded protein response

The endoplasmic reticulum controls a variety of cell processes like calcium homeostasis, protein folding and glycosylation, and lipid biosynthesis (Basseri & Austin, 2012; Schönthal, 2012). A number of cellular stress conditions, such as nutrient deprivation, hypoxia, alterations in glycosylation status, and disturbances of Ca\(^{2+}\) flux, result in the accumulation and aggregation of unfolded and/or misfolded proteins in the endoplasmic reticulum lumen and cause endoplasmic reticulum stress. All of these signals can lead to the activation of an adaptive stress response termed the unfolded protein response (UPR). The UPR attempts to increase the folding capacity of the endoplasmic reticulum through the induction of key proteins involved in chaperoning, protein folding, and degradation pathways. While the UPR is fundamentally a protective response, excessive or prolonged UPR can result in cell death, predominantly through induction of apoptosis.

Transduction of the UPR pathway is mediated through three endoplasmic reticulum transmembrane proteins that function as sensors of endoplasmic reticulum stress: PKR-like endoplasmic reticulum kinase (PERK), inositol requiring enzyme 1 (IRE1α), and activating transcription factor 6 (ATF6). All three of the stress sensors contain luminal domains that sense the accumulation of unfolded proteins within the endoplasmic reticulum lumen (Gardner, Pincus, Gotthardt, Gallagher, & Walter, 2013). In the absence of stress, glucose-regulated protein 78 (GRP78) is bound to all three, maintaining the sensors in an inactive configuration and because of this gatekeeping ability, GRP78 has been coined the “master regulator” of the UPR (Luo & Lee, 2013). Accumulation of unfolded proteins within the endoplasmic reticulum lumen will cause GRP78 to dissociate from the three sensors in an attempt to refold the protein; once released by GRP78, the three sensors are free to initiate the UPR signal transduction pathways.

The first response of the UPR is translational attenuation—this is largely mediated by PERK. The cytosolic kinase domain of PERK, upon oligomerization, can be activated by autophosphorylation. Activated PERK then phosphorylates protein eukaryotic initiation factor 2 alpha (eIF2α) which represses protein synthesis and prevents further influx of
endoplasmic reticulum client proteins (Teske et al., 2011). In the event of sustained PERK signalling, the pro-apoptotic transcription factor C/EBP-homologous protein (CHOP) is upregulated. This downregulates Bcl-2, induces the expression of some apoptotic BH3-only members of the BCL-2 family, and upregulates growth arrest.

IRE1α is a kinase that dimerizes and autophosphorylates under endoplasmic reticulum stress conditions. This leads to the activation of the cytosolic RNase domain, due to a conformational change (Walter & Ron, 2011). Active IRE1α catalyzes the excision of an intron in the unspliced transcription factor X box-binding protein 1 (XBP-1) mRNA, resulting in the translation of the spliced XBP-1 isoform, which then translocates to the nucleus to induce the upregulation of its target genes. The overall result is the production of proteins involved in endoplasmic reticulum-associated degradation, the entry of proteins into the endoplasmic reticulum and protein folding, among other functions (Jiang, Niwa, & Koong, 2015). Spliced XBP1 also modulates phospholipid synthesis, which is required for endoplasmic reticulum membrane expansion under endoplasmic reticulum stress (Hetz et al., 2011).

The second response is the upregulation of the expression of endoplasmic reticulum chaperones by ATF6 to increase the protein-folding capacity in endoplasmic reticulum (Teske et al., 2011). Once released from GRP78, ATF6 is cleaved in the Golgi to yield a cytosolic fragment that then translocates to the nucleus to regulate transcription of UPR-responsive genes such as XBP-1 and CHOP (Kaufman, 2002). Although both the PERK and ATF6 pathways can upregulate CHOP, the PERK pathway predominates through selective upregulation of translation of ATF4, which subsequently induces transcription of CHOP and other genes involved in autophagy, apoptosis, amino acid metabolism and antioxidant responses (Li, Guo, Tang, Jiang, & Chen, 2014).

If the overload of unfolded or misfolded proteins in the endoplasmic reticulum is not resolved, prolonged activation of the UPR would lead to programmed cell death. Three known proapoptotic pathways emanating from the endoplasmic reticulum are mediated by IRE1, caspase-12, and PERK/CHOP (Kaufman, 2002; Ron, 2002).
Caspase-12 is an endoplasmic reticulum-associated proximal effector of the caspase activation cascade, and cells defective in this enzyme are partially resistant to endoplasmic reticulum stress-induced apoptosis (Nakagawa et al., 2000). Interestingly, most humans have a premature stop codon in this gene that leads to the transcription of a truncated, non-functional form of caspase-12 (McIlwain, Berger, & Mak, 2013). The reason for this was due sepsis, an illness due to an abnormal immune network in response to invading pathogens, that led to the positive selection of this stop codon polymorphism (Xue et al., 2006). The active form of caspase-12 however, is present in some individuals of African descent (Chen, Wilson, Dahmer, Quasney, Waterer, Feldman, & Wunderink, 2014). Consequently, these individuals have increased vulnerability to sepsis. As an alternative to caspase-12 in humans, caspase-4 is involved in endoplasmic reticulum stress-induced cell death pathways (Li et al., 2013).

CHOP is a transcription factor that induces expression of genes favoring apoptosis in response to endoplasmic reticulum stress (Ron, 2002). Prolonged UPR activation also leads to expression of transcription factor ATF4 through the PERK-eIF2α pathway. ATF4 then induces expression of CHOP, which binds to the promoter of death receptor 4 (DR4) and death receptor 5 (DR5) genes and upregulate their expression, thus promoting caspase-8 activation and cell death (Iurlaro & Muñoz-Pinedo, 2016).

UPR activation represents a hallmark of several human cancers, together with the upregulation of GRP78. In fact, UPR activation enables cancer cells to survive, adapts to adverse environmental conditions, and leads to growth arrest driving dormancy, which promotes resistance to conventional chemotherapy (Avril, Vauléon, & Chevet, 2017). Cancer cells often display elevated levels of endoplasmic reticulum stress markers compared with their non-malignant counterparts, suggesting that they exist in a state of chronic, low level endoplasmic reticulum stress as an adaptive mechanism, permitting cell growth advantages and resistance to death under adverse conditions, such as hypoxia and low nutrient environments. Accordingly, this difference in endoplasmic reticulum stress status of normal and malignant cells provides an opportunity for therapeutic intervention as it may sensitize cancer cells to inducers of apoptosis.
1.4 The family of cadherins in cancer

The ability of cells to form cell contacts, adhere to the extracellular matrix, change morphology, and migrate is essential for development, wound healing, metastasis, cell survival and the immune response (Martin, Ye, Sanders, Lane, & Jiang, 2013). Cadherins are the major cell adhesion molecules responsible for Ca\(^{2+}\)-dependent cell-cell adhesion in vertebrate tissues and form homophilic interactions with same molecules on other cells, thus allowing cell-cell interaction (Perrais, Chen, Perez-Moreno, & Gumbiner, 2007).

The first three cadherins that were discovered were named according to the main tissues in which they were found: epithelial (E)-cadherin is expressed on many types of epithelial cells; neural (N)-cadherin on nerve, muscle, and lens cells; and placental (P)-cadherin on cells in the placenta and epidermis (Alberts et al., 2002). While these cadherins are largely expressed in the aforementioned tissues, they are also present in other tissues. Two of the cadherins, E and N-cadherin, are of particular interest to us due to their role in tumor progression and metastasis.

E-cadherin is generally considered the prototype of all cadherins because of its early identification and thorough characterization in both normal and pathological conditions. Loss of this cadherin has mainly been implicated in cancer progression and has been shown to play an important role in the transition of epithelial tumors from a benign to an invasive state (Wong, Fang, Chuah, Leong, & Ngai, 2018); in experimental animal models, epithelial-mesenchymal transition (EMT) has been observed with the loss of E-cadherin, suggesting that it promotes metastasis (Yang et al., 2004). E-cadherin expression is also modulated by estrogen; in breast carcinoma cells, estrogen withdrawal or estrogen antagonists led to increased E-cadherin levels (Li et al., 2003). However, this effect may be indirect since estrogen response elements have not been found in the E-cadherin promoter. In addition, E-cadherin has a much wider implication in human cancer biology. The cadherin 1 (CDH1) gene, which encodes for E-cadherin, functions as a tumor suppressor gene and CDH1 germline mutations are associated with a hereditary tumor syndrome (Liu & Chiu, 2014). Thus, loss of E-cadherin can initiate tumor development.
N-cadherin on the other hand, is upregulated in more invasive and less differentiated breast cancer cell lines that lack E-cadherin expression (Mrozik, Blaschuk, Cheong, Zannettino, & Vandyke, 2018). This type of cadherin is also expressed in endothelial cells and plays an essential role in the maturation and stabilization of normal vessels and tumor-associated angiogenic vessels (Mariotti et al., 2007). In addition, expression of N-cadherin in epithelial cells induces changes in morphology to a fibroblastic phenotype, rendering the cells more motile and invasive (Derycke & Bracke, 2004).

Typically, the selective loss of E-cadherin or upregulation of N-cadherin confers a more invasive and metastatic phenotype (Wong, Fang, Chuah, Leong, & Ngai, 2018); this switch in cadherin type is observed in aggressive tumours, which often displays EMT in which cells have a stroma-oriented cellular adhesion profile with increased tumor cell motility and invasive properties (Gravdal et al., 2007).

1.5 Cell death and the Bcl-2 family

1.5.1 Apoptosis in cancer

Cell death plays an important role in the development of tissues and organisms. Dysregulated cell death is prevalent in many human diseases, including cancer, autoimmune diseases, stroke and neurodegeneration (Elmore, 2007). As such, identifying control points in cell death pathways would provide rational targets for the development of therapeutics (Danial & Korsmeyer, 2004). There are many ways a cell can die. This study however, focuses on apoptosis, a form of programmed cell death.

Apoptosis is the most prevalent form of cell death in mammals, and its molecular signalling pathway is well-defined (Tsujimoto, 2003). Morphologically, apoptotic cells are characterized by the blebbing of the plasma membrane, condensation of the cytoplasm and nucleus, and cellular fragmentation into membrane apoptotic bodies (Steller, 1995; Wyllie et al., 1972). At the molecular level, several common features are often associated with apoptosis: loss of mitochondrial membrane potential ($\Delta\Psi_m$), cytochrome c release, activation of caspases, and exposure of phosphatidylserine to the extracellular space (Kroemer, Dallaporta, & Resche-Rigon, 1998).
1.5.2 The Bcl-2 family

The B-cell lymphoma 2 (Bcl-2) family of proteins, which consists of pro-apoptotic and anti-apoptotic members, controls the commitment of a cell to apoptosis by regulating mitochondrial outer membrane permeabilization (MOMP) (Brunelle & Letai, 2009). Members of this family share specific conserved regions, known as Bcl-2 homology (BH) domains. BH domains contribute at multiple levels to the structure and function of these proteins. Particularly, the BH3 domain is the site at which pro-apoptotic proteins engage with and neutralize the prosurvival proteins. BH3 mimetics bind to the BH3 domain of prosurvival members, causing the displacement of proapoptotic members. Once the proapoptotic members are displaced, they are free to activate pore-forming proteins, which will induce MOMP and trigger apoptosis. Various cancer cells depend on anti-apoptotic Bcl-2 family members for survival so Bcl-2 antagonism through BH3 mimetics has emerged as a promising cancer therapeutic (Zhang, Ming, & Yu, 2007). In 2016, ABT-199 (Venetoclax), a BH3-mimetic Bcl-2 inhibitor, was approved by the FDA for the treatment of chronic lymphocytic leukemia (Roberts et al., 2016).

The Bcl-2 family is divided into three classes: pro-apoptotic proteins that form pores to induce MOMP; pro-apoptotic BH3-only proteins that directly or indirectly activate the pore-forming proteins; and the anti-apoptotic proteins that inhibit MOMP by binding to the pore-forming proteins or sequestering the pro-apoptotic proteins (Kale, Osterlund, & Andrews, 2018). The pore forming proteins are closely associated with the mitochondrial membrane, and when activated by BH3-only proteins, promote the permeabilization of the outer mitochondrial membrane, releasing apoptotic factors into the cytoplasm and initiating apoptosis. Both the pore-forming and BH3-only proteins are inhibited by direct interaction with anti-apoptotic Bcl-2 family members. The dynamic balance that occurs
between anti-apoptotic and pro-apoptotic members determines whether the cell commits apoptosis (Strasser, Cory, & Adams, 2011).

**Figure 4.** The Bcl-2 family regulates MOMP.

The family is divided into 3 classes: anti-apoptotic pore-forming proteins (green), BH3-only proteins which can directly (blue, activators) or indirectly (orange, sensitizers) activate the pore forming proteins, and anti-apoptotic proteins (grey). Pore forming proteins include: Bax and Bak. BH3-only sensitizers include: Bad, Noxa, Bik, and Bmf. BH3-only activators include Bim, Bid, and Puma. Pore-forming proteins can induce MOMP, causing the release of pro-apoptotic factors to be released into the cytoplasm, causing subsequent caspase activation and cell death.
1.5.3 Apoptotic signalling pathways

Mammalian cells possess two apoptotic signalling pathways that govern the activation of caspases: the intrinsic and extrinsic pathways. In the intrinsic pathway, most apoptotic stimuli transmit death signals to the mitochondria and induce MOMP, which leads to the release of pro-apoptotic mitochondrial proteins, such as cytochrome c and Smac, into the cytoplasm. Cytochrome c is a hemeprotein that is part of the electron transport chain in mitochondria. After its release, cytochrome c associates with apoptotic protease activating factor-1 (Apaf-1), which activates the caspase cascade to execute apoptotic cell death. Smac is a mitochondrial protein that binds to inhibitor of apoptosis proteins, freeing caspases to activate apoptosis.

The mammalian family of caspases can be divided into three groups: initiator caspases (2, 8, 9, 10), effector caspases (3, 6, 7) and inflammatory caspases (1, 4, 5, 11, 12). The first group initiates the apoptotic signals while the second group perform the proteolysis that leads to subsequent DNA fragmentation in apoptosis. The third group is not involved in apoptosis but is involved in inflammatory cytokine signaling and other types of cell death such as pyroptosis (McIlwain, Berger, & Mak, 2013).

In the extrinsic pathway, the engagement of death receptors, such as Fas and tumor necrosis factor-α, directly activates caspase-8 that activates downstream caspases, such as caspase-3 or caspase-7, which induce apoptotic cell death. However, the extrinsic and intrinsic pathways are linked; caspase-8 can also cleave the BH3-only proapoptotic protein, Bid, into its active form, truncated Bid (tBid), which activates Bax and Bak, the pore-forming proteins that initiate MOMP (Kalkavan & Green, 2018).

It is important to note that MCF-7 cells do not express caspase-3 because of a 47-base pair deletion within exon 3 of the CASP-3 gene (Jänicke et al., 1998). This deletion results in the skipping of this exon during pre-mRNA splicing and introduction of a premature stop codon at position 42 that completely abrogates translation of the CASP-3 mRNA (Jänicke et al., 1998). Although MCF-7 cells lack caspase-3, they execute apoptosis via caspase 6 and 7.
1.5.4 The pro-survival Bcl-2 and its role in breast cancer

Given their role in preventing cell death, it is not surprising that deregulation of genes encoding either anti-apoptotic or pro-apoptotic Bcl-2 family members is a common feature of many cancers (Hata, Engelman, & Faber, 2015). In fact, the founding member of this family, Bcl-2, was discovered because of its involvement in chromosomal translocations observed in non-Hodgkin’s lymphomas (Tsujimoto et al., 1985 as referenced in Hata, Engelman, & Faber, 2015). Overexpression of the pro-survival protein Bcl-2 is also common in breast cancer and has been targeted by BH3 mimetics in order to sensitize primary tumors to chemotherapy (Oakes et al., 2012).

Bcl-2 is upregulated by estrogens in breast cancer, through two estrogen-responsive elements located within its gene (Perillo, Sasso, Abbondanza, & Palumbo, 2000). Consequently, Bcl-2 expression in breast cancer may be an indicator of estrogen receptor functional activity. While high levels of myeloid leukemia cell-1 (Mcl-1), another pro-survival Bcl-2 family member, predicts poor patient outcome in breast cancer, the prognostic role of Bcl-2 plays depends on the molecular subtype of the breast cancer. The current consensus is that Bcl-2 is an independent favorable prognostic marker for only luminal A breast cancer (Eom et al., 2016). While this may be the case, it is noteworthy that a significant number of patients with Bcl-2-positive disease relapse and die (Dawson et al., 2010). Regardless of its prognostic value, overexpression of Bcl-2 makes the cancer cells more resistant to cell death stimuli and may modulate the sensitivity of breast cancer cells to therapeutic agents like tamoxifen. Moreover, breast cancer cells with acquired anti-estrogen resistance exhibit significantly higher expression of Bcl-2 than anti-estrogen sensitive breast cancer cells (Crawford et al., 2007).

1.6 The PPAR family of proteins

1.6.1 Subtypes and function

In addition to the Bcl-2 family of proteins, peroxisome proliferator-activated receptors (PPARs) are also of interest in cancer research. The PPARs are a family of nuclear hormone
receptors that govern energy homeostasis and metabolism. There are three subtypes: α, δ, and γ, each having different tissue specificity.

PPARα is primarily expressed in the heart, liver, skeletal muscles, intestines, and macrophages (Moreno, Farioli-Veccioli & Ceru, 2004). Fibrates, synthetic ligands of PPARα, are a class of lipid-lowering drugs and are extensively used in the treatment of hyperlipidemia; activation of PPARα upregulates genes involved in lipid oxidation and promotes plasma triglyceride clearance (Pawlak, Lefebvre & Staels, 2015).

PPARγ on the other hand, is highly expressed in adipose tissue and plays roles in glucose and lipid uptake, glucose oxidation, adipogenesis, and insulin sensitivity (Olefsky & Saltiel, 2000). Thiazolidinediones are a class of drugs that increase insulin sensitivity through activating PPARγ and have been used to treat patients with Type II diabetes (Saltiel & Olefsky, 1996).

Finally, PPARδ is expressed in a wide range of tissues, including metabolically active sites such as liver, muscle, and fat; its near-ubiquitous expression raised early speculation that it may have a general housekeeping role (Kliewer et al., 1994). Compared to the other subtypes, PPARδ has not been extensively studied and its metabolic roles are only recently being elucidated. PPARδ has also been demonstrated to be involved in many different biological activities including lipid metabolism, skeletal muscle lipid oxidation, inflammation, neuronal differentiation, and mitochondrial respiration (Chen et al., 2015). PPARδ knockout mice exhibit placental defects, decreased adipose mass, defective myelination, and altered inflammatory responses (Barak et al., 2002).

Overall, PPARs have the potential to influence multiple intracellular pathways through regulating the transcription of their target genes. In addition to its current use in treating metabolic diseases, agonists and antagonists of the PPARs have potential as therapeutic agents in cancer. For example, PPARδ controls cell proliferation and angiogenesis through upregulating vascular endothelial growth factor (VEGF) transcription. Angiogenesis is essential for cancer development and growth, as the cells need blood vessels for nutrients and oxygen (Carmeliet, 2005). PPARγ can downregulate cyclooxygenase-2 (COX-2) transcription, which is associated with an increased production of prostaglandin E2.
(PGE2), one of the major products of COX-2 which is known to modulate cell proliferation, cell death, and tumor invasion in many types of cancer including colon, breast, and lung (Sobolewski et al., 2010).

1.6.2 PPAR ligands and activation

The family of PPARs consists of ligand-activated transcription factors that bind to fatty acids and their metabolites (Willson et al., 2000). Activation of PPARs involves ligand-binding, heterodimerization with the retinoic X receptor (RXR), interaction with PPAR response elements (PPREs) on the DNA, and recruitment of co-regulatory proteins (Mangelsdorf et al., 1995). An interesting feature of the PPAR ligand-binding cavity is its size, which is 3 to 4 times larger than that of the other nuclear receptors (Grygiel-Gorniak, 2014), allowing them to accommodate and bind a variety of natural and synthetic ligands.

Synthetic ligands of PPARα and PPARγ are widely used in clinical practice. These include fibrates and thiazolidines, which are used to treat hyperlipidemia and type II diabetes, respectively.

Natural ligands of PPARs include fatty acids and eicosanoids, which are derivatives of polyunsaturated fatty acids. Overall, these fatty acids act as PPAR agonists that regulate the transcription of genes involved in glucose and lipid homeostasis (Krey et al., 1997; Plutzky, 2000; Neschen et al., 2007).

Figure 5. PPARs are a family of nuclear transcription factors.
PPARs (in green) work by heterodimerizing with RXR (in orange) to bind to specific sequences on the DNA, PPREs (blue), to regulate gene transcription.

1.6.3 Synthetic PPARδ agonists and their mechanisms of actions

GW0742 is a synthetic PPARδ agonist but displays 300 to 1,000-fold selectivity for PPARδ versus other PPARs (Sznaidman et al., 2003). It acts by altering a number of genes like Fos, Junb, Egr1, Egr2, with Angptl4 emerging as its main target gene (Kojonazarov et al., 2013). Comparisons of the PPARδ:GW0742 complex with published structures of PPARs in complex with α and γ selective agonists and pan agonists suggests that two residues (Val312 and Ile328) in the buried hormone binding pocket play special roles in PPARδ selective binding and experimental and computational analysis of effects of mutations in these residues confirms this and suggests that bulky substituents that line the PPARα and γ ligand binding pockets as structural barriers for GW0742 binding (Batista et al., 2012).

1.6.4 Synthetic PPARδ antagonists and their mechanisms of action

DG172 exhibits high binding affinity and potent antagonistic properties, enhancing transcriptional co-repressor recruitment and down-regulating transcription of PPARδ target genes like angiopoietin-like 4 (ANGPTL4) (Lieber et al., 2012). ANGPTL4 is a secretory protein that promotes the migration of different cell types in vitro and inhibits anoikis, a form of anchorage-dependent cell death (Liao et al., 2017).

PT-S58, a cell-permeable diaryl-sulfonamide, acts as a pure competitive PPARδ antagonist targeting the ligand binding site of PPARδ while not allowing co-regulator interactions (Levi et al., 2013; Naruhn et al., 2011).

GSK0660 is the first PPARδ antagonist described in the literature. It reduces the expressions of ANGPTL4, C-C motif chemokine ligand 8 (CCL8), and C-X-C motif chemokine ligand 10 (CXCL10) (Savage et al., 2015). Both CCL8 and CXCL10 are chemokines that play a role in immunoregulatory and inflammatory processes (Esche, Stellato, & Beck, 2005).
GSK3787 is a selective and irreversible PPARδ antagonist that inhibits carnitine palmitoyltransferase 1A (CPT1A) gene transcription, which is involved in the translocation of long chain fatty acids across the mitochondrial membranes for β-oxidation (McGarry & Brown, 1997). Its binding to PPARδ is irreversible because it forms a covalent bond with a cysteine residue in the ligand binding domain (Shearer et al., 2010).

Despite the apparent importance of PPARδ in cancer biology, there are presently no PPARδ antagonists available for clinical use and selective antagonists have only recently been described. Here, the definition of antagonist is a compound that has opposing effects on the target genes of PPARδ. The antagonist I used in this project is a synthetic compound known as NXT1511, which lowers the expression of PPARδ’s signature genes, 3-Phosphoinositide Dependent Protein Kinase 1 (PDPK1) and catalase (CAT) (Wang et al., 2016). Whether the alterations in the expression of PPARδ signature genes are a direct or indirect effect of the compound is not clear. PDPK1 activates protein kinase B (PKB) and the constitutive activation of PKB contributes to cancer progression by promoting proliferation and increased cell survival (Harris, 2003). On the other hand, CAT plays an important role in cellular protection against oxidative stress by scavenging H₂O₂ generated from peroxisomal fatty acid β-oxidation (Kim & Yang, 2013).

1.6.5 PPARδ and its role in breast cancer

PPARδ, the least well-characterized subtype of the PPARs, is overexpressed in more than half of all invasive breast cancers (Glazer et al., 2017). Not only does it protect keratinocyte (Zhu et al., 2014), muscle (Salvadó et al., 2014), and heart (Cheang et al., 2017) cell lines from endoplasmic reticulum stress, but it has also been recently shown to protect breast cancer cell lines from conditions like hypoxia, glucose deprivation, and endoplasmic reticulum stress drugs (Wang et al., 2016). However, the mechanism of PPARδ’s protection is not well understood. While this is the case, one can infer how PPARδ might work by looking at the more well-characterized subtype, PPARγ; PPARγ and PPARδ have opposing actions either by direct competition (Shi et al., 2002), coactivator competition (Gustafsson et al., 2009) and/or ligand-dependent activation and repression (Adhikary et
al., 2011). Activation of PPARγ has been shown to upregulate the gene expression of BRCA1, suggesting its role as a tumor suppressor (Pignatelli et al., 2003).

Concerning the interaction between PPAR and ER pathways, the PPAR/RXR heterodimer has been shown to bind to ERE-related palindromic sequences (Keller et al., 1995) as well as estrogen receptors being able to negatively interfere with PPRE-mediated transcriptional activity (Wang & Kilgore, 2002). It was also demonstrated that PPARγ could upregulate the transcription of phosphatase and tensin homolog (PTEN), which can inactivate PI3K survival signalling by dephosphorylating the intermediate, phosphatidylinositol 3, 4, 5-trisphosphate (PIP3). In 2005, Bonofiglio et al. showed that breast cancer cells that were stimulated with Rosiglitazone, a PPARγ agonist, showed an increase in PTEN protein as well as an inhibition of AKT phosphorylation and cellular growth. This is significant because this crosstalk between PPARγ and the ER may guide future breast cancer therapeutics. Furthermore, a vital mechanism underlying endocrine therapy resistance is the collateral activation of the PI3K/AKT/mTOR pathway so a theory for PPARδ’s protection may be through its negative regulatory effect on PPARγ.

1.7 Comparing Bcl-2 and PPARδ

1.7.1 Rationale for this project

While there are many reasons to study PPARδ in breast cancer, this project focuses on the ability of PPARδ to protect cells from endoplasmic reticulum stress. In particular, comparing Bcl-2 and PPARδ mediated protection in MCF-7 cells, an ER+ cell line, is of interest to us since the overexpression of either one of these proteins can protect the cells from stress and render it more resistant to cell death.

1.7.2 Separating Bcl-2’s two pathways of protection

Comparing Bcl-2 and PPARδ protection is a bit complex however, because Bcl-2 is located at both the mitochondria and the endoplasmic reticulum and protects through two different pathways. Bcl-2 at the mitochondria can sequester pro-apoptotic Bcl-2 family members and prevent mitochondrial outer membrane permeabilization and subsequent caspase
activation, while Bcl-2 at the endoplasmic reticulum can interact with IP₃ receptors to prevent calcium release from the endoplasmic reticulum and Ca²⁺-mediated apoptosis (Rong et al., 2009). These two pathways of protection may be separated through fusion proteins of Bcl-2: Bcl2-acta and Bcl2-cb5, which target the mitochondria and the endoplasmic reticulum, respectively, through tail anchor targeting sequences at the C-terminus of the protein (Zhu et al., 1996). Bcl2-acta is a fusion protein with the cytoplasmic domain of Bcl-2 and the insertion sequence from the mitochondria specific insertion sequence from the ActA protein from Listeria. Bcl2-cb5 on the other hand, is a fusion protein with the cytoplasmic domain of Bcl-2 and the insertion sequence from the endoplasmic reticulum specific isoform of cytochrome b5. Bcl-2’s two routes of protection can be differentiated by the order of two events during apoptosis: loss of the mitochondrial membrane potential (Δψₘ) and the release of cytochrome c. While Bcl2-cb5 effectively protects cells against drugs where the loss of the Δψₘ precedes cytochrome c release, Bcl2-acta protects cells against drugs where cytochrome c release precedes the loss of the Δψₘ (Annis et al., 2001). As such, these fusion proteins allow us to compare PPARδ’s protection to Bcl-2’s distinct paths of protection.

1.7.3 MCF-7 cell lines as models for patients with resistant cells

In this project, patients that would be resistant to endocrine therapy are modeled by MCF-7 cells that either overexpress PPARδ, Bcl-2 (three variants: wt (wild type), acta (mitochondrial-targeting), cb5 (endoplasmic reticulum-targeting)), or both proteins. In addition to confirming PPARδ’s protection to endoplasmic reticulum stress, the overarching goal, is to find out whether patients with high PPARδ expression have to be treated differently than patients with high Bcl-2 expression. My hypothesis is that PPARδ and Bcl-2 share a common pathway of protection—in other words, a drug that targets this pathway could potentially be used to treat patients regardless of their cancer cells’ PPARδ or Bcl-2 expression levels.
1.8 Background on drugs used to induce endoplasmic reticulum stress and apoptosis

To test the separate pathways of Bcl-2 protection, a panel of drugs was needed to induce either endoplasmic reticulum stress-mediated or mitochondrial-mediated apoptosis. The drug panel used in this project included the following drugs: tumor necrosis factor-alpha + cycloheximide (TNF-α + CHX), thapsigargin (TG), tunicamycin (TN), staurosporine (STS), and actinomycin D (ActD). This group of drugs can be divided into two groups based on whether they induce mitochondrial-dependent apoptosis or endoplasmic reticulum stress-induced apoptosis in Table 1. The drug mechanisms are discussed in further detail in the following section.

<table>
<thead>
<tr>
<th>Endoplasmic reticulum stress-dependent</th>
<th>Mitochondrial-dependent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thapsigargin (TG)</td>
<td>Tumor necrosis factor (TNF-α) + Cycloheximide (CHX)</td>
</tr>
<tr>
<td>Tunicamycin (TN)</td>
<td>Staurosporine (STS)</td>
</tr>
<tr>
<td></td>
<td>Actinomycin D (ActD)</td>
</tr>
</tbody>
</table>

**Table 1.** The drug panel separated into 2 classes: drugs that induced apoptosis through endoplasmic reticulum stress and drugs that induced mitochondrial dependent apoptosis.

1.8.1 Mechanisms of actions for the drug panel

TNF-α + CHX induce apoptosis through a pathway that involves caspase-8 activation. Typically, TNF-α activates cell survival and cell death pathways simultaneously but through co-treatment with a protein synthesis inhibitor such as CHX, the response to TNF-α can be switched to apoptosis; CHX treatment results in the downregulation of the caspase-8 inhibitor, c-Flip, promoting caspase-8 activation (Wang et al., 2008). Caspase-8 then cleaves Bid, a member of the Bcl-2 family, into truncated Bid (tBid) which
translocates to the mitochondria and activates Bax. Bax then oligomerizes in MOMP and triggers the release of cytochrome c and subsequent caspase activation. This release of cytochrome c through tBid is antagonized through overexpression of Bcl-2 at the mitochondria.

STS is a pan-kinase inhibitor that induces apoptosis through caspase-dependent and caspase-independent pathways (Belmokhtar et al., 2001). Staurosporine can activate the mitochondrial caspase-dependent apoptotic pathway through Bax, an effector of the Bcl-2 family that forms pores on the mitochondrial outer membrane and subsequently causes the release of apoptotic factors (Zhang, Gillespie, & Hershey, 2004). Although STS is a promiscuous drug with many off-target effects, it serves as a positive control for cell death.

ActD is a transcription inhibitor that binds to guanine residues and inhibits DNA-dependent RNA polymerase (Kleeff et al., 2000). It is thought to induce apoptosis by preventing the transcription of cyclin factors and anti-apoptotic genes, leading to cell cycle arrest and cell death (Lu et al., 2015).

TG is a sarcoplasmic/ endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump inhibitor that induces endoplasmic reticulum stress by depleting Ca^{2+} levels in the endoplasmic reticulum. Consequently, Ca^{2+}-dependent chaperone proteins are unable to function properly and the result is an accumulation of misfolded proteins.

TN is an N-linked glycosylation inhibitor that blocks the initial step of glycoprotein biosynthesis in the endoplasmic reticulum. Treatment with TN causes accumulation of unfolded glycoproteins in the endoplasmic reticulum, leading to endoplasmic reticulum stress.

Both TG and TN induce endoplasmic reticulum stress and are therefore protected by overexpression of Bcl-2 at the endoplasmic reticulum. Since the mitochondria are the converging point of apoptosis, overexpression of Bcl-2 at the mitochondria can also protect against these drugs. As mentioned in section 1.5.2, a BH3 mimetic typically mimics the BH3 domain of pro-apoptotic Bcl-2 family members, thereby inhibiting anti-apoptotic proteins by occupying their BH3 binding groove and sensitizing cells to apoptosis. This is
what has been published but my data suggests that the use of the BH3 mimetic, ABT-199, which specifically inhibits Bcl-2, is unable to sensitize cells that overexpress Bcl-2 to apoptosis caused by endoplasmic reticulum stress-inducing drugs.

In addition, it is interesting to note that both TG and TN elicit a transient state known as “mitochondrial hyperpolarization”, where the $\Delta \psi_m$ becomes more negative. This can be observed by staining cells with tetramethylrhodamine ethyl ester (TMRE), a dye that is dependent on the $\Delta \psi_m$; during mitochondrial hyperpolarization, more of this cationic dye accumulates in the mitochondria, leading to more TMRE signal as measured using fluorescent microscopy.

1.8.2 Adding tamoxifen to the drug panel as a query compound

Tamoxifen is an estrogen receptor antagonist; its core mechanism of action is to prevent the pro-survival and proliferative effects through direct competition with estrogen. It is typically used as an adjuvant therapy after primary resection of estrogen receptor-positive breast tumor to decrease the risk of recurrence. Although the primary mechanism of action of TAM is through the inhibition of estrogen receptor, research over the years has revealed mechanisms that are independent of the estrogen receptor.

For example, tamoxifen can exert anti-proliferative effects through direct interaction with protein kinase C epsilon (PKC$\varepsilon$), which plays a role in tumor cell differentiation and growth (Jain & Basu, 2014). Tamoxifen can also modulate calmodulin (CaM), an important Ca$^{2+}$-binding protein that binds with a variety of proteins in response to increases in intracellular Ca$^{2+}$ and mediates a variety of signalling pathways ranging from proliferation, differentiation, and apoptosis (Berchtold & Villalobo, 2014). Inhibition of the Ca$^{2+}$ calmodulin signalling pathway is an effective way to activate apoptosis in epithelial cells and it has been shown that tamoxifen binds to and inhibits CaM (Lam, 1984). Other ways tamoxifen inhibits cell growth and induces apoptosis in estrogen receptor-positive breast cancer cells is through inhibiting estrogen-induced c-myc expression (Bogush, Dudko, Bogush, Polotsky, Tjulandin, & Davydov, 2012). In addition, tamoxifen treatment of Bcl-2 overexpressing clones was found to induce apoptosis via activation of c-Jun N-terminal kinase (JNK), p38 kinase and phosphorylation of c-Jun (Moodbidri & Shirshat, 2005).
Furthermore, oxidative stress, mitochondrial permeability transition, ceramide generation, and changes in the fluidity of the cell membrane play vital roles in TAM-induced apoptosis (Mandelkar & Kong, 2001).

1.9 High-Content Analysis

High-Content Analysis (HCA) is an established tool that involves three main automated components: image acquisition, image analysis, and data analysis. Throughout these three components, a series of steps is followed with varying degrees of user interaction depending on the application and the software being used for image analysis. First, the cells in the image are segmented, providing the software algorithms with the information it needs for downstream processing of individual cell measurements. Next, the areas of interest are defined by the user based on a multitude of parameters such as texture, morphology and intensity. After areas of interest are defined, measurements are collected. These measurements, often referred to as features, are dictated by the type of data desired from the samples in order to answer the research question being asked.

Automation, not only of the image acquisition but also of the analysis, allows millions of cells to be analyzed and reveals the heterogeneity of responses that exist within cell populations. HCA has evolved over the past decade to the extent that it is now widely used in basic research and in drug discovery for compound and genetic screening. In many instances, HCA complements and provides significant advantages over existing approaches, bringing additional data that can be used to make scientific conclusions (Zock, 2009).

Firstly, fully automated imaging gives more cellular information and unbiased quantitative image analysis exploits the full potential of fluorescence microscopy. Also, the ability to see what is going on at individual cell resolution makes the measurements more accurate and reliable (Vogt et al., 2003; Gasparri et al., 2004). Furthermore, HCA enables the large-scale and simultaneous quantification and correlation of multiple phenotypic responses and physiological reactions (Denner, Schmalowsky, & Prechtl, 2008). For example, single-cell data cellular events such as protein expression can be correlated with the cell cycle or other
key cellular processes, producing correlations that can be difficult or impossible to answer using traditional assays.

Secondly, screening can be performed in more physiologically and biologically relevant systems than in classical biochemical assays. Unlike traditional Western blotting, which requires cell lysis and fractionation, HCA is more efficient and allows for in situ analysis of protein expression and posttranslational modifications with intact cells. Compared to biochemical assays, visual assays performed with HCA probe a chemical’s activity on a cellular phenotype rather than simply testing for binding to a particular isolated protein that may be, in the end, less physiologically relevant (Carpenter, 2007).

Thirdly, as HCA technologies improved over the years, its traditional application in secondary screening has shifted to frontline primary drug screening (Haney et al., 2006; Bickle, 2008). This particularly important in drug screening assays, where liver toxicity is one of the major reasons for drug non-approval and withdrawal in clinical trials. Conventional assays have not been reliable in predicting cytotoxicity because of low sensitivity in detecting potential human hepatotoxicity. However, measurements of mitochondrial damage, oxidative stress, and intracellular glutathione captured by HCA were ranked as the three most important features that contribute to the prediction of hepatotoxicity (Zanella, Lorens, & Link, 2010). With HCA, the researcher is able to evaluate the efficacy of potential therapeutics by monitoring the physiological state of cells through the simultaneous analysis of multiple cellular parameters in the context of an intact biological system.

Lastly, more conditions, whether it be timepoints, drug treatments, or cell lines, can be accommodated within a single experiment through the use of multi-well plates. HCA generates a greater volume of content-rich information in the data sets compared to non-imaging read-outs, allowing a number of questions to be answered simultaneously. Overall, this tool can be harnessed to examine the complex effects of compounds or other reagents in physiologically relevant models, not only against their intended targets, but also against other cellular targets and pathways (Giuliano, Johnston, Gough, & Taylor, 2006).
1.9.1 Classification of cell state using multiparametric, phenotypic analysis

The combination of confocal microscopy and multi-parametric analysis allow us to assess cell stress and death based on automated image analysis. Considering the many measurable intracellular changes that occur before, during, and after apoptosis, combining a variety of measurements from each cell can provide a more accurate reflection of the cell’s state and cell death detection (Galluzzi et al., 2011).

Using fluorescent dyes, we can easily visualize the events that occur during apoptosis including mitochondrial outer membrane permeabilization, loss of the mitochondrial transmembrane potential ($\Delta \Psi_m$), phosphatidylserine externalization, and nuclear condensation. In this experiment, a combination of morphology, texture, and intensity features were used to distinguish cells that were alive, stressed, and dead or apoptotic. Binary cell death analysis often excludes intermediate states, such as a cell that is under stress but has not yet undergone apoptosis. Biochemical assays like immunoblotting are often used to detect the presence of endoplasmic reticulum stress by probing for downstream targets such as CHOP and PERK but are labor intensive and slow. So, by taking advantage of mitochondrial hyperpolarization (discussed in further detail in section 3.1.1) being an event that precedes cell death (Gergely et al., 2002) and is an indirect indicator of endoplasmic reticulum stress (Chan et al., 2006), we can use TMRE staining to accommodate three different cell states based on the cell’s phenotype. Not only is this image-based method more efficient and offers a rich collection of information from each cell, but it also has direct clinical applications; Opperman et al. published a paper in 2016, where they used image-based screening to identify patient specific drug combinations to treat patients with chronic lymphocytic leukemia.

Furthermore, multiparametric analysis and phenotypic classification allows us to use a tool compound screen to identify drugs that can be specifically protected by PPARδ or Bel-2. Especially if other cell lines are tested with different concentrations of each drug, it would be a daunting task to rely on immunoblotting or flow cytometry assays. Multiparametric analysis of drug combinations and their effect on a given cell population allows for a better understanding patient responses and prediction of an optimal therapeutic route for each
patient. Along with machine learning to quantify and speed up the analysis, there is potential to identify therapeutics in a future of personalized and precision medicine.

1.9.2 Random forests classifier

Random Forests, devised by L. Breiman in the early 2000s (Breiman, 2001), is one of the most successful machine learning algorithms. This supervised learning procedure, influenced by the early work of Amit and Geman (1997), Ho (1995), and Dietterich (2000), operates according to the simple but effective “divide and conquer” principle: sample fractions of the data, grow a randomized tree predictor on each small piece, then aggregate these predictors together (Beau & Scorned, 2016).

**Figure 6.** Schematic of the Random Forest algorithm.
Random Forests grows many decision trees from a random subset of the data. In a decision tree, each internal node represents a test on an attribute (e.g. whether a cell is positive for AnnexinV/Alexa488 staining or not), each branch represents the outcome of the test, and the terminal node represents a class label (decision taken after computing all attributes). Decision trees identify the features that are most discerning when it comes to identifying classes. The final class is decided based on the majority voting of the ensemble of trees.

The idea behind Random Forests is to generate multiple decision trees from random subsets of data. In this way, each of those trees offers some group of biased classifiers. Random Forest provides additional randomness to the model during the branching of the tree; instead of searching for the most important feature while splitting a node, it searches for the best feature among a random subset of features. This results in a wide diversity and generally results in a better model.

What has greatly contributed to the popularity of forests is the fact that it can be applied to a wide range of prediction problems and has few parameters to tune. Aside from being simple to use, the method is generally recognized for its accuracy and its ability to deal with a large number of input variables.

1.10 Background on transfection techniques

Transfection of eukaryotic cells is a routine but sometimes tricky procedure. There are several transfection reagents available on the market, but sometimes the old methods are the best. In the process of deciding what transfection method to use, I did preliminary experiments where I tried several methods including calcium phosphate coprecipitation, Mirus reagents, and polyethylenimine (PEI) transfection.

One of the simple, fast and cheap transfection methods for eukaryotic cells is calcium phosphate mediated transfection. Its main advantage is that, since Ca$^{2+}$ is a small ion and part of the culture medium, cell viability is not a problem. The exact mechanism of calcium phosphate mediated transfection is not known, but what we do know is that calcium, being poorly soluble in culture medium, forms microprecipitates in the presence of phosphate ions. These microprecipitates are believed to have a positive effect on transfection
efficiency by creating a complex that binds strongly to the surface of the cell monolayer and enhances uptake of DNA through endocytosis. However, this method does not work for all cell lines and in our case, it was not very effective so other approaches were tested.

Another option was using Mirus transfection reagents. This is available on the market and the one I tested was the TransIT-X2 system, which is a non-liposomal polymeric system that enables high efficiency transfection of many cell types, including primary cells. Unfortunately, this method was observed to be rather toxic to the MCF-7 cells and is expensive to use.

Finally, DNA can be introduced into a host cell by transfection with polyethylenimine (PEI), a stable cationic polymer (Boussif et al., 1995). PEI condenses DNA into positively charged particles that bind to anionic cell surfaces. Consequently, the DNA: PEI complex is endocytosed by the cells and the DNA released into the cytoplasm (Sonawane et al., 2003). In the end, this method was found to be the best due to its minimal effect on cell death, transfection efficiency, and low cost.

2 Materials and Methods

2.1 Cell culture and mycoplasma testing

MCF-7 cells were cultured in DMEM containing 10% FBS and 1% P/S at 37°C with 5% CO₂. Cell cultures were tested routinely for mycoplasma contamination. The protocol published by (Anne Hopert et al., 1993) was used for mycoplasma testing.

2.2 Bcl-2 overexpressing cell lines

Bcl-2hi cell lines were made by infecting MCF-7 cells with a construct containing either empty vector (control; logged in the lab’s database as plasmid #655), Bcl2-wt (wild type Bcl-2; logged in the lab’s database as plasmid #656), Bcl2-acta (mitochondrial-targeting Bcl-2; logged in the lab’s database as plasmid #663), Bcl2-cb5 (endoplasmic reticulum-targeting Bcl-2; logged in the lab’s database as plasmid #664) in the mammalian expression vector pRc/CMV (Invitrogen). Fusion genes of Bcl-2 that targeted the mitochondria or
endoplasmic reticulum were made by replacing the C-terminal of Bcl-2 with either acta or cb5 (Zhu et al., 1996). Stable cell lines were kept under neomycin selection (0.5mg/mL). Western blots were performed to check the overexpression of Bcl-2 in these cell lines.

2.3 Generating PPARδ overexpressing cell lines

Human PPARδ full cDNA was obtained from Addgene (Cambridge, MA, USA) and sub-cloned into the XhoI and NotI sites of lentiviral pLemiR plasmid. mCerulenean3 was from another one of our lab’s plasmid encoding mC3-BaxS184E. PPARδ and mC3 primers were designed and used to amplify PPARδ and mC3 transcripts.

<table>
<thead>
<tr>
<th>Primers for mC3</th>
</tr>
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<tbody>
<tr>
<td><strong>Forward</strong></td>
</tr>
<tr>
<td>AGACTAGTATGTTAGCAAGGGCGAGGAGCT</td>
</tr>
<tr>
<td><strong>Reverse</strong></td>
</tr>
<tr>
<td>AATCTATGGCCGCTTGTACAGCTCGTCCATGCGAGAG</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Primers for PPARδ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward</strong></td>
</tr>
<tr>
<td>ATAGATGGCGCCGCAAATGGAGCAGCCACAGGAGGAAGCC</td>
</tr>
<tr>
<td><strong>Reverse</strong></td>
</tr>
<tr>
<td>GGATCCTCATATGACATGTCCCTGTAGATCTCC</td>
</tr>
</tbody>
</table>

Table 2. Primers used for amplifying PPAR and mC3 transcripts.

Ligation reaction between PPARδ and mC3 fragments was performed to create fusion gene with mC3 at the N terminus of PPARδ. This was then inserted back into the SpeI and BamHI sites of the original pLVX-EF1α-IRES-Puro vector. Fusion construct of PPARδ was made where the N terminus of PPARδ was tagged with a cyan fluorescent protein, mCerulenean3 (mC3) and expressed in the lentiviral expression vector, pLVX-EF1α-IRES-Puro (stored in the lab’s plasmid bank and logged in the lab’s database as plasmid #3881). The sequencing results for mC3-PPARδ is shown in Table 3. HEK293T cells were grown
to 50% confluency. On the day of transfection, HEK293 cells had media replaced with fresh media (DMEM+10% FBS+1% P/S). Two Eppendorf tubes were prepared with the following constitutions: 1) 400μL OptiMEM + 6μL Turbofect and 2) 2μg construct + 1.5μg PAX2 (packaging plasmid) + 0.5μg VSVG (envelope protein). These tubes were brought to the BSL2+ tissue culture room and mixed. This mixture was incubated at room temperature for 20 min before it was added dropwise to the HEK293T cells. Cells were incubated (37°C, 5% CO₂) and the media was replaced after 4 hrs. Cells were incubated for another 48 hrs before the supernatant was collected and filtered with a 0.2μm filter. The day before infection, the cell lines were seeded at 200,000 cells/mL in a 6-well plate. 200μL of the viral supernatant was added per well of the 6-well plate. Following 24 hrs of incubation, the media was removed and replaced with fresh media. Cells were grown for 3 days before proceeding with selection with puromycin media (0.5μg/mL). Following selection with puromycin media, the cells that overexpressed both Bcl-2 and PPARδ were cultured in media containing both puromycin and neomycin at the concentrations mentioned previously. CMV (empty vector control), Bcl2-wt hi (wild type Bcl-2), Bcl2-acta hi (mitochondria-targeting Bcl-2), and Bcl2-cb5 hi (endoplasmic reticulum-targeting Bcl-2) cell lines were infected with this fusion construct of PPARδ. 4 new cell lines generated were CMV/mC3-PPARδ hi, Bcl2-wt hi/mC3-PPARδ hi, Bcl2-acta hi/mC3-PPARδ hi, and Bcl2-cb5 hi/mC3-PPARδ hi.
<table>
<thead>
<tr>
<th>Sequence for mC3-PPARδ</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATGGTGAGCAAGGCAGGAGCTGGTTACCCGGGCTGGTGCCCACCCATTGGTCGAGCT</td>
</tr>
<tr>
<td>GGACCGGCAGCTAAGCCGACCAGCTGCCGTCGAGCCGGGCGAGGCGGAGGCGATG</td>
</tr>
<tr>
<td>CCACTACGGCAAGCTGAACCCTTACGCTCTCTCTGCTGACTGCTGCTGGAGTTCG</td>
</tr>
<tr>
<td>AGTCCGCCATGCCGAAGGCTACGCAGCTGCAAGATTCAGAAGAAGAACCGCAACAAGTGCCAGTACTGCCGCTTCCAGAA</td>
</tr>
<tr>
<td>GTGCTGCAACGCAGGGCAAGGCAAGGAGCCCCAGAGCTCAATGGGGGACCACAGCATGCACTTCCTTCCAGCAGCTACACAGACCTCTCCCGGAGCTCCTCGCCACCCTCACTGCTGGACCAA</td>
</tr>
<tr>
<td>CTGAGAAGAGGAAGCCCGACCTCTCCGAGGTCCGGGAAGAGGGAGAAAGAGGAAGTGGCAGAGGAGAAGGAGCCCCAGAGCTCAATGGGGGACCACAGCATGCACTTCCTTCCAGCAGCTACACAGACCTCTCCCGGAGCTCCTCGCCACCCTCACTGCTGGACCAA</td>
</tr>
<tr>
<td>CGTCTATCTCACGCCAACAGAGAACAGAGAACAGGCATCAAGGCCAACAGGAGGGAAGCCCCTGAGGTCCGGGAAGAGGAGAAAGAGGAAGTGGCAGAGGAGAAGGAGCCCCAGAGCTCAATGGGGGACCACAGCATGCACTTCCTTCCAGCAGCTACACAGACCTCTCCCGGAGCTCCTCGCCACCCTCACTGCTGGACCAA</td>
</tr>
<tr>
<td>ACTGCAACATGAGGCAATGGAGCTGGTGCAGTCCGCCAGGCACTACAGCAAGGGAAGTTGGTGAATGGCCTGCCTCCCTACAAGGAGATCAGCGTGCAAGTCTTCTACCGCTGCCAGTGCACCACAGTGGAGACCGTGCGGGAGCTCACTGAGTGGCGGGGACAAGGCATCGGGCTTCCACTACGGTGTTCATGCATGTGAGGGGTGCAAGGGCTTCTTCCGTCG</td>
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<td>AGTCCGCCATGCCGAAGGCTACGCAGCTGCAAGATTCAGAAGAAGAAGAACCGCAACAAGTGCCAGTACTGCCGCTTCCAGAA</td>
</tr>
<tr>
<td>ATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCTCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGACCCACCACGAGACATTGTGGCAGGCAGAGAAGGGGCTGGTGTGGAAGCAGTTGGTGAATGGCCTGCCTCCCTACAAGGAGATCAGCGTGCAAGTCTTCTACCGCTGCCAGTGCACCACAGTGGAGACCGTGCGGGAGCTCACTGAGTGGCGGGGACAAGGCATCGGGCTTCCACTACGGTGTTCATGCATGTGAGGGGTGCAAGGGCTTCTTCCGTCG</td>
</tr>
<tr>
<td>AGTCCGCCATGCCGAAGGCTACGCAGCTGCAAGATTCAGAAGAAGAAGAACCGCAACAAGTGCCAGTACTGCCGCTTCCAGAA</td>
</tr>
</tbody>
</table>
Table 3. Sequencing data for mC3-PPARδ.
The mC3 sequence is in blue while the PPARδ sequence is in yellow.

2.4 Testing the drug panel with Bcl-2\textsuperscript{hi} cell lines

For this thesis, the superscript “hi” refers to overexpression of a particular protein (e.g. Bcl2-wt\textsuperscript{hi} means the cell line expresses high levels of wild type Bcl-2 protein). Bcl-2\textsuperscript{hi} cell lines (Bcl2-wt\textsuperscript{hi}, Bcl2-acta\textsuperscript{hi}, Bcl2-cb5\textsuperscript{hi}) were treated with the following panel of drugs: Tumor necrosis factor (TNF)-\textalpha + cycloheximide (CHX), thapsigargin (TG), tunicamycin (TN), Actinomycin D (ActD), and tamoxifen (TAM). Media containing 0.2% DMSO was used as a control. The concentrations used were TNF-\textalpha (3nM) + CHX (10\mu g/mL), TG (10nM), TN (30\mu M), ActD (30nM), and TAM (14\mu M). These concentrations are the final concentrations on the cells. The concentrations of the drugs used, with the exception of cycloheximide, were based on a cell death screening assay paper published by the David Andrews’ lab at Sunnybrook (Collins et. al, 2015). The concentration of cycloheximide used here is sufficient to inhibit protein synthesis (Adams & Cooper, 2007).

2.5 Staining, drug dosing, and image collection

The cell lines were seeded in a 384-well plate at a concentration of 2000 cells/40\mu L per well. After 24 hrs of letting the cells settle, 5\mu L of 10x drugs and 5\mu L of 10x ABT-199 working solution were added to each well. The cells were incubated with the drugs for 24
and 48 hrs before staining and imaging. On the day of imaging, cells were stained with 50μL of a 2x staining solution that consists of DMEM media (10% FBS, 1% P/S) mixed with 1:1000 dilutions of AnnexinV/Alexa Fluor 488, TMRE (10μM), and DRAQ5 (5mM). AnnexinV conjugated to the green fluorophore, Alexa 488 was made in the lab by PhD student, Elizabeth Osterlund. Cells were imaged using the 20x water objective in confocal mode on the Opera Phoenix.

2.6 Random Forests classification

Intensity, morphology, and texture features were extracted from cells from all three channels (DRAQ5, AnnexinV, and TMRE). Using the MATLAB script written by Dr. Santosh Hariharan, cells were classified into either one of three classes: Alive (DMSO), Dead/Apoptotic (TNF-α+CHX), or Stressed (TG/TN/TAM, cells with a TMRE intensity 2 standard deviations above DMSO control). Random Forest feature reduction was used to pick out the 20 most important features that were used to separate the 3 classes.

2.7 Testing mC3-PPARδ cell lines with the drug panel

All 8 cell lines (CMV (empty vector control), Bcl2-wt\textsuperscript{hi} (wild type Bcl-2), Bcl2-acta\textsuperscript{hi} (mitochondria-targeting Bcl-2), and Bcl2-cb5\textsuperscript{hi} (endoplasmic reticulum-targeting Bcl-2) with or without mC3-PPARδ overexpression) were seeded into two 384-well plates. The cells were dosed with the drug panel mentioned previously for 24 and 48 hrs before staining and imaging. The results were then analyzed with the multiparametric 3-way classification method described before.

2.8 Sensitizing PPARδ\textsuperscript{hi}/Bcl-2\textsuperscript{hi} cells with Bcl-2 inhibitor and PPARδ antagonist

Cells were treated with the same drug panel but now with the addition of DMSO media (control), a Bcl-2 inhibitor (ABT-199, 1μM), a PPARδ antagonist (NXT1511, 3μM), or both the Bcl-2 inhibitor and the PPARδ antagonist. Drug treatment was left for 48 hrs before staining and imaging. Same 3-way classification was used for analyzing the results.
2.9 ABT-199 combination with drug panel

Cells were treated with the same drug panel with either DMSO media (control) or the Bcl-2 inhibitor, ABT-199 at 1μM. Drug treatment was left for 48 hrs before staining and imaging. Same 3-way classification was used for analyzing the results.

2.10 Transient transfection with mC3-Bcl-2 plasmids

MCF-7 cells were grown until they were 50-70% confluent and actively growing on a 10cm dish. The following protocol is for 36 wells of a 384-well dish. Adjust the calculations accordingly for more or fewer wells. Polyethylemine (PEI) and OptiMEM media were warmed up to room temperature before use. The following plasmids were used: control (mC3 only; logged in the lab’s database as plasmid #3036), mC3-Bcl2-wt (mC3-tagged wild type Bcl-2; logged in the lab’s database as plasmid #3006), mC3-Bcl2-acta (mC3-tagged mitochondrial-targeting Bcl-2; logged in the lab’s database as plasmid #3008), and mC3-Bcl2-cb5 (mC3-tagged endoplasmic reticulum-targeting Bcl-2; logged in the lab’s database as plasmid #3010). These pVITRO plasmids (InvivoGen) have a Blasticidin resistance marker. They can be transfected in mammalian cells and the genes of interest are expressed at high levels. They allow the ubiquitous and constitutive co-expression of two genes of interest.

For each plasmid, make an Eppendorf tube with plasmid (calculate for 100 ng/well) + 8μL PEI + 200μL OptiMEM. Incubate this mixture for 20 min at room temperature. Trypsinize the dish of MCF-7 cells and resuspend them to a concentration of 100,000 cells/mL. 2mL of cell suspension needed for each plasmid of interest. Mix the transfection mixture with the cells. Pipette 60μL/well into the 384-well plate. Incubate overnight and replace media with 40μL (DMEM + 10% FBS + 1%P/S) the next day. Following the media change, the cells were dosed with the following drugs: TNF-α+CHX, ActD, TN, TG, and TAM. These cells were then dosed with 10x drugs (same drug panel as described previously) with or without ABT-199 (1μM), incubated for 24 hrs, stained, and imaged. During image analysis, cells that exhibited fluorescence intensity in the cyan fluorescence protein channel above the background level were used so that only cells that were positive for mC3-Bcl-2 were
used in the analysis. A two-way classifier was used to classify cells as dead or alive based on nuclear condensation, TMRE signal, and AnnexinV staining.

2.11 Immunofluorescence experiment with N & E-cadherin

All 8 cell lines were seeded into a 384-well plate. Aspirate media and wash once with PBS. Aspirate PBS and then add 4% paraformaldehyde and incubate for 15 min at room temperature. Aspirate and rinse with PBS. Add 50 mM ammonium chloride/PBS for 10 min. Add 0.5% Triton 100 in PBS for 5 min. Aspirate and rinse with PBS. Add 3% BSA in PBST (PBST = PBS + 0.1% Tween 20). Aspirate and add primary antibody in 0.3% BSA/PBST for 1 hr at room temperature. N-cadherin (Cell Signalling, D4R1H) and E-cadherin (BD Transduction, #610182) antibodies were used at 1:200 and 1:50 dilution, respectively. Aspirate and wash 3 times with PBS. Add secondary antibody in 0.3% BSA/PBST for 30 min at room temperature. Secondary antibodies were used at 1:1000 dilutions; α-rabbit conjugated with Alexa 488 was used for N-cadherin and α-mouse conjugated with Alexa 568 was used for E-cadherin. Aspirate and wash 3 times with PBS. Cells were stained with DRAQ5 for imaging. Wells with secondary antibody alone were included as controls.

2.12 Statistics used

Results are reported as means ± standard error of the mean from n=3, where n is the number of biological replicates. P values were determined by analysis of variance (ANOVA) for multiple comparisons. The ANOVA test was used to determine whether there were any statistically significant differences between the means of three or more independent groups. Values of p < 0.05 was considered statistically significant. Asterisks are used to represent statistically significant results as follows: * for p<0.05, ** for p<0.01, *** for p<0.005, and **** for p<0.0001. GraphPad Prism software version 8.0 was used to plot the graphs and to calculate statistics.

Tukey’s test was used as a post-hoc analysis in section 3.4 to determine if there was synergistic or additive protection among the cell lines against different drug treatments.
This test compares all possible pairs of means to figure out which groups in the whole dataset differ. It uses the “Honest Significant Difference,” a number that represents the distance between groups, to compare every mean with every other mean (Tukey & Cleveland, 1984).

Bonferroni correction was used for the ABT-199 results in section 3.6. This is a multiple-comparison post-hoc analysis that uses t-tests to perform pairwise comparisons between group means. The overall error rate is controlled by setting the error rate for each test to the experiment-wise error rate divided by the total number of tests. In this way, the observed significance level is adjusted for the fact that multiple comparisons are being made.

3 Results and Data Interpretation

3.1 Confirming Bcl-2’s protection through different pathways: endoplasmic reticulum and mitochondria

The overarching goal of this project is to compare Bcl-2 and PPARδ mediated protection from the induction of cell death in MCF-7 cells. As mentioned before, it has been published that Bcl-2 prevents apoptosis by different programmed cell death pathways depending on its localization in the cell. These spatially distinct pathways are defined by the order of two events: loss of the $\Delta\psi_m$ and release of cytochrome c. When Bcl-2 is overexpressed at the mitochondria, it protects the cell against cytotoxic agents that induce mitochondrial-dependent apoptosis. In these cases, the release of cytochrome c often precedes the loss of the $\Delta\psi_m$. On the other hand, overexpression of Bcl-2 at the endoplasmic reticulum can protect the cells against drugs that induce endoplasmic reticulum stress. These drugs typically cause the loss of the $\Delta\psi_m$ before cytochrome c release is observed. Our lab has the tools to study these two distinct pathways of protection through MCF-7 cell lines that overexpress the following Bcl-2 proteins: Bcl2-wt (wild type, targets to both mitochondria and endoplasmic reticulum), Bcl2-acta (targets to mitochondria), and Bcl2-cb5 (targets to endoplasmic reticulum).
However, before I compared Bcl-2 and PPARδ mediated protection, I confirmed that the Bcl-2 mutants are functioning in MCF-7 cells as expected. The first experiment was done with the four MCF-7 cell lines: CMV (empty vector control), Bcl2-wt\(^{hi}\) (wild type Bcl-2), Bcl2-acta\(^{hi}\) (mitochondria-targeting Bcl-2), and Bcl2-cb5\(^{hi}\) (endoplasmic reticulum-targeting Bcl-2). The panel of drugs used included the following drugs, whose mechanisms of actions were discussed in section 1.8.1.

### 3.1.1 Mitochondrial hyperpolarization observed with endoplasmic reticulum stress drugs

Mitochondrial hyperpolarization (↑Δψ\(_m\)) is tightly connected to ROS production and release (Ivanova et al., 2017); early stages of this state have been shown to coincide with peak generation of ROS (Chan et al., 2006). With mitochondrial hyperpolarization and extrusions of H\(^+\) ions from the mitochondrial matrix, the cytochromes within the electron transport chain become more reduced, which favors the generation of ROS (Nagy, Knock, & Perl, 2003). ROS are key inducers of mitochondrial permeability transition pore opening, which triggers the release of apoptogenic factors and caspase activation (Crompton & Costi, 1988).

Mitochondrial hyperpolarization has also been attributed to Ca\(^{2+}\) buffering during endoplasmic reticulum stress, where rapid depletion of endoplasmic reticulum Ca\(^{2+}\) levels can lead to surges in cytoplasmic Ca\(^{2+}\) levels that can trigger apoptosis; hyperpolarized mitochondria provide an increased driving force for mitochondrial Ca\(^{2+}\) uptake (Morciano
et al., 2016), which serves to prevent cytosolic overload upon release from the endoplasmic reticulum. Although reversible, sustained mitochondrial hyperpolarization and excessive mitochondrial Ca$^{2+}$ can trigger the loss of mitochondrial integrity and cell death (Hajnoczky et al., 2000; Deniaud et al., 2008; Filippin et al., 2003).

**Figure 7.** Images of mitochondrial hyperpolarization in MCF-7 cells.
MCF-7 cells were treated with either DMSO, TNF-α + CHX, or TG. These are grayscale images from the TMRE channel. DMSO image serves as a reference image for normal TMRE staining of active mitochondria. TNF-α + CHX causes a loss of the mitochondrial membrane potential, hence there is no TMRE signal in that image. TG induces mitochondrial hyperpolarization ($\uparrow \Delta \psi_m$), seen by the brighter TMRE signal compared to DMSO.

Given its relation to endoplasmic reticulum stress and observable phenotype, I can take advantage of this and use $\uparrow \Delta \psi_m$ as an indirect indicator of endoplasmic reticulum stress. Traditional methods of probing endoplasmic reticulum stress involve Western blotting for downstream effectors of the UPR like CHOP and PERK. Not only is this time consuming and laborious, but it makes it difficult to screen large libraries of drugs. By using phenotypic analysis through fluorescent microscopy, I can easily test different concentrations of various drugs as well as cell lines in a timely manner.

3.1.2 Stains used for imaging

The 3 stains used for imaging included Alexa 488 (conjugated to AnnexinV), deep red anthraquinone 5 (DRAQ5), and tetramethylrhodamine ethyl ester (TMRE). AnnexinV binds phosphatidylserine, a phospholipid which flips from the cytosolic side of the cell to the outside during apoptosis. Phosphatidylserine is typically restricted to the cytosolic side of the cell via Flippase, a transmembrane transporter protein. Upon apoptosis, the enzyme scramblase catalyzes the exchange of phosphatidylserine from the cytosolic side to the external side, where its presence serves as a signal for macrophages to engulf the dying cell. AnnexinV is not inherently fluorescent; to use this as a staining for apoptotic cells, it
was conjugated to a green fluorophore, Alexa 488. This signal is sensitive to timing and is not always observed when cells are imaged at one timepoint.

Fortunately, dead cells are also captured by measuring chromatin condensation using DRAQ5. DRAQ5 intercalates with DNA and serves as a nuclear stain. Its fluorescence increases in cells undergoing cell death as chromatin condensation occurs. The morphology and local intensity changes in DRAQ5 can be used to accurately identify cells that have condensed nuclei, another measure of cell death. However, chromatin condensation is not sufficient to conclude that cell death was due to apoptosis.

Finally, TMRE is a mitochondrial dye that is dependent on the membrane potential of the mitochondrial inner membrane. Loss of this staining can result from disruption of the mitochondrial membrane that generally leads to cell death via apoptotic factors released from the mitochondria that leads to subsequent caspase activation. The TMRE distribution was measured for the control cells and 2 standard deviations above the mean of this distribution was used as a threshold to identify cells that had mitochondria with $\Delta \psi_m$ (cells with intense TMRE staining in the images). Mitochondrial hyperpolarization was used as an indirect measure of endoplasmic reticulum stress as drugs like TG, TN, and TAM elicited this phenotype consistently.

**Figure 8.** Three states of the cells defined by the stains used for imaging. Cells were stained with a combination of DRAQ5 (nuclear stain), TMRE (mitochondrial stain that is dependent on the membrane potential), and AnnexinV conjugated with Alexa Fluor 488 (apoptotic marker). Above are three images of MCF-7 cells stained with these 3
dyes. The image on the left has healthy cells with round, intact nuclei and an active mitochondrial membrane potential, as indicated by the TMRE dye. In the middle image, the TMRE staining is particularly intense; this reflects a state known as mitochondrial hyperpolarization, which is hypothesized to result from mitochondrial Ca2+ buffering in times of endoplasmic reticulum stress. This phenotype serves as an indirect measure of endoplasmic reticulum stress for our analysis. Finally, the image on the right shows three apoptotic cells, each characterized by the ring of AnnexinV on the cell membrane.

3.1.3 Training controls for the experiment

Out of the drug panel, controls were identified for training classifiers used for subsequent analyses. Control cells treated with TNF-α + CHX were used to train the dead/apoptotic class. DMSO treated wells for all the cell lines were used to train the alive class. Cells treated with either TG/TN/TAM that exhibited TMRE intensity 2 standard deviations above DMSO treated cells were used to train the stressed class with mitochondrial hyperpolarization being used as an indirect indicator of stress.

![Figure 9](image_url)

**Figure 9.** Images of the control group of MCF-7 cells treated with the drug panel. Purple represents DRAQ5 staining, red represents TMRE staining, and green represents AnnexinV staining. Images from specific treatments were used to train a 3-way classifier that sorts cells into 3 classes: apoptotic/dead, ↑Δψm, and alive. On the left are the control cells (DMSO) were used to train the alive class. CMV cells treated with TNF-alpha + CHX
were used to train the dead/apoptotic class. Cells in these images usually had one of the following phenotypes: i) AnnexinV positive ii) Condensed nuclei iii) TMRE-negative. Finally, of the cells treated with TG, those that had TMRE intensity 2 standard deviations above DMSO treated cells were used to train the $\Delta \psi_m$ class.

A combination of morphology, intensity, and texture features were extracted from confocal cell images using the software, Columbus (PerkinElmer, version 2.3.0), which allows for cell segmentation and feature extraction. The features from each of the training groups made up a feature profile for their respective class. All the other images collected from the experiment had each cell sorted into one of the 3 classes based on how closely its features matched one of the 3 reference feature profiles. Dr. Santosh Hariharan, a previous PhD in Dr. David Andrews’ lab wrote a MATLAB script for the analysis used in this project. This classification was done through Random Forests, a machine learning algorithm. Random forest feature reduction was also used to pick out the features that were most important in separating the cells into these 3 classes.

3.1.4 Workflow of experiments

Figure 10 summarizes the workflow of the imaging experiments.
**Figure 10.** Workflow of drug panel experiment.

1) Cell lines were seeded in a 384-well plate and settled overnight in the incubator at 37°C, 5% CO₂

2) Cells were incubated with different concentrations of the drugs for 24 or 48 hrs

3) The cells were stained with a combination of dyes (DRAQ5, TMRE, AnnexinV/Alexa488). The plate was incubated for 30 min 37°C, 5% CO₂ before it was imaged using an automated confocal microscope (Opera Phoenix).

4) The images collected were used for multiparametric analysis, which can be broken down into 3 steps:

   i) Cell segmentation (Different regions of interest were identified with the stains: DRAQ5 images were used to identify the cell nuclei and cytoplasmic masks. TMRE images were used to identify the mitochondria.)

   ii) Feature extraction (For every cell in the image, a combination of morphology, texture, and intensity features were extracted from nuclear, cytoplasmic, and cell membrane regions)

   iii) For each well, which represents a certain drug treatment, the cells in the well were subjected to a 3-way classification. The results of the 3-way classification are summarized by the pie chart; cells that look alive are shown by the blue section, cells that are stressed are shown by the red section, and cells that are dead/apoptotic are shown by the green section.

3.1.5 Optimizing concentrations and time points for imaging experiments

For the initial experiments with a drug titration, the range in the concentrations of the drugs used were based on a cell death screening assay paper published by Dr. David Andrews’ lab at Sunnybrook (Collins et al., 2015). In subsequent experiments, only one concentration of each drug was used. The chosen dose for the endoplasmic reticulum stress drugs was one where hyperpolarization was observed at the endpoint whereas the dose for the apoptotic drugs was one where the control (CMV) cells were killed but the Bcl-2ʰⁱ cells showed protection.
In the preliminary experiments, drug titrations for each drug were performed and the cells were imaged at both the 24 and 48 hr time points. Once the experimental protocol was optimized and the Bcl-2 mutants were protecting as expected, subsequent experiments used one concentration for each drug with the treatment lasting for 48 hrs. This was a time point where protection was still observed through the Bcl-2hi cells compared to the controls.

**Figure 11.** Titration of the drug to find the optimal dose and time point.

The pie charts above show the 3-way classification results for control cells that were treated with different doses of ActD for either 24 or 48 hours. As the dose was increased, there are more ↑Δψ\text{m} and dead cells, seen by the large proportion of red and green in the pie charts. Cells at 48 hours show more death for the higher concentrations of the drug.

### 3.1.6 Results of the 3-way classification

I expected Bcl2-acta\text{hi} cells to protect against drugs that induce mitochondrial-dependent apoptosis, Bcl2-cb5\text{hi} cells to protect against drugs that induce endoplasmic reticulum stress-dependent apoptosis, and finally Bcl2-wt cells to protect against all drugs, though to a lesser extent than the ones with Bcl-2 localized to one compartment. Western blots were performed to assess the levels of Bcl-2 in these cell lines; the cells that overexpressed Bcl-2 had comparable levels of protein. Three independent replicates were done of this experiment and the results were as expected. Figure 12 summarizes the results for one replicate of this experiment at the 48 hr imaging time point.
Figure 12. 3-way classification results for the experiment testing Bcl-2 protection.

These results are from the 48 hr time point. Each row represents a different drug treatment and each column represents the different cell lines: CMV (empty vector control), Bcl2-wt\(^{\text{hi}}\) (wild type Bcl-2; “WT”), Bcl2-acta\(^{\text{hi}}\) (mitochondria-targeting Bcl-2; “Mito”), and Bcl2-cb5\(^{\text{hi}}\) (endoplasmic reticulum-targeting Bcl-2; “ER”). The drug panel included drugs that can be separated into 2 classes: 1) drugs that induce mitochondrial-dependent cell death (TNF-\(\alpha\) (3ng/mL) + CHX (10\(\mu\)g/mL), STS (30nM), ActD (30nM)), which are outlined in the green box and 2) drugs that induce endoplasmic reticulum stress-dependent cell death (TG (10nM), TN (30\(\mu\)M)), which are outlined in the red box. The control was media containing a small percentage of DMSO (0.2%), the solvent used to dissolve the drugs. The three colours in the pie charts represent the proportion of cells that were classified as alive.
(blue), $\uparrow \Delta \psi_m$ (red), and dead/apoptotic (green). The number above each pie graph represents the number of cells that were classified in total.

The different colours on the pie graph represent the results of the 3-way classification: blue (alive), red ($\uparrow \Delta \psi_m$), and green (apoptotic/dead). The first row shows cells in the media control; the classification results show a largely blue pie chart, meaning that the majority of the cells are alive. This is expected in the absence of any drug treatment. In the following three rows, cells were treated with the drugs that induce mitochondrial-dependent apoptosis (TNF-$\alpha$+CHX, STS, and ActD). Here, pie charts are largely green for the control cells, indicating that the cells have died from this treatment. However, cells that have overexpression of Bcl-2 at the mitochondria, Bcl2-acta$^{hi}$ and Bcl2-wt$^{hi}$ cells, are protected from these types of drugs, as shown by the larger proportion of red and blue in their pie charts. Bcl2-cb5$^{hi}$ cells, which overexpress Bcl-2 in the endoplasmic reticulum, are not protected from TNF-$\alpha$+CHX, confirming that this treatment induced mitochondrial-dependent cell death. However, Bcl2-cb5$^{hi}$ cells showed slight protection against ActD and STS compared to control cells. This suggests ActD and STS also elicit mitochondrial-independent cell death pathways.

Finally, the last two rows represent cells treated with the endoplasmic reticulum stress drugs. Bcl2-cb5$^{hi}$ and Bcl2-wt$^{hi}$ cells showed protection against TN and TG, as expected. In addition, while Bcl2-acta$^{hi}$ have Bcl-2 at the mitochondria mainly, it was still very effective against the drugs that elicited endoplasmic reticulum stress. The mitochondria are a point of convergence in the apoptosis pathways induced by various drugs (Bola & Letai, 2016), and should therefore protect cells against agents that induce cell death upstream of or through the mitochondrial pathway. However, my data suggests that Bcl-2, regardless of its localization, may mediate resistance to apoptosis against endoplasmic reticulum stress-inducing drugs independently of its BH3 domain, which is required to sequester pro-apoptotic BH3-only Bcl-2 family members that induce cell death through mitochondrial pathways.
In summary, Bcl2-acta\textsuperscript{hi} and Bcl2-wt\textsuperscript{hi} cells showed protection against all drugs in the panel whereas Bcl2-cb5\textsuperscript{hi} cells’ protection was more restricted to the endoplasmic reticulum stress drugs. Overall, I confirmed that the Bcl-2 mutants are working as expected.

3.2 Generating PPAR\textsuperscript{\delta\textsubscript{hi}} cell lines to model patients with high Bcl-2 and/or PPAR\textsuperscript{\delta} levels

Having confirmed the functional properties of the mutant versions of Bcl-2 in MCF-7 cells, the next step was to generate cell lines that overexpressed PPAR\textsuperscript{\delta}. A fusion protein was made in which the cerulean fluorescent protein, mCerulean3 (mC3), was expressed in frame as an N-terminal fusion to PPAR\textsuperscript{\delta}. The fusion construct was expressed with the HIV-1-based lentiviral expression vector, pLVX-EF1\textalpha-IRES-Puro. This vector was chosen because it has the EF1\textalpha promoter, which would not compete with the CMV promoter currently present in the Bcl-2\textsuperscript{\textsubscript{hi}} cell lines.

The four cell lines I had before were CMV (empty vector control), Bcl2-wt\textsuperscript{hi} (wild type Bcl-2), Bcl2-acta\textsuperscript{hi} (mitochondria-targeting Bcl-2), and Bcl2-cb5\textsuperscript{hi} (endoplasmic reticulum-targeting Bcl-2). The four new cell lines created through lentiviral infection are mC3-
PPARδ\textsuperscript{hi}, mC3-PPARδ\textsuperscript{hi}/Bcl2-wt\textsuperscript{hi}, mC3-PPARδ\textsuperscript{hi}/Bcl2-acta\textsuperscript{hi}, and mC3-PPARδ\textsuperscript{hi}/Bcl2-cb5\textsuperscript{hi}.

\textbf{Figure 13.} Plasmid map of the lentiviral expression vector, pLVX-EF1\textalpha-IRES-Puro. 5’ LTR is in magenta, EF1\textalpha promoter in orange, mC3 in cyan, PPARδ in yellow, IRES in dark blue, Puromycin marker in green, and 3’ LTR in dark purple.

The infected cells were sorted twice using flow cytometry. The four original cell lines: CMV (empty vector control), Bcl2-wt\textsuperscript{hi} (wild type Bcl-2), Bcl2-acta\textsuperscript{hi} (mitochondria-targeting Bcl-2), and Bcl2-cb5\textsuperscript{hi} (endoplasmic reticulum-targeting Bcl-2) are Neomycin resistant while the new cell lines that express mC3-PPARδ are both Neomycin and Puromycin resistant. mCerulean3 was chosen because of its bright, photostable nature as well as its performance as a fusion protein in living cells. The fusion protein allows us to correlate PPARδ expression with the endoplasmic reticulum stress protection in future imaging experiments. Figure 14 shows images of unstained mC3-PPARδ\textsuperscript{hi} cells through

![Nuclear localization](image)
the cyan fluorescent protein channel on the confocal microscope. These cells show correct localization, given that PPARδ is a nuclear hormone receptor.

**Figure 14.** Image of unstained MCF-7 cells that overexpress the fusion construct of mCerulean3, tagged to the N terminus of PPARδ. This image was captured by modifying the GFP channel settings (433nm excitation/475nm emission) to detect mC3 fluorescence on the Opera Phenix confocal microscope (PerkinElmer) using the 60x water objective. The fusion protein of PPARδ displays nuclear localization.

3.2.1 Verifying overexpression of PPARδ through fluorescent microscopy and Western blots

When all 8 cell lines were probed for PPARδ, I noticed that the cells which overexpressed mC3-PPARδ and mitochondria-targeting Bcl-2 showed the greatest amounts of the fusion protein. This is seen in the Western blots as well as the images from fluorescence microscopy. Figure 15 shows the distributions of CFP intensity for the 8 cell lines.

**Figure 15.** The images of the 8 cell lines using the modified GFP channel settings (433nm excitation/475nm emission) to detect mC3.
The top row includes the cells that do not overexpress mC3-PPARδ. The bottom row includes the cell lines that overexpress mC3-PPARδ. Cells outlined in a yellow border are negative for mC3 and cells outlined in a green border are positive for mC3. MCF-7 cells that overexpressed both mC3-PPARδ and Bcl-2 at the mitochondria showed the highest intensity. These are unstained images taken with the 20x water objective.

![Graph showing the distribution of CFP intensity for the 8 cell lines.](image)

**Figure 16.** Histograms showing the distribution of CFP intensity for the 8 cell lines.
The x-axis shows the mC3 intensity and the y-axis shows the number of cells with that particular mC3 intensity. The vertical red dashed line represents the threshold for CFP intensity for cells that are considered mC3 positive. The dashed lines represent the cell lines overexpressing mC3-PPARδ.

**Figure 17.** Western blots for the cell lines to detect the relative levels of Bcl-2 and PPARδ.

First image from top to bottom:

i) Blot for Bcl-2 levels: Slight differences in size are observed for the different Bcl-2 mutants. This is expected because of the additional amino acids added for the mitochondrial (amino acids 613-638 of *Listeria* ActA) and endoplasmic reticulum-targeting (amino acids 100-134 from cytochrome b5 hydrophobic tail) mutants, resulting in a molecular weight. PPARδ<sup>hi</sup> cell lines expressed similar Bcl-2 levels compared to their counterparts.

ii) A GFP antibody was used to detect overexpression of mC3-PPARδ. Cell lines with both PPARδ and Bcl-2 acta (Mito) overexpression had the highest levels of PPARδ.
iii) GAPDH was used as the loading control.

Second image:
i) Blot for PPARδ with antibody that detects the C-terminal region of PPARδ. Both mC3-PPARδ (77kDa) and endogenous PPARδ (50kDa) can be detected in the PPARδhi cell lines
ii) GAPDH was used as the loading control.

3.3 Immunofluorescence experiment with E & N-cadherin to assess cell invasiveness

As mentioned before in section 1.4, a switch from E to N cadherin is observed in aggressive tumors. PPARδ upregulation in breast cancer cells is associated with more aggressive clinical behaviour. An immunofluorescence experiment was performed where the 8 cell lines were probed with antibodies for N and E-cadherin to test for any changes in invasive properties in our cell lines.

3.3.1 Results for immunofluorescence experiment with the 8 cell lines

A decrease in E-cadherin expression was noted in PPARδhi cells, supporting what has been reported in the literature where PPARδ overexpression leads to a more invasive and metastatic phenotype. In the cell lines that did not overexpress PPARδ, there was a wider range in E-cadherin expression. Among the 8 cell lines, N-cadherin expression did not change much.

In addition, the Bcl-2hi cells did not exhibit any significant differences in E or N-cadherin expression compared to control cells and is in accordance with the literature. After all, MCF-7 cells are generally not considered to be an aggressive cell line and Bcl-2 expression is associated with better outcomes of metastatic and early breast cancer treated with either hormone therapy or chemotherapy (Tsutsui et al., 2006; Gasparini et al., 1995). Moreover, this observation is supported by the results of previous studies that showed that Bcl-2 is inversely correlated with proliferative markers, such as Ki-67, and HER2-overexpression, and hence plays an anti-proliferative role despite its anti-apoptotic effect (Knowlton et al., 1998; Mitrović et al., 2014).
Figure 18. Graphs showing the correlation of E and N-cadherin with respect to CFP mean. CFP mean is used to measure mC3-PPARδ expression. Cells with a CFP greater than 300 are deemed as mC3-PPARδ+. This threshold is noted by the red dotted line. The cells that are mC3-PPARδ+ have a diminished E-cadherin expression whereas those cells that do not overexpress PPARδ have a wider range in E-cadherin expression. On the other hand, N-cadherin expression is not much different across all 8 cell lines.
Figure 19. Bar graph of the E/N-cadherin intensity ratio for the 8 cell lines. The mC3-PPARδ cells have an overall lower ratio due to a lower expression of E-cadherin.
To see whether the overexpression of PPARδ changes the cell’s invasiveness, the 8 cell lines were probed with antibodies for N (green) and E (yellow)-cadherin. A decrease in E-cadherin expression was noted in PPARδ hi cells, suggesting that PPARδ overexpression leads to a more invasive and metastatic phenotype. Cells were stained with DRAQ5 (purple) to visualize the nuclei. Images were taken using the 20x water objective on the confocal microscope.

3.4 Comparing PPARδ hi and Bcl-2 hi cells

Having confirmed that the Bcl-2 hi cell lines were working, I proceeded to test the newly made mC3-PPARδ hi cell lines against the drug panel. The goal was to see if PPARδ and Bcl-2 protect through different pathways. If they did, additive protection should be observed in cell lines that overexpress both proteins compared to the cell lines that only overexpress one of the proteins. As mentioned in section 1.8.2, tamoxifen was added to the drug panel as our query drug, as it was not known whether it induced apoptosis through
more mitochondrial or endoplasmic reticulum stress-dependent pathways but is frequently used to treat estrogen receptor positive breast cancer. As MCF-7 cells express estrogen receptors and die in the absence of estrogen, the cell lines established here should be a good model for understanding cell death in response to tamoxifen.

3.4.1 Media controls for the experiment

This experiment was repeated 3 times. Figure 21 shows the 3-way classification results for the controls for these experiments. Each dot represents the average result for the 3 experiments. The open black circles represent the cell lines that do not overexpress mC3-PPARδ while the solid black circles represent the cell lines that overexpress mC3-PPARδ. There are three separate graphs in the figure, plotting either the percentage of dead, alive, or stressed (↑Δψₘ phenotype) cells based on the 3-way classification.

![Graphs showing the 3-way classification results for the media controls in the experiment.](image)

**Figure 21.** Graphs showing the 3-way classification results for the media controls in the experiment.
Top: % of alive cells. Bottom left: % of dead/apoptotic cells. Bottom right: % of stressed cells. Cells are around 80% alive at the time of imaging with 20% of the cells stressed or dead. The open black circles represent mC3-PPARδ− cells while the solid black circles represent mC3-PPARδ+ cells.

3.4.2 Tamoxifen elicits endoplasmic reticulum stress that is protected through overexpression of either Bcl-2 or PPARδ

In the early titration experiments with tamoxifen for MCF-7 cells, cell death was observed at concentrations higher than 30µM after 24 hours of treatment. Moreover, the dose response curve resembles a step function; there was an abrupt shift from cells being stressed to dead once the concentration was above 30µM. The dose chosen for the drug panel was 14µM, which elicits mitochondrial hyperpolarization similar to TG and TN. This is a clinically relevant dose because patients are often given 20 mg of tamoxifen per day and the average concentration of 4-hydroxytamoxifen in serum is observed to be approximately 8µM (Kisanga et al., 2004). So, even at a higher than average concentration of tamoxifen, cells that overexpressed Bcl-2 or PPARδ were protected against endoplasmic reticulum stress elicited by this drug, as seen in Figures 22 and 23. In tissues, the tamoxifen concentration is around 10 times higher than serum levels, and will ultimately result in apoptosis (Kisanga et al., 2004). As mentioned before in section 1.3.1, tamoxifen is metabolized by the liver into more active forms; the tamoxifen used in these experiments is 4-hydroxytamoxifen, which is the more potent form that has a higher binding affinity to the estrogen receptor.
The following 8 cell lines were tested: CMV (empty vector control), Bcl2-wt\textsuperscript{hi} (wild type Bcl-2), mC3-PPAR\textdelta\textsuperscript{hi}, Bcl2-wt\textsuperscript{hi}/mC3-PPAR\textdelta\textsuperscript{hi}, Bcl2-acta\textsuperscript{hi} (mitochondria-targeting Bcl-2), Bcl2-acta\textsuperscript{hi}/mC3-PPAR\textdelta\textsuperscript{hi}, Bcl2-cb5\textsuperscript{hi} (endoplasmic reticulum-targeting Bcl-2), and Bcl2-cb5\textsuperscript{hi}/mC3-PPAR\textdelta\textsuperscript{hi}. Cells were treated with the panel of drugs for 48hrs before being imaged. Based on the 3-way classification results, TAM elicited more of a $\Delta\psi_m$ phenotype that was rescued by either Bcl-2 or PPAR\textdelta overexpression following 24 hrs of drug treatment. In Figure 22, the control cells are mostly stressed—shown by the large proportion of the pie graph that is red. With Bcl-2 overexpression, a lot more cells are alive, shown by the large proportion of blue. Finally, with mC3-PPAR\textdelta overexpression, there are more cells that are alive compared to control cells but there are also more dead cells compared to Bcl2-wt cells.

**Figure 22.** 3-way classification results for cells treated with tamoxifen (14\textmu M) for 24hrs using the three cell lines: control, Bcl2-wt\textsuperscript{hi}, and mC3-PPAR\textdelta\textsuperscript{hi}. Control cells show a large proportion of cells that are classified as stressed (red). Bcl2-wt\textsuperscript{hi} and mC3-PPAR\textdelta\textsuperscript{hi} cells on the other hand, have fewer cells that are stressed.

Figure 23 shows graphs of the percentage of stressed cells based on the 3-way classification for TAM and TG treatment for all the cell lines. The results shown here are for the 24 hr timepoint, where the Bcl-2\textsuperscript{hi} cells still had fewer cells with a $\Delta\psi_m$ phenotype compared to control.
Figure 23. Percentage of stressed cells through TAM and TG treatment.

TAM and TG treatments cause endoplasmic reticulum stress that is protected via PPAR or Bcl-2. The open black circles represent mC3-PPARδ- cells while the solid black circles represent mC3-PPARδ+ cells. The two treatments shown here are TAM (14µM) and TG (10nM) at the 24 hr timepoint.

In general, cells overexpressing PPARδ had fewer hyperpolarized cells compared to their respective counterparts and the cells that overexpressed both PPARδ and Bcl2-acta/Bcl2-cb5 had the least number of hyperpolarized cells following TG or TAM treatment, supporting mitochondrial hyperpolarization as a useful indirect measure of endoplasmic reticulum stress.

By the 48 hrs of drug treatment however, the cell lines only overexpressing Bcl-2 showed similar levels of stressed cells compared to control, although they still had fewer dead cells than control. However, the cells that overexpressed PPARδ maintained a low percentage of stressed cells even at 48hrs, seen in the next figure.
Figure 24. Percentage of stressed cells via TAM and TG treatments at 48hrs. The open black circles represent mC3-PPARδ⁻ cells while the solid black circles represent mC3-PPARδ⁺ cells. The two treatments shown here are TAM (14µM) and TG (10nM) at the 48 hr timepoint.

3.4.3 Missing differences in protection with 2-way classification

The reason why 3-way classification was used in the analysis is because intermediate stages are missed with just classifying cells as dead or alive. In a 2-way classification, cells that are stressed but not dead would look more similar to cells that are alive; given two options, the computer would classify the stressed cells into the alive group, resulting in an overestimation of cells that are unaffected by the drug treatment. In Figure 25, TG treatment is used as an example and three graphs show either the percentage of cells that are dead, stressed, or alive. Looking the graph plotting the percentage of dead cells due to TG treatment, there does not appear to be any protection through the overexpression of PPARδ. In contrast, if the percentage of cells that are stressed with TG treatment is considered, it should be noted that in every case where PPARδ is overexpressed, there is a lower proportion of cells that are stressed.
Figure 25. Capturing differences in protection through 3-way classification that would otherwise be missed in 2-way classification.

The 8 cell lines were treated with TG (10nM) for 24 hrs. The graphs above show the percentage of dead, stressed, and alive cells. The open black circles represent mC3-PPARδ⁻ cells while the solid black circles represent mC3-PPARδ⁺ cells. There seems to be no protection through mC3-PPARδᵢBi cells by just looking at the graph plotting the percentage of dead cells. However, if the percentage of stressed and alive cells is considered, the mC3-PPARδᵢBi cells are protected from TG treatment.

3.4.4 PPARδ protects cells against endoplasmic reticulum stress drugs

PPARδ overexpressing cells protected similarly to cells overexpressing Bcl-2 at the endoplasmic reticulum, represented by cells overexpressing the fusion protein, Bcl2-cb5. mC3-PPARδᵢBi cells were protected against TG and TN compared to control cells, supporting what was published in the literature (Salvadó et al., 2014; Wang et al., 2016; Cheang et al., 2017). Figure 26 shows the results for TG and TN at 48hrs, shown again by the three separate graphs.
Figure 26. 3-way classification results for the 8 cell lines treated with TG (10nM) for 48 hrs.

The graphs above show the percentage of dead, stressed, and alive cells. The open black circles represent mC3-PPARδ- cells while the solid black circles represent mC3-PPARδ+ cells.

Looking at the graph plotting the percentage of dead cells, the Bcl-2hi cells are protected compared to the control cells, seeing as they have a lower percentage of dead cells. However, comparing the mC3-PPARδhi cells to those that do not overexpress mC3-PPARδhi, no significant differences can be seen. As mentioned in the previous section, the protective effects of overexpressing PPARδ become more evident when the percentage of stressed cells is plotted.
Figure 27. 3-way classification results for the 8 cell lines treated with TN (30µM) for 48 hrs.

The graphs above show the percentage of dead, stressed, and alive cells. The open black circles represent mC3-PPARδ− cells while the solid black circles represent mC3-PPARδ+ cells.

Here, I have shown that PPARδ can protect cells from endoplasmic reticulum stress agents like TG and TN. However, as mentioned in the previous section, TAM also elicits mainly endoplasmic reticulum stress that is protected through either Bcl-2 or PPARδ overexpression. The significance of these results is that patients with cancer cells overexpressing either one of these proteins would be difficult to treat because their cancer cells are able to evade or remain in a state of endoplasmic reticulum stress that prevents them from dying.

The results for TAM treatment for 48 hrs are shown in Figure 28. Looking at the percentage of dead cells, control cells have more dead cells compared to the Bcl-2 hi cells. Comparing among the controls, cells that overexpress PPARδ show 10-20 % more dead cells.
However, they still have a much lower percentage of cells that are stressed compared to control, as well as more cells that are alive.

**Figure 28.** 3-way classification results for the 8 cell lines treated with TAM (14µM) for 48 hrs.

The graphs above show the percentage of dead, stressed, and alive cells. The open black circles represent mC3-PPARδ− cells while the solid black circles represent mC3-PPARδ+ cells.

3.4.5 PPARδ’s protection against drugs that induce mitochondrial-dependent death

So far, I have shown the results for the endoplasmic reticulum stress drugs and TAM and confirmed that both PPARδhi and Bcl-2hi cell lines are protected from these agents. It appears that ↑Δψm is reduced only transiently for the cells that overexpressed Bcl-2 alone. Moreover, the concentration of each drug used resulted in only very low levels of cell death and were selected to highlight ↑Δψm. The remaining drugs are those that induce
mitochondrial-dependent death. While PPARδ has been shown to protect against endoplasmic reticulum stress in the literature, it is not clear whether it can protect against mitochondrial-dependent forms of cell death. Drugs that induce this form of death are best protected by Bcl-2 at the mitochondria, modeled by Bcl2-actaahi cells (Annis et al., 2001). Bcl2-wtahi cells are also protected against these drugs because they have Bcl-2 at both the mitochondria and endoplasmic reticulum. On the other hand, Bcl2-cb5ahi cells are not expected to protect against these drugs very well, especially those that induce mostly mitochondrial-dependent death like TNF-α+CHX.

First, the results for TNF-α+CHX treatment in Figure 29. Considering the percentage of dead cells, the Bcl2-wtahi and Bcl2-actaahi cells show significantly fewer dead cells compared to control. The Bcl2-cb5ahi cells are not protected as expected and have similar levels of dead cells compared to control. Cells overexpressing PPARδ alone show a lower percentage of dead cells compared to control but is still comparable to Bcl2-cb5ahi cells. In combination with the different forms of Bcl-2 however, there is not much of a change in the proportion of dead cells.

Considering the percentage of stressed cells, PPARδ overexpression resulted in significantly fewer stressed cells only for the Bcl2-actaahi cells. In addition, there were also more cells that were classified as alive in mC3-PPARδahi/Bcl2-actaahi cells compared to Bcl2-actaahi cells. The result here, where the overexpression of PPARδ provided additional protection only for the mC3-PPARδahi/Bcl2-actaahi cells and not the Bcl2-wtahi or Bcl2-cb5ahi cells suggests that its protection is different from both the ER and mitochondrial routes of protection through Bcl-2. Although the mC3-PPARδahi/Bcl2-actaahi cells have much higher PPARδ levels, as seen in the microscopy images shown in section 3.2.1 and may explain the greater protection against the drug panel, the following two results with STS and ActD show additional protection when PPARδ is overexpressed any one of the Bcl-2 mutants, supporting that Bcl-2 and PPARδ are indeed cooperating.

Here, the need for 3 classes becomes apparent again; if only the percentage of stressed cells were considered in Figure 29, it may seem as though the control cells and the Bcl2-cb5ahi cells are less stressed than the Bcl2-wtahi and Bcl2-actaahi cells. The reason why there are
fewer cells with $\Delta \psi_m$ mitochondria for the control and Bcl2-cb5<sup>hi</sup> groups is because the cells are dead.

**Figure 29.** 3-way classification results for the 8 cell lines treated with TNF-\(\alpha\) (3ng/mL) + CHX (10\(\mu\)g/mL) for 48 hrs.

The graphs above show the percentage of dead, stressed, and alive cells. The open black circles represent mC3-PPAR\(\delta\)- cells while the solid black circles represent mC3-PPAR\(\delta\)+ cells.

The next drug is the pan-kinase inhibitor, STS, which elicits death through both mitochondrial-dependent and mitochondrial-independent mechanisms. As shown in the early experiments, STS can be protected by all three Bcl-2 mutants, with Bcl2-wt<sup>hi</sup> and Bcl2-acta<sup>hi</sup> cells faring better. Here, mC3-PPAR\(\delta\)<sup>hi</sup> cells provided additional protection for the control, Bcl2-wt<sup>hi</sup>, and Bcl2-acta<sup>hi</sup> cells in terms of percentage of dead cells. Within the control group, PPAR\(\delta\)<sup>hi</sup> cells lower the percentage of dead cells from approximately 65% to 40%, as seen in Figure 30. This is similar to the Bcl2-wt<sup>hi</sup> cells but instead of the cells
dying, more cells acquire hyperpolarized mitochondria. When both PPARδ and Bcl2-wt are overexpressed, the protection from STS is decent. It is as though Bcl-2 is protecting from death while PPARδ is removing stress.

Among the Bcl-2hi cell lines, Bcl2-wt hi and Bcl2-actahi cells were better protected against STS compared to Bcl2-cb5 hi cells but overall, they all had lower percentages of dead cells compared to control. In terms of percentage of stressed cells, the Bcl-2hi cell lines had slightly higher levels of stressed cells with STS treatment, but this level was lowered by the overexpression of PPARδ. Finally, for the percentage of cells that are alive, the Bcl-2hi cell lines show a slightly higher percentage compared to control and in each case, this percentage was larger for those cell lines overexpressing PPARδ.

**Figure 30.** 3-way classification results for the 8 cell lines treated with STS (30nM) for 48 hrs.
The graphs above show the percentage of dead, stressed, and alive cells. The open black circles represent mC3-PPARδ⁻ cells while the solid black circles represent mC3-PPARδ⁺ cells.

Finally, there is ActD, a drug that inhibits transcription. As shown in the previous experiments, this treatment is protected through either Bcl2-wt hi or Bcl2-acta hi cells. This is seen to be true in Figure 31, where control and Bcl2-cb5 hi cells show similar levels of cell death. With the overexpression of PPARδ, the percentage of dead cells only change significantly for Bcl2-acta hi cells and not as much for Bcl2-wt hi or Bcl2-cb5 hi cells. This suggesting that PPARδ protects in a manner similar to Bcl-2 at the endoplasmic reticulum since the mutants that overexpress Bcl-2 at the endoplasmic reticulum do not show additional protection with PPARδ. However, the percentage of stressed cells is lowered for every Bcl-2 hi cell line with the overexpression of PPARδ, which shows that PPARδ overexpression prevents cells from transitioning to the stressed state. This was also seen for ActD treatment and the other endoplasmic reticulum stress drugs. In general, there is also a higher percentage of cells that are alive for the mC3-PPARδ hi cells.
**Figure 31.** 3-way classification results for the 8 cell lines treated with ActD (30nM) for 48 hrs.
The graphs above show the percentage of dead, stressed, and alive cells. The open black circles represent mC3-PPARδ⁻ cells while the solid black circles represent mC3-PPARδ⁺ cells.

3.4.6 Different pathways of protection: Bcl-2 prevents cell death while PPARδ prevents mitochondrial hyperpolarization

Figure 32 summarizes the results for one of the replicates of the experiment where the 8 cell lines were treated with the drug panel for 48hrs. The different columns represent the 8 cell lines and the rows represent the drug treatment. Pie charts were used for easier visualization of the results. From a first glance, the first three rows, which represent the drugs that induce mitochondrial-dependent death, show a largely green pie chart for the control cells (first column)—this means that the cells are dead or apoptotic. For the latter three rows, which include the endoplasmic reticulum stress drugs and TAM, the pie charts for the control cells are mostly red, meaning the cells are stressed.

The odd columns are the cell lines that do not overexpress PPARδ and the even columns are the cell lines that do overexpress PPARδ. For each drug treatment, there is markedly more protection for the cells that overexpress both Bcl-2 and PPARδ compared to the cell lines with just one protein overexpressed, suggesting that Bcl-2 and PPARδ protect through different pathways. Moreover, cells that overexpressed Bcl2-acta and PPARδ were resistant to every treatment and exhibited the most protection. This is observed in the sixth column of the figure, where the pie chart is mostly blue, meaning the cells are alive and resistant to the drug treatment. The most additive protection was observed with this cell line. This however, may be because this cell line had the highest levels of mC3-PPARδ, as seen in the Western blots.
Figure 32. Summary of the 3-way classification results for the 8 cell lines treated with the whole drug panel.

The eight MCF-7 cell lines: control, Bcl2-wt\textsuperscript{hi} (wild type Bcl-2; “WT”), Bcl2-acta\textsuperscript{hi} (mitochondria-targeting Bcl-2; “Mito”), Bcl2-cb5\textsuperscript{hi} (endoplasmic reticulum-targeting Bcl-2; “ER”), mC3-PPAR\(\delta\)-hi, mC3-PPAR\(\delta\)-hi/Bcl2-wt\textsuperscript{hi}, mC3-PPAR\(\delta\)-hi/Bcl2-acta\textsuperscript{hi}, and mC3-PPAR\(\delta\)-hi/Bcl2-cb5\textsuperscript{hi} were treated with the following 6 drugs: Tumor Necrosis Factor-alpha + Cycloheximide (TNF-\(\alpha\) + CHX), thapsigargin (TG), tunicamycin (TN), staurosporine (STS), actinomycin D (ActD), and tamoxifen (TAM). The addition of PPAR\(\delta\) to the Bcl-2\textsuperscript{hi} cells offered additional protection, suggesting that Bcl-2 and PPAR\(\delta\) were working through different pathways. In addition, cells that overexpress Bcl2-acta and PPAR\(\delta\) were resistant to nearly every treatment. The results shown here are from the 48 hr drug treatment experiment.

3.5 Targeting protection through PPAR\(\delta\) and Bcl-2 with a PPAR\(\delta\) antagonist and a Bcl-2 inhibitor

Having established that PPAR\(\delta\) and Bcl-2 protected MCF-7 cells from the panel of drugs described previously, I determined whether the addition of ABT-199, the Bcl-2 inhibitor,
and NXT1511, the PPARδ antagonist, could sensitize the resistant cell lines (i.e. those that overexpress both Bcl-2 and PPARδ) to the drug treatments.

3.5.1 ABT-199 sensitizes Bcl-2<sup>hi</sup> cells to TNF-α+CHX and ActD, but not TAM

Initially, I tested all eight cell lines with the following three drugs: TNF-α+CHX, ActD, and TAM. In addition to the three drugs, cells were also treated with either: plain media, NXT1511 (3uM) media, ABT-199 (1uM) media, or media with both NXT1511 and ABT-199.

With the addition of ABT-199, Bcl2-wt<sup>hi</sup> and Bcl2-acta<sup>hi</sup> cells could no longer protect the cells from TNF-α+CHX treatment. For cells treated with ActD, ABT-199 takes away part of the protection offered by Bcl-2 overexpression, though not to the same extent as TNF-α+CHX treatment. However, no significant losses in protection were seen when ABT-199 was used in combination with TAM. This was surprising because treatment with ABT-199 was expected to prime the cells for death by sequestering anti-apoptotic Bcl-2 proteins, freeing pro-apoptotic proteins and sensitizing cells to apoptosis. The observation that the combination of TAM and ABT-199 did not result in increased killing raised the possibility of a BH3-independent mechanism of protection through Bcl-2 for TAM treatment.
Figure 33. ABT-199 sensitizes Bcl-2<sup>hi</sup> cells to TNF-α + CHX and ActD, but not TAM. Images of two cell lines, CMV and Bcl2-acta<sup>hi</sup> (Mitochondria-targeting Bcl-2; “Mito”), are shown under the 8 different conditions (Media, TNF-α (3ng/mL) + CHX (10ug/mL), ActD (30nM), or TAM (14uM) with/without ABT-199 (1uM)). Bcl2-acta<sup>hi</sup> (Mito) cells show protection against the 3 different drugs compared to control cells in the absence of ABT-199. This protection is lost when ABT-199 is added for TNF-α + CHX and ActD but not TAM treatment of Bcl2-acta<sup>hi</sup> (Mito) cells. The images shown represent one field in one well of a 384-well plate. The pie graphs next to the images represent the 3-way classification results for all the cells in the wells of a particular treatment/condition and accounts for all nine fields of view in the well and is therefore a more accurate representation of the outcome of the drug treatment. The number above the pie graph shows the total number of cells that were classified.

3.5.2 ABT-199 unable to sensitize Bcl-2<sup>hi</sup> cells to other endoplasmic reticulum stress drugs

To determine whether this phenomenon was specific to TAM, another experiment was performed using the whole drug panel with or without ABT-199 with just the Bcl-2<sup>hi</sup> cell
lines. The results from these experiments showed once again that ABT-199 was ineffective for drugs protected by Bcl2-cb5 at the endoplasmic reticulum (TG, TN, and TAM) but effective for drugs protected by Bcl-2 and Bcl2-acta but not Bcl2-cb5 (TNF-α+CHX and ActD). The phenomenon where ABT-199 was ineffective at sensitizing Bcl-2 hi cells to TAM was consistent in subsequent experiments.

Also, ABT-199 did not provide additional killing when used in combination with endoplasmic reticulum stress drugs like TG and TN. That is, ABT-199 was ineffective for drugs that were protected by Bcl-2 at the endoplasmic reticulum. However, ABT-199 in combination with drugs like TNF-α+CHX and ActD provided additional killing and sensitized Bcl2-wt and Bcl2-acta drugs to the treatments.

3.5.3 ABT-199 phenomenon independent of Bcl-2 localization

In addition, for the endoplasmic reticulum stress-inducing drugs, ABT-199 was ineffective regardless of the localization of Bcl-2. ABT-199 binds to the pocket on Bcl-2 that binds to the BH3-domain of pro-apoptotic proteins and thereby prevents Bcl-2 mediated inhibition of these pro-apoptotic proteins. Therefore, this result suggests that there is a mechanism by which Bcl-2 inhibits cell death independent of sequestering pro-apoptotic BH3-domain containing proteins. This is significant because traditionally, the BH3 domain is targeted in cancers where there is an overexpression of Bcl-2. The results from these experiments suggest that this BH3-independent protection through Bcl-2 must be targeted to sensitize the cells to the drug panel. This was repeated three times and the same phenomenon was observed. Similar results were obtained when other clones of MCF-7 cells expressing the
Bcl-2 variants were tested, demonstrating that protection from cell death by a mechanism not inhibited by ABT-199 was not a clone specific effect.

Figure 34. 3-way classification results showing ABT-199 ineffectively sensitizing Bcl-2^{hi} cells to endoplasmic reticulum stress drugs like TG.

The following 4 cell lines were used: CMV (empty vector control), Bcl2-wt^{hi} (wild type Bcl-2; “WT”), Bcl2-acta^{hi} (mitochondria-targeting Bcl-2; “Mito”), and Bcl2-cb5^{hi} (endoplasmic reticulum-targeting Bcl-2; “ER”). Cells were treated with either DMSO media, TNF-α (3ng/mL) + CHX (10µg/mL), or TG (10nM) with or without ABT-199 (1µM) for 48hrs. More cells are dead when ABT-199 is used in combination with TNF-α + CHX for the Bcl-2hi cell lines (Bcl2-wt and Bcl2-acta) whereas when ABT-199 is used in combination with TG, there are no significant changes in cell death.
3.5.4 Results with the PPARδ antagonist, NXT1511

As for NXT1511, the PPARδ antagonist, it did not seem to have an effect on protection based on the 3-way classification. In the preliminary experiments with the antagonist, a concentration of 1µM was used. Because of the lack of effect observed, I used a higher concentration of 3µM. Even at this concentration, no loss of protection through PPARδ via the antagonist could be seen based on the 3-way classification results. A possible explanation for the antagonist not working may be due to the effect of transgenic PPARδ not being transcriptional but rather, affecting molecular complexes to which it binds. I did not pursue further experiments with the antagonist and decided to follow up on the ABT-199 result in more detail.

![3-way classification results for the two cell lines: control and Bcl2-wt\(^{hi}/mC3\)-PPARδ\(^{hi}\) cells. These cells were treated with the three drugs: TNF-α+CHX, TG, and TAM. On top of that, there were four media conditions: DMSO, ABT-199 (1µM), NXT1511 (3µM), and both ABT-199 + NXT511. ABT-199 effectively removed Bcl-2](image)

**Figure 35.** Attempting to sensitize cells that overexpress PPARδ and Bcl-2 with a PPARδ antagonist and a Bcl-2 inhibitor.
protection from the Bcl2-wt\textsuperscript{hi}/mC3-PPAR\(\delta\textsuperscript{hi}\) cells for TNF-\(\alpha\)+CHX but was unable to provide additional killing for TG or TAM. On the other hand, NXT1511, the PPAR\(\delta\) antagonist, did not render the Bcl2-wt\textsuperscript{hi}/mC3-PPAR\(\delta\textsuperscript{hi}\) cells more sensitive to any of the drug treatments.

3.6 Titration of PPAR\(\delta\) agonist: optimal range for PPAR\(\delta\) activity?

Since the overexpression of PPAR\(\delta\) was observed to be protective for MCF-7 cells, an experiment that was performed was to titrate a PPAR\(\delta\) agonist in combination with the drug treatments. The rationale for this experiment was to see if I can specifically activate PPAR\(\delta\) and increase the cell’s ability to protect against the drug panel. Considering the cells are cultured in media containing FBS, there are already natural ligands for PPAR\(\delta\) in the media, which can activate the receptors. However, I hypothesized that higher doses of the agonist would offer the cells greater levels of protection through PPAR\(\delta\).

3.6.1 Culturing the cells in media containing the agonist, GW0742

In the first pilot experiments, the agonist, GW0742, was added at the same time as the drug treatments. Figure 36 shows the three-way classification results for PPAR\(\delta\textsuperscript{hi}\) cells following 48hrs of incubating with the drugs (TAM, TG, and TNF-\(\alpha\)+CHX) and PPAR\(\delta\) agonist after 48 hours. Even with doses ranging from 1-10\(\mu\)M of the agonist, there was no observed improvement in protection with increasing doses of the agonist.
Figure 36. Increasing doses of the PPARδ agonist did not increase protection against the drug panel.

PPARδ^{hi} cells were treated with increasing doses of the agonist, GW0742 (columns from left to right: 0, 1, 3, 10µM) with or without drugs (TAM, TG, or TNF-α+CHX) for 48hrs. No increases in protection were seen with the drug treatment in the presence of the agonist.

To see if the agonist needed a longer time to take effect, I cultured the cells in media containing the agonist and then subjected it to the drug treatments. The four cell lines: CMV, Bcl2-wt^{hi}, mC3-PPARδ^{hi}, and Bcl2-wt^{hi}/mC3-PPARδ^{hi} were cultured in DMEM media containing 1µM of PPAR agonist, GW0742, for 5 days before a simple experiment involving treating the cells with TG to see if there would be an increase in protection for cells overexpressing PPARδ with increasing doses of the agonist. Morphological changes were observed after 5 days of being cultured in the GW0742 media. The cells that already
overexpressed PPARδ showed more drastic changes than their non-overexpressing PPARδ counterparts did. These cells were smaller and grew in tight, packed colonies.

**Figure 37.** Morphology changes in the cell lines following PPARδ agonist treatment. The four cell lines: control, Bcl2-wt hi, mC3-PPARδ hi, and Bcl2-wt hi/mC3-PPARδ hi were cultured in media containing 1µM of the agonist, GW0742, for 5 days. The top row shows cells being cultured in regular DMEM media for 5 days and the bottom panel shows cells being cultured in the agonist media. Overall, all four cell lines shrunk in cell area, though the effects were more pronounced in the cells that overexpressed PPARδ. These mC3-PPARδ hi cells grew in tight, packed colonies after the agonist treatment, indicated by the red arrows.

Figure 38 shows the 3-way classification results for the four cell lines: CMV, mC3-PPARδ hi, Bcl2-wt hi, and mC3-PPARδ hi/Bcl2-wt hi. These cells were treated with the drug panel for 48hrs after being cultured in media containing 1µM of the agonist, GW0742, for 5 days.
Incubation with the PPARδ agonists did not improve the protection of cell lines overexpressing mC3-PPARδ to the drug panel.

CMV, mC3-PPARδ,hí, Bcl2-wtí, and mC3-PPARδ,hí/Bcl2-wtí cells were treated with either TNF-α+CHX, TG, or TAM for 48hrs after being cultured in 1µM of the agonist, GW0742, for 5 days. The numbers on top of the pie charts represent the total number of cells that were analyzed and classified.

Contrary to what I expected, the cells that already overexpressed PPARδ performed worse than the cells that did not overexpress PPARδ, suggesting that hyperactivity of PPARδ may be pushing the cells closer to death rather than protecting them. When treated with 10nM of TG for 48 hrs, the PPARδ cells that showed protection in the past experiments were now performing worse after being cultured with the agonist. Surprisingly, the cells that did not overexpress PPARδ showed slightly better protection after being cultured in media containing the agonist. These results seem to suggest that there is an optimal range of PPARδ activity that is protective for the cells.

**Figure 38.** Incubation with the PPARδ agonists did not improve the protection of cell lines overexpressing mC3-PPARδ to the drug panel.
3.7 Transient transfection with mC3-Bcl-2 plasmids to exclude possibility of cell culture-selection of ABT-199 resistant cells

To test whether the absence of additional killing when endoplasmic reticulum stress-inducing drugs were used in combination with ABT-199 was due to gradual selection of ABT-199 resistant cells through cell culture, MCF-7 cells were transiently transfected with mC3-tagged Bcl-2 plasmids via PEI inverse transfection and subjected to the same drug panel treatment with and without ABT-199 for 24 hrs. mC3-tagged Bcl-2 plasmids were used to allow cells that expressed fluorescently tagged Bcl-2 to be selected by setting an intensity threshold for cyan fluorescence during image analysis.

3.7.1 Results of the transient transfection experiments

As mentioned in section 1.10, several transfection techniques were tested, and the PEI transfection method was chosen to be the most suitable option. Using the PEI transfection protocol described in section 2.10, MCF-7 cells were inversely transfected with the following four plasmids: mC3 (control), mC3-Bcl2-wt (mC3-tagged wild type Bcl-2), mC3-Bcl2-cb5 (mC3-tagged endoplasmic reticulum-targeting Bcl-2), and mC3-Bcl2-acta (mC3-tagged mitochondrial-targeting Bcl-2).
Figure 39 shows one replicate of the experiments where the transiently transfected cells were treated with the drug panel (TNF-α+CHX, ActD, TG, TN, TAM) with or without ABT-199. Cells in media alone showed a background death around 30%. Death here is defined through a 2-way classification. Cells that are negative for TMRE staining, positive for AnnexinV/Alexa488 staining, or have condensed nuclei are considered dead. Compared to the controls (mC3 alone), the cells that were positively transfected with mC3-Bcl-2 were protected against the different drugs. As with the stable cell lines, the observation held, where Bcl2-wt\textsuperscript{hi} and Bcl2-acta\textsuperscript{hi} cells showed protection against TNF-α+CHX and ActD treatments but this protection was lost in the presence of ABT-199. On the other hand, all the Bcl-2 overexpressing cells showed no significant changes in protection when ABT-199 was combined with drugs like TAM, TN, and TG. This is seen in the bottom row of the figure.
**Figure 39.** MCF-7 cells transiently transfected with the mC3-plasmids: control (“-”), Bcl2-wt (wild type Bcl-2; “WT”), Bcl2-acta (mitochondria-targeting Bcl-2; “Mito”), or Bcl2-cb5 (endoplasmic reticulum-targeting Bcl-2; “ER”) plasmids via inverse PEI transfection. Following 24 hrs after transfection, the cells were dosed with either TNF-α (3ng/mL) + CHX (10µg/mL), TG (10nM), TAM (14µM), TN (30µM), and ActD (30nM). The y-axis of each graph shows the percentage of cells that were classified as dead based on nuclear condensation, loss of TMRE, and AnnexinV positivity. The white bars represent cells in the DMSO media, and the black bars represent the cells in the ABT-199 media for each drug treatment. The phenomenon where ABT-199 was ineffective for endoplasmic reticulum stress drugs as well as tamoxifen was observed for transiently transfected cells. For the TNF-α+CHX and ActD treatment, the addition of ABT-199 removes Bcl2-wt and Bcl2-acta’s protection against the drug treatment.

This experiment was repeated 3 times and the same results were obtained. The results of the three replicates are plotted in Figure 40, with each dot representing the average of the replicate.
Figure 40. 3 repeats of the transient transfection experiment.
The y-axis shows the percentage of cells that are classified as dead based on nuclear condensation, TMRE negativity, and/or AnnexinV positivity. Each dot represents the average of a particular replicate. The open black circles represent the DMSO media condition and the solid black circles represent the ABT-199 media condition.

4 Discussion and Future Directions

4.1.1 Using a tool compound screen to identify compounds protected solely by Bcl-2 or PPARδ

Both PPARδ and Bcl-2 have been reported to protect cells from endoplasmic reticulum stress and are promising targets in various diseases. For this reason, the overarching goal of this project was to see whether patients with cells exhibiting high Bcl-2 expression have to be treated differently from patients with cells exhibiting high PPARδ expression. Results from this project show that PPARδ overexpression protects cells from a variety of apoptosis and endoplasmic reticulum stress-inducing agents.

These results are partly novel; the data in this thesis not only confirms PPARδ-mediated protection against TG and TN but also shows that overexpression of PPARδ protects cells against TAM and apoptotic agents such as STS, ActD, and TNF-α + CHX. In addition, while cells that overexpress PPARδ or Bcl-2 share overlap in what drugs they can protect against, there is a difference in their protection; cells that overexpressed both proteins showed additional protection compared to cells that overexpressed either protein alone. Furthermore, overexpression of both proteins seemed to cooperate in the following manner: PPARδ prevented mitochondrial hyperpolarization while Bcl-2 prevented cell death.

Though the mechanisms were not explored in this project, the results suggest that a patient, whose cancer cells overexpress both these proteins, may require these separate pathways to be targeted in order to get effective killing and clearance of cancer cells. The drugs that were used to test PPARδ and Bcl-2-mediated protection was limited. As a next step,
performing a screen with a tool compound library will help identify compounds that can be protected through PPARδ only or Bcl-2 only. In a second screen, the selected compounds should be tested again with or without ABT-199. If ABT-199 does not remove the protection for drugs protected by PPARδ only, then PPARδ is different from Bcl-2. Hopefully, targeting pathways mediated by Bcl-2 and PPARδ in parallel with standard hormone therapy would likely make for more effective therapies.

4.1.2 ABT-199’s inability to sensitize Bcl-2^hi^ cells to drug treatments points to a possible new target: the BH4 domain of Bcl-2

Tamoxifen is the standard therapy for estrogen receptor-positive breast cancer, but patients often acquire resistance to this drug. As a result, combination therapy is under investigation for this treatment. With the success of ABT-199 in chronic lymphocytic leukemia, there is an increasing interest in targeting Bcl-2 family proteins in various types of cancers. For example, there are currently clinical trials testing the combination of tamoxifen with ABT-199 in order to prime breast cancer cells for apoptosis and elicit better cell death responses. However, the data in this project show that with a clinically relevant dose of tamoxifen, cells exhibited endoplasmic reticulum stress that can be protected through either the overexpression of Bcl-2 or PPARδ.

One of the novel findings in this project was the observation that ABT-199 did not succeed in effectively killing the resistant Bcl2-overexpressing cells when used in combination with tamoxifen, suggesting that targeting the BH3 domain of Bcl-2 may not be sufficient. This is significant as the BH3 domain is the most well characterized and traditionally targeted in cancers overexpressing Bcl-2. Importantly, this phenomenon was observed regardless of the localization of Bcl-2, suggesting a BH3-independent route of protection by Bcl-2. Moreover, this observation was not limited to tamoxifen; ABT-199 was also ineffective for drugs that induced endoplasmic reticulum stress. While ABT-199 sensitized Bcl-2 overexpressing cells to drugs treatments like TNF-α + CHX and ActD, it was ineffective against drugs that could be protected by overexpression of Bcl-2 at the endoplasmic reticulum. This result was also observed for MCF-7 cells that were transiently transfected with the various Bcl-2 variants.
Overexpression of Bcl-2 is associated with therapy resistance in various human cancers. Traditional approaches target the BH3 domain of Bcl-2; however, the BH4 domain represents a promising therapeutic target in light of its involvement in various cellular functions. The BH4 domain of Bcl-2, though not as well known, has many functions in addition to binding to IP$_3$R at the endoplasmic reticulum to prevent Ca$^{2+}$-initiated apoptosis. For example, Ras and Raf-1 are both proteins that can associate with the BH4 domain of Bcl-2 and mediate resistance to apoptosis (Liu et al., 2003, Wang et al., 1996). With ABT-199 being ineffective for drugs that are protected by Bcl-2 at the endoplasmic reticulum, it would be worth exploring BH4 mutations in the future to see if Bcl-2 overexpressing cells are then sensitized to tamoxifen in the presence of ABT-199. It may be that the endoplasmic reticulum stress pathways and the apoptotic pathways have to be targeted simultaneously in order get effective killing.

One of the weaknesses in this data was not being able to confirm whether the Bcl-2 overexpressing cells were protecting in a BH4-dependent manner. As a future experiment, making BH4 mutations in our current cell lines and subjecting these cells to the same combination treatments with ABT-199 will confirm if this is a BH4-mediated route of resistance through Bcl-2. Cell lines with a mutated BH4 domain but an intact BH3 domain should not be protected from drugs that induce endoplasmic reticulum stress. Another step that can be taken is to make BH3 mutations. One would expect cell lines overexpressing Bcl-2 with a mutated BH3 domain but an intact BH4 domain to behave similarly to PPARδ overexpressing cells, since they would be protected from drugs that induce endoplasmic reticulum stress. Since tamoxifen is the current accepted estrogen receptor-positive form of endocrine therapy in breast cancer today, understanding the mechanisms regulating cell survival under stress conditions is crucial in circumventing resistance to current therapies.

### 4.1.3 Controversy surrounding PPARδ agonists: protective or tumorigenic?

From the results shown with the PPARδ agonists and antagonist experiments, there seems to be an optimal range of PPARδ activity that is protective for the cell. This is interesting because this could explain the discrepancy in cancer studies with PPARδ; whether PPARδ is oncogenic or tumor suppressive may depend on the levels of PPARδ activity.
Agonist regulation of PPARδ has been implicated in regulating cell growth and tumor formation, but considerable controversy surrounds this area of research, and both carcinogenic and tumor suppressor activities have been attributed to PPARδ agonists (Youssef & Badr, 2011; Peters, Gonzalez, & Müller, 2015; Palomer et al., 2018). A reason why these studies with PPARδ have often been controversial is perhaps due to the different levels of PPARδ ligands, coactivators, and corepressors. Furthermore, the cell line and tissues that PPARδ is expressed in will change the context and influence PPARδ activity. Therefore, in order to assess whether PPARδ is protective or tumorigenic, I need a controllable system where I can limit the variation in these external variables.

Future work that can be done to confirm whether the protection observed is truly through PPARδ overexpression is to use shRNA to silence PPARδ activity and test its protection against the same drug panel. If the effect is specific to PPARδ, PPARδhi cells that have been treated with the shRNA along with the drug should be not show protection and exhibit a similar level of cell death compared to control cells. This would also allow us to separate the amount of protection that is offered via Bcl-2 and PPARδ for the double-overexpressing cell lines. I only tested one antagonist but there are also other PPARδ antagonists available such as DG172, PT-S58, GSK0660, and GSK3787. Like NXT1511, these ligands act through their inhibitory effect on the basal expression of PPARδ target genes and an increased recruitment of transcriptional corepressors (Naruhn et al., 2011).

Despite the fact that preclinical studies have provided evidence that PPARδ agonists possess therapeutic value in the treatment of several metabolic diseases, no FDA-approved agonists for PPARδ currently exist (Yang & Long, 2018). This is in contrast to PPARα and PPARγ, which have FDA-approved agonists that are standard of care for metabolic disorders, such as hyperlipidemia and diabetes (Monsalve, Pyrarasani, Delgado-Lopez, & Moore-Carrasco, 2013). Although clinical trials have revealed that short-term administration of the PPARδ agonists like GW501516 had beneficial effects in patients with metabolic syndrome, preclinical long-term studies in mice have indicated that PPARδ agonists might have pro-tumorigenic effects, thus dampening enthusiasm for these compounds in human trials (Palomer et al., 2018). Nevertheless, uncovering the cellular
and molecular mechanisms by which PPARδ drives its effects may lead to new therapeutic insights, especially in those cancers with high PPARδ activity.

4.1.4 Other assays to assess invasiveness and metastatic potential

Measuring E and N-cadherin expression is just one way to assess invasiveness. Based on the tools available in our lab, an immunofluorescence assay was a fairly simple and accessible experiment to perform. To explore changes in metastatic potential and cell invasiveness, there are other methods available such as scratch assays, transmembrane assays, microfluidic chamber-based assays, and cell exclusion zone assays.

Scratch assays involve using a needle to etch away cells from a given area of a confluent monolayer to form a cell-free zone into which cells at the edges of the scratch can migrate. The advantages of this assay are that it can be performed in various types of plates and images can be taken throughout the assay to capture the morphology and the movement of the cells. However, there are also drawbacks to this system. The confluency of the monolayer and the size of the scratch can vary among replicates, making it hard to maintain consistency in experiments. In addition, scratching also damages the underlying extracellular matrix and causes the release of factors that might influence the assay (Hulkower & Herber, 2011).

Transmembrane assays offer the ability to analyze migration in response to a chemotactic gradient and can be used with adherent as well as non-adherent cells (Hulkower & Herber, 2011). Here, a membrane is suspended in a well and cells are added to the top part of the membrane. A chemoattractant added the bottom side of the membrane forces cells to migrate across the membrane and the fraction of cells that have traversed to the other side is counted. The disadvantage of this assay is that a non-physiologic polycarbonate or polypropylene membrane is used. However, matrix proteins can be applied to this to better mimic physiological conditions. Still, this assay is challenging to set up and because the gradient is non-linear and equilibrates between both compartments over time, it is difficult to visualize the cells during the experiments.
Microfluidic chamber-based assays, on the other hand, are advantageous if you have limited or expensive reagents. Here, you have a device with two ports: a small and large one. Cells are added to the small port and test reagents are added to the large port. Due to surface tension in the device channel, a gradient will be set up. Cells are then imaged to measure migration in response to the reagent. Because of the small volumes, this assay has increased labor costs because the growth media for the cells need to be replaced daily (Echeverria et al., 2010). Additionally, care must be taken to maintain the humid environment surrounding the microchannel plates to avoid evaporation (Echeverria et al., 2010).

Finally, cell exclusion zone assays spare cells or the extracellular matrix from being damaged, unlike scratch assays. This assay also allows continuous visual assessment of the cells throughout the experiment and the ability to acquire multiplexed data unlike the transmembrane assays where the filter restricts observation (Kramer, Walzl, Unger, Rosner, Krupitza, Hengstschläger, & Dolznig, 2013).

4.1.5 Testing other estrogen receptor-positive breast cancer cell lines

In this project, MCF-7 cells were used to model estrogen receptor-positive breast cancer patients. To see if the observed phenomenon is specific to MCF-7 cells, it would be worth testing other estrogen receptor-positive breast cancer cell lines such as T47D, CAMA1, MDA-MB, and BT474. After all, cell lines only capture a subpopulation of the tumours they are purported to model. To see how extendable this result is, it would also be worth testing other cancer cell lines in parallel.

5 Conclusion and Summary

The ability for cancer cells to survive under unfavourable conditions and stress allows them to evade cell death, making them resistant to drug treatments. Both PPARδ and Bcl-2 have been reported to protect cells from endoplasmic reticulum stress and are promising targets in various diseases. For this reason, the overarching goal of this project was to see whether patients with cells exhibiting high Bcl-2 expression have to be treated differently from
patients with cells exhibiting high PPARδ expression. Overall, there are several conclusions that can be made from the results of these experiments.

Firstly, patients with cancer cells exhibiting high PPARδ or Bcl-2 expression will be more resistant to tamoxifen therapy since these cells are better able to evade stress and bypass apoptosis. This is supported by the results where cell lines overexpressing either one of these proteins were protected against various drugs like TNF-α+CHX, ActD, STS, TG, TN, and TAM.

Secondly, patients have to be treated differently based on their Bcl-2 and PPARδ status. This is supported by the results where additional protection seen in cells overexpressing both proteins, suggesting that they work through different pathways. Targeting both pathways in parallel with standard hormone therapy would likely make for more effective therapies.

Thirdly, cancer cells with higher PPARδ are more likely to form aggressive tumours, supported by the lower E/N-cadherin ratio observed in PPARδ^hi cell lines. This confirms what is already published in the literature.

Fourthly, the use of ABT-199 will be ineffective with tamoxifen unless it is combined with a drug that can remove protection from endoplasmic reticulum stress. This is justified through the results that show how ABT-199 sensitizes Bcl-2^hi cells to TNF-α+CHX and ActD treatment but not TAM, or the endoplasmic reticulum stress drugs, TG and TN.

Lastly, the benefits of using multiparametric analysis are highlighted in the experiments testing differences in PPARδ and Bcl-2 protection. As shown in the data, intermediate phenotypes, such as cells with hyperpolarized mitochondria, were overlooked if one simply asked whether cells were alive or dead.

To conclude, intrinsic or acquired therapeutic resistance remains a major obstacle to an endocrine therapy in estrogen receptor-positive breast cancer. Thus, understanding possible pathways of resistance is vital in order to develop therapeutic strategies that will enhance and broaden responsiveness. This thesis used multiparametric analysis to confirm and compare the protection of two proteins, PPARδ and Bcl-2, in MCF-7 cells. In addition,
Bcl-2 was revealed to have a novel BH3-independent route of protection against ABT-199 in the presence of TAM and endoplasmic reticulum stress drugs. As such, future therapies may need to target both Bcl-2 and PPARδ’s ability to evade endoplasmic reticulum stress in order to get enhanced responses in resistant patients.
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