PHENOTYPIC PROFILING OF P53 WILDTYPE & MUTANT BREAST CANCER CELL LINES

PHENOTYPIC AND CHEMOTHERAPY RESPONSE PROFILING OF P53 WILD-TYPE AND MUTANT HUMAN BREAST CANCER CELL LINES

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree Master of Science

McMaster University MASTER OF SCIENCE (2015) Hamilton, Ontario (Biochemistry)

TITLE: Phenotypic and chemotherapy response profiling of p53 wild-type and mutant human breast cancer cell lines

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NUMBER OF PAGES: XI, 70

ABSTRACT

Anthracycline-based chemotherapy is the mainstay neoadjuvant therapy for breast cancer. However, it is efficacious in only 60% of patients while carrying substantial toxicity. The application of a predictive marker of response may spare predicted 'poor responders' from the toxicity. Previously, we demonstrated a gene expression signature that predicts chemotherapy resistance which is linked to TP53 integrity. Further investigation showed that p53 signatures predict response in only ER+ tumors. We hypothesized that the loss of p53 confers an elevated chemotherapy sensitivity in ER+ breast tumors. We engineered isogenic p53 mutant ER+ breast cancer cell lines and assayed their cell cycle kinetics and chemotherapy sensitivity. Our results demonstrated that the loss of p53 is necessary to abrogate p53-mediated cell cycle arrest and produce an increase in apoptosis. Therefore, p53 signatures may be utilized as a predictive marker of response for patients with ER+ breast tumor and spare 'poor responders' from toxicity. Since ER+ p53 wild-type breast tumors are associated with anthracycline resistance, new anticancer drugs against that subgroup of tumors are needed. Phenotypic drug screening approach, which do not focus on isolated targets but instead classify compounds by their impact on cell physiology, is highly suitable for this purpose. Current cell-based phenotypic assays require fixation and staining for phenotypic markers, which reduce screen throughput and introduce potential variations and artifacts. Here we describe a high-content live-cell phenotypic assay, which streamlines the process of cytological profiling and provides a consistent platform for empirically evaluating drug action. Importantly, when combined with chemical similarity clustering, the phenotypic assay provided an inference of structure-activity relationships.

Finally, a small-scale phenotypic screen of natural products enabled classification of unknown compounds against the cytological profiles of commercial compounds. Hence, the phenotypic screen provides a new and robust opportunity for accelerating the evaluation of compound activity during high-throughput drug screens.

ACKNOWLEDGEMENTS

First and foremost, I wish to express my sincerest appreciation to my supervisor, Dr. Jonathan Draper, for introducing me to the vast and exciting field of breast cancer research. I am very grateful for your support, guidance and valuable advice throughout my master's study. Most of all, thank you for believing in my potential.

I would also like to thank my committee members, Dr. John Hassell and Dr. Kristin Hope for their time, encouragement and insightful comments. I wish to extend a special thank you to our collaborator, Dr. Robin Hallett, for his expertise and feedback which are invaluable to my success and the success of these projects.

I wish to thank my past and present colleagues in the Draper lab for their patience, support and friendship. In particular, I want to thank Rohan Nadkarni. You have been like a brother to me, sharing stressful times, hilarious moments, and sibling banters. I am happy and thankful to have made a lifelong friend. I also want to thank Sonam Bhatia, Dr. Soumeya Abed, Amanda Hrenczuk, and Garrett Bullivant.

I would like to thank Dr. Nathan Magarvey for providing the nature products for the screen and Michael Skinnider from his lab for generating the chemical cluster networks.

I would also like to thank the members of the Hassell lab, William Gwynne, Kayla Driver, Adele Girgis-Gabardo for their generous assistance and valuable input towards my projects.

Thanks also go to all members of the SCCRI especially Laura de Rooij, Ryan Mitchell, Enio Polena, Saleem Mahammad, Borko Tanasijevic and Jennifer Russell for providing me with their scientific expertise, personal and career advice, memorable anecdotes, encouragement and laughter.

Finally, a very special thank you to my mother and father for their unconditional love and support throughout my life. You have given me the strength, confidence and freedom to pursue my dreams.

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LIST OF ABBREVIATIONS

AR	Androgen receptor
BC	Breast cancer
Cas9	CRISPR associated protein 9
cDNA	complementary DNA
CRISPR	Clustered regularly interspaced short palindromic repeats
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
FUCCI	Fluorescent ubiquitination-based cell cycle indicator
H2B	Histone 2B
HER2	Human epidermal growth factor receptor 2
HTS	High-throughput screen
IF	Immunofluorescence
IHC	Immunohistochemistry
KO	Knockout
MoA	Mode of action
ORF	Open reading frame
pCR	Pathological complete response
PR	Progesterone receptor
SAR	Structural-activity relationship
SER	Saddle-edge-ridge (texture analysis)
TAX	Docetaxel (Taxotere)

CONTRIBUTIONS

Dr. Jonathan Draper – involved in the conception of both the p53-chemotherapy response and the phenotypic profiling projects, provided critical feedback for both projects

Dr. Robin Hallett – involved in the conception of the p53-chemotherapy response project and provided the bioinformatics result that forms the rationale for that project; provide critical feedback for the p53 and phenotypic screening projects

Dr. Soumeya Abed – performed IHC staining of explanted tumors slides for the p53-chemotherapy response project

Amanda Hrenczuk – generated the lentiGuide-BFP vector, lentiGuide plasmids and viruses for the CRISPR genetic screen associated with the phenotypic profiling project; scanned the IHC slides from the explanted tumors and assisted with cell culturing for the p53-chemotherapy response project

Garrett Bullivant – generated the western blots for p53-chemotherapy response project

Rohan Nadkarni – performed IF staining, imaging for the cell cycle kinetics assay associated with the p53-chemotherapy response project; assisted with cell culturing, transfections, lentiCas9 virus production and Cas9-MCF7 generation for the phenotypic profiling project

William Gwynne – helped monitoring, anesthetize and administer doxorubicin to mice for the p53-chemotherapy response project

Michael Skinnider – generated the chemical structure networks for the phenotypic profiling project

Dr. Nathan Magarvey – provided the microbial natural products for the phenotypic screen

INTRODUCTION

Breast Cancer

Cancer is one of the most critical health problems and also a leading cause of death worldwide (Stewart and Wild 2014; Canadian Cancer Statistics 2015). It is a heterogeneous and complex system of related diseases, and as such, despite advances in targeted therapeutics, cancer treatment remains a formidable challenge. Cancer is characterized by uncontrolled cell division, which can lead to tumor formation, and some cases can metastasize to distant sites.

Breast cancer has the highest incidence among women in Canada, with 1 in 9 females expected to develop breast cancer in their lifetime (Canadian Cancer Statistics 2015). In 2015, an estimated 25,000 new breast cancer patients will be diagnosed, accounting for 26% of all new female cases. Despite advances in therapy, breast cancer remains the secondly most deadly type of cancer. In 2015, 1 in 7 of all cancer patients succumbing to breast cancer. The cause of breast cancer is complex and not fully understood. Familial breast cancer due to germline mutations of tumor suppressor genes BRCA1, BRCA2 and/or TP53 accounts for approximately 5-10% of all breast cancer cases (Fackenthal and Olopade 2007).

Molecular classification of breast cancers

Breast cancer is a heterogeneous group of tumors that differ in clinical behavior and response to therapy (Niemeier et al. 2010). Analysis at the molecular level by gene-expression profiling has revealed that each breast tumor has its own unique molecular portrait, providing the basis for a molecular taxonomy of this disease (Perou et al. 2000).

Based on this molecular taxonomy, breast tumors are broadly divided into 2 groups: ER-positive and ER-negative, according to their expression of the estrogen receptor (ER). The first broad group, the ER-positive tumors, contribute to 75% of breast cancers (Niemeier et al. 2010). This group is subdivided into two subgroups: luminal A and luminal B. The second broad group, the ER-negative tumors, contribute to 20-25% of breast cancers (Niemeier et al. 2010). This group is subdivided into three subgroups: HER2 (ERBB2)-overexpressing (HER2+), basal-like, and normal-like. More recent studies revealed 2 additional albeit less common subtypes: claudin-low (Prat et al. 2010) and molecular apocrine (Doane et al. 2006; Sanga et al. 2009).

Table 1. Molecular subtype of breast cancer based on gene-expression profiling

Molecular subtype	Gene-expression profile	Frequency (%)
Luminal A	ER+, PR+, HER2–, AR+/–	50-60 %
Luminal B	ER+, PR+, HER2+, AR+/–	10-20 %
HER2+	ER–, PR–, HER2+,	15-20 %
Basal-like	ER–, PR–, HER2–, AR–	10-15 %
Normal-like	ER–, PR–, HER2–	<10 %
Claudin-low	ER–, PR–, HER2–	
Molecular apocrine	ER–, PR–, HER2–, AR+	

Treatment of breast cancer

Breast cancer treatment aims to control cancer progression and eliminate cancer cells. Breast cancer treatment is generally classified into two groups: local therapy and systemic therapy (Fig. 1) (NCCN 2013). Local therapy mainly involves surgery that is aimed to remove the tumor, while preserving normal breast tissue. In some instances, when the tumor is massive, mastectomy may be one of treatment options. Local therapy is often aided by radiation or chemotherapy as a method to shrink the tumor before the surgery.

Systemic therapy consists of chemotherapy and hormonal therapy, which targets cancer cells throughout the body. Chemotherapy can be given before the surgery (neoadjuvant) to shrink a tumor that is inoperable in its existing state or after the surgery (adjuvant) to reduce the risk of tumor recurrence and prolong disease-free survival (Early Breast Cancer Trialists' Collaborative Group (EBCTCG) 2005). The most common chemotherapy combinations used to treat breast cancer are anthracyclines and taxanes (NCCN 2013). Anthracyclines, most notably doxorubicin (DOX), are topoisomerase II inhibitors. During DNA replication, topoisomerase II causes transient double-strand DNA breaks, unwinds the DNA and ligates them, thereby relieving tension. Anthracyclines act by stabilizing a reaction immediate; they covalently link to topoisomerase II while the DNA strands are cut thus impeding DNA resealing. Topoisomerase II-mediated DNA damage can be followed by cell cycle arrest in G1, G2, and apoptosis (Perego 2001, Zunino 2001).

Anthracycline-based regimens are the preferred neoadjuvant chemotherapy for breast cancer patients because anthracycline-containing regimens were shown to be significantly more effective at preventing recurrence (0.89 to 1, $p = 0.001$) and increasing survival (0.84 to 1, $p < 0.00001$) than the standard CMF regimens (Early Breast Cancer Trialists' Collaborative Group (EBCTCG) 2005). An established standard of measuring clinical response to neoadjuvant chemotherapy is a pathological complete response (pCR) (Kuerer et al. 1999). pCR is defined as the absence of residual invasive disease in the breast and in the axillary lymph nodes at the completion of the neoadjuvant treatment (Kuerer et al. 1999). It is recognized that patients that had a pCR with neoadjuvant therapy tend to have a significantly better prognosis. In a study examining the response of breast cancer

patient to DOX-based neoadjuvant chemotherapy, the 5-year overall and disease-free survival rates were significantly higher in the group who had a pCR than in the group who had not (88% to 61%; $p < 0.01$) (Kuerer et al. 1999). Hence, patients that achieve a pCR are at much lower risk for subsequent distant disease recurrence. However, more recent large randomized long-term studies have revealed that among breast cancer patients who had anthracycline chemotherapy, 60% achieved long-term disease-free survival and only 20% had a pCR (Evans et al. 2005; Mansi et al. 2010).

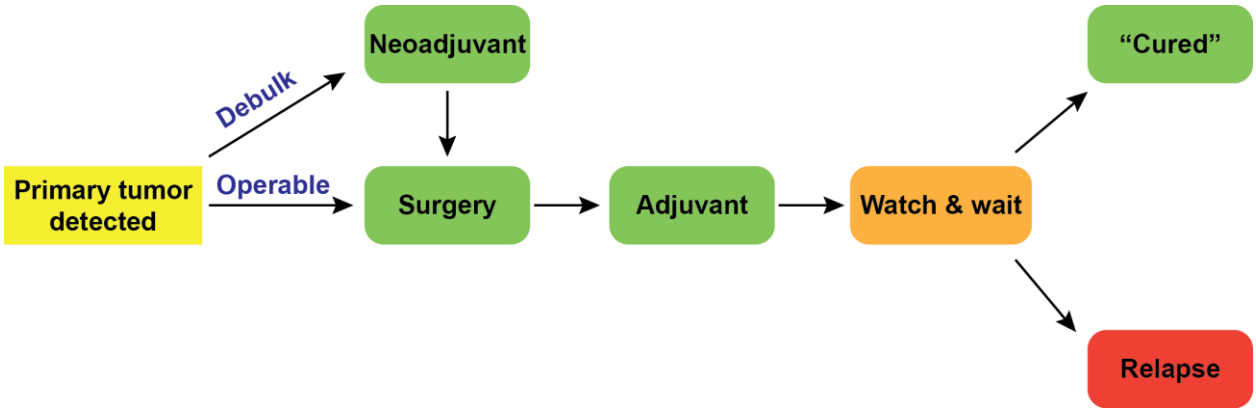


Figure 1. Treatment of breast cancer.
This figure is adapted from (Waddell 2013)

Cell cycle kinetics dictate chemotherapy response and is linked to TP53 integrity

Given that chemotherapy fails to provide a durable cure for the majority of cancer patients, it is evident that additional insight to the mechanisms associated with chemotherapy resistance is necessary (Hallett 2015). Genes that express differentially in breast tumors before and after neoadjuvant chemotherapy treatment were identified and compared to the follow-up patient response data (Hallett 2015). Following chemotherapy, tumors that show a decrease in cell cycle related gene expression are found to be associated with

chemotherapy resistance and poor clinical response (Fig. 2) (Hallett 2015). The gene expression signature that predicted these changes proved to be a reliable predictor of response for patients with breast, ovarian, and colon tumors to chemotherapy (Hallett 2015). Moreover, the decrease in cell cycle related gene expression is linked to p53-mediated G1/G0 cell cycle arrest. Thus suggesting that the chemotherapy resistance is associated with the functional p53 activity (Fig. 2) (Hallett 2015).

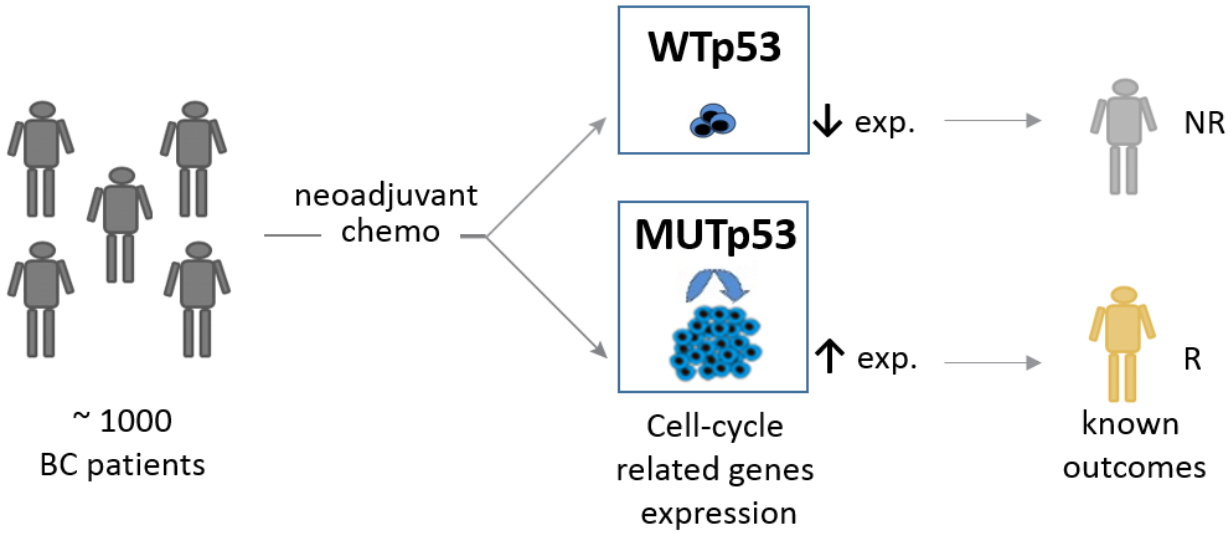


Figure 2. Cell cycle kinetics dictate chemotherapy response and is linked to TP53 integrity

TP53 gene

The TP53 gene is the most frequently mutated gene in breast cancer (Muller and Vousden 2013). Its occurrence is highly associated with molecular tumor subtypes and ER expression (Dumay et al. 2012; Coates et al. 2012). Mutations in TP53 are less frequent in ER+ tumors (17% of luminal A, 41% of luminal B) and more frequent in ER– tumors (50% of HER2 amplified, 69% of molecular apocrine, and in 88% of basal-like) (Philippe

Bertheau et al. 2013). The p53 tumor suppressor protein, encoded by the TP53 gene, is a transcription factor that when activated as part of the cellular stress response, regulates suites of genes involved in cellular processes including the cell cycle, apoptosis, and senescence to maintain the integrity of the genome (Brosh and Rotter 2009). In response to DNA damage, wild-type p53 mediates a G1 cell cycle arrest through the transactivation of p21Cip1/Waf1, a cyclin-dependent kinase inhibitor. p21Cip1/Waf1 inhibits the phosphorylation and activation of Cdk2 associated with Cyclin D or Cyclin E, which in turn prevents the phosphorylation of one of the critical downstream protein targets, retinoblastoma protein (Rb). Rb, in its hypophosphorylated state, binds and sequesters E2F. E2F is a transcription factor that is required for cells to enter S phase (Johnson et al. 1993). Thus, DNA damage can trigger a p53 mediated p21 induced G1 cell cycle arrest that blocks cells from S-phase entry. In addition, p53 can induce apoptosis in susceptible cells in which the damage is beyond repair thereby protects the tissue against transmission of DNA abnormalities. The current model of an apoptotic cascade begins with cell death signals reaching the BH3-domain proteins (Fridman and Lowe 2003). BH3-domain proteins bind and activate to pro-apoptotic proteins, Bax, and Bak, which in turn drive the release of cytochrome C by the mitochondria. Cytochrome C activates the Apaf-1/caspase-9 pathway leading ultimately to caspase 3 activation and cell death (Fridman and Lowe 2003). Mutations in p53 adversely affect its ability to bind regulatory DNA sequences of pro-apoptotic genes thus preventing a cascade of downstream effects. Mutation of the p53 gene increases the risk of developing breast cancer and affects the biology of cancer cells and their response to therapy (Olivier, Hollstein, and Hainaut 2010).

Investigate the role of p53 status in ER+ breast cancer and response to chemotherapy

Given that TP53 status is related to cell cycle kinetics and changes in cell cycle kinetics is related to chemotherapy response, further investigation between TP53 integrity and chemotherapy response is necessary. Evaluation of reported gene signatures that predict p53 mutation (p53 signatures) (Fig. 4A) show that they have significant capacity to identify p53 mutations in a TCGA breast cancer cohort (Fig. 4B&C) and predict patient response to neoadjuvant chemotherapy (Fig. 4D–F), indicating at relationship between p53 status and patient response (Hallett and Huang, unpublished). However, the signatures are also significantly associated with breast cancer subtype and estrogen receptor (ER) status (Fig. 5A&B). A stratified analysis of the ER+ or ER– only cohort reveals that the signatures predict response in ER+ tumors (Fig. 5C&D) but not ER– tumors (Fig. 5E–G). Hence, the data suggests that p53 predictive signatures are only able to indicate response in the ER+ tumors. Based on the preliminary result, an investigation into the role of p53 and chemotherapy sensitivity in ER+ breast cancer cell lines and tumors is launched.

Drug discovery

Although there has been an enormous increase in our knowledge concerning the molecular pathogenesis of cancer and mechanisms associated with chemotherapy resistance in the past two decades, our ability to control, much less cure, cancer has been disappointing (Ruddon 2010). Anthracycline chemotherapy, the mainstay neoadjuvant treatment that produces a relatively superior outcome, is efficacious in only 60% of treated BC patients (Evans et al. 2005; Mansi et al. 2010). Latest information indicates that wild-type p53 is

associated with chemotherapy resistance in BC patients with ER+ tumors (Hallett 2015; Hallett and Huang unpublished). As such, new anticancer agents effective for the group of BC patients with ER+ p53 wild-type tumors are urgently needed. Development in personalized medicine where drugs are tailored to groups of patients who share a common response to a therapeutic agent is also necessary. Unfortunately, oncological research has one of the poorest records in terms of novel drugs in clinical development (Kamb, Wee, and Lengauer 2007). So why is cancer drug discovery so difficult? Based on an insightful article by Kamb *et al.*, there are broadly three elements linked to the poor success in anticancer drug discovery and development: targets, drugs and patients (Kamb, Wee, and Lengauer 2007). First, anticancer drug targets are divided between those that have essential functions and those that have non-essential functions. Drug targets that have an essential function directly impact the survival of a cell. As a result, drugs that modulate these targets usually have a narrow therapeutic window, and becomes toxic once outside of the beneficial dose range. In contrast, non-essential targeting drugs act on a drug target that is non-essential in normal cells but is essential for tumor cells. However, it is limited by the discovery of selective tumor-depend druggable targets. Even in cases where such drugs are developed, the accumulation of mutations may enable tumor cells to lose their dependency on the non-essential target. Over time tumors may become drug resistance. Next, an optimal target is incomplete without a corresponding drug. There are two categories of anticancer drugs: selective and multi-targeted. Selective drugs are beneficial such that they require a lower therapeutic dose and few to none off-target effects making the clinical data easier to interpret. However, due to target specificity, prolong use of selective drugs may

cause tumors to acquire drug resistance. Conversely, multi-targeted drugs that inhibit several effectors or pathways are less susceptible to drug resistance. Nevertheless, they are prone to off-target effects. In the clinic, increasing the dose of multi-targeted drugs is associated with improved efficacy but also increased toxicity and unforeseen side-effects. Lastly, cancer is an exceptionally heterogeneous disease and patient variability adds additional complications, hence compounding the complexity of anticancer drug discovery. Genetic variability in patients can affect their pharmacokinetic and pharmacodynamic responses to anticancer agents. Also, tumors found in the same location in different patients can originate from different cell types and location within their body, have entirely distinct gene expression profile, and response to therapy.

In spite of the challenges facing anticancer drug discovery, there has been much exciting development in the field associated with a host of disciplines and technologies. Advances in organic chemistry have facilitated that complete synthesis of complex natural products with powerful pharmacological activities (Pors et al. 2009). Combinatorial chemistry has expanded greatly the numbers of drug-like compounds which can be analyzed for anticancer activities (Lam 1997). Publicly accessible databases of compounds, such as PubChem, ChemSpider, DrugBank, and ChemBank, provide chemical structure, physiological effect and biological mode of actions on tens of millions of compounds (Lazo, Brady, and Dingledine 2007). Advances in automated liquid handling platforms have enabled high-throughput screening of hundreds of thousands of compounds for bioactivity (Linask and Lo 2005; Pegan et al. 2010). Similarly, high content platforms have enabled researchers to directly assess compounds for cellular actions (Perlman et al. 2004).

Ultimately, the development in these various disciplines integrates and benefit the process of new anticancer drug discovery.

Two main strategies of drug discovery – targeted-based versus phenotypic screen

High-throughput drug screening has been the mainstay of drug discovery for pharmaceutical and biotechnology companies over the last two decades (Swinney and Anthony 2011). Fundamentally, there are two main types of screening strategies that are typically employed to identify new drug candidates at a preclinical stage: target-based screening and phenotypic screening. (Fig. 3) (Schenone et al. 2013) Target-based screen is conducted with the goal of finding a modulator, usually an inhibitor, of a molecular target that is believed to be critical for the cancer phenotype (Drews 2000) It often requires testing a vast number of compounds against a single target protein, whereby the active hits then undergo more optimization via chemical methods (Drews 2000). However, a landmark study on new molecular entities (NME) has revealed that the target-based approaches trail behind the traditional phenotype-based approaches according to the number of first-in-class small molecule drugs generated (Swinney and Anthony 2011). An NME is a drug that contains an active moiety that has not been approved by the FDA previously. Between 1999 and 2008, 37% of all NMEs are discovered via phenotypic screening compared to 23% by target-based approach (Swinney and Anthony 2011). A crucial limitation of target-based methods is the fact that many compounds are discovered to interactive with multiple targets (Mestres et al. 2009). As a result, compounds identified using target-based approaches often undermine the one drug, one target dogma which is held to be the cornerstone of target-based methods (J. Lee and Bogyo 2013). This limitation has lead

facilitated a paradigm shift and combined with the latest technological development in proteomics, genomics, and image-based methods have catalyzed a renewed interest for phenotypic screening methods (J. Lee and Bogyo 2013). In contrast, phenotypic screen relies on observing phenotypic changes of cells, organs, or organisms produced by chemical substances (J. Lee and Bogyo 2013). An important benefit of phenotype-based methods is that it provides an unbiased approach to discover active compounds in a complex biological systems (J. Lee and Bogyo 2013). Since phenotypic screening occurs in a physiologically pertinent settings of cells, organs, or organisms, the results in this context offer a more direct view of the desired responses while highlighting potential side effects (J. Lee and Bogyo 2013). Moreover, phenotypic screens can bring about the discovery of multiple proteins or pathways which may be formerly associated with a given biological output. Thus, narrowing down and distinguishing the molecular targets of active hits from phenotypic screens are important processes that are necessary to comprehend underlying mechanisms and optimize active compounds further (J. Lee and Bogyo 2013). However, subsequently determining the relevant molecular targets has often proven slow or impossible (Swinney and Anthony 2011).

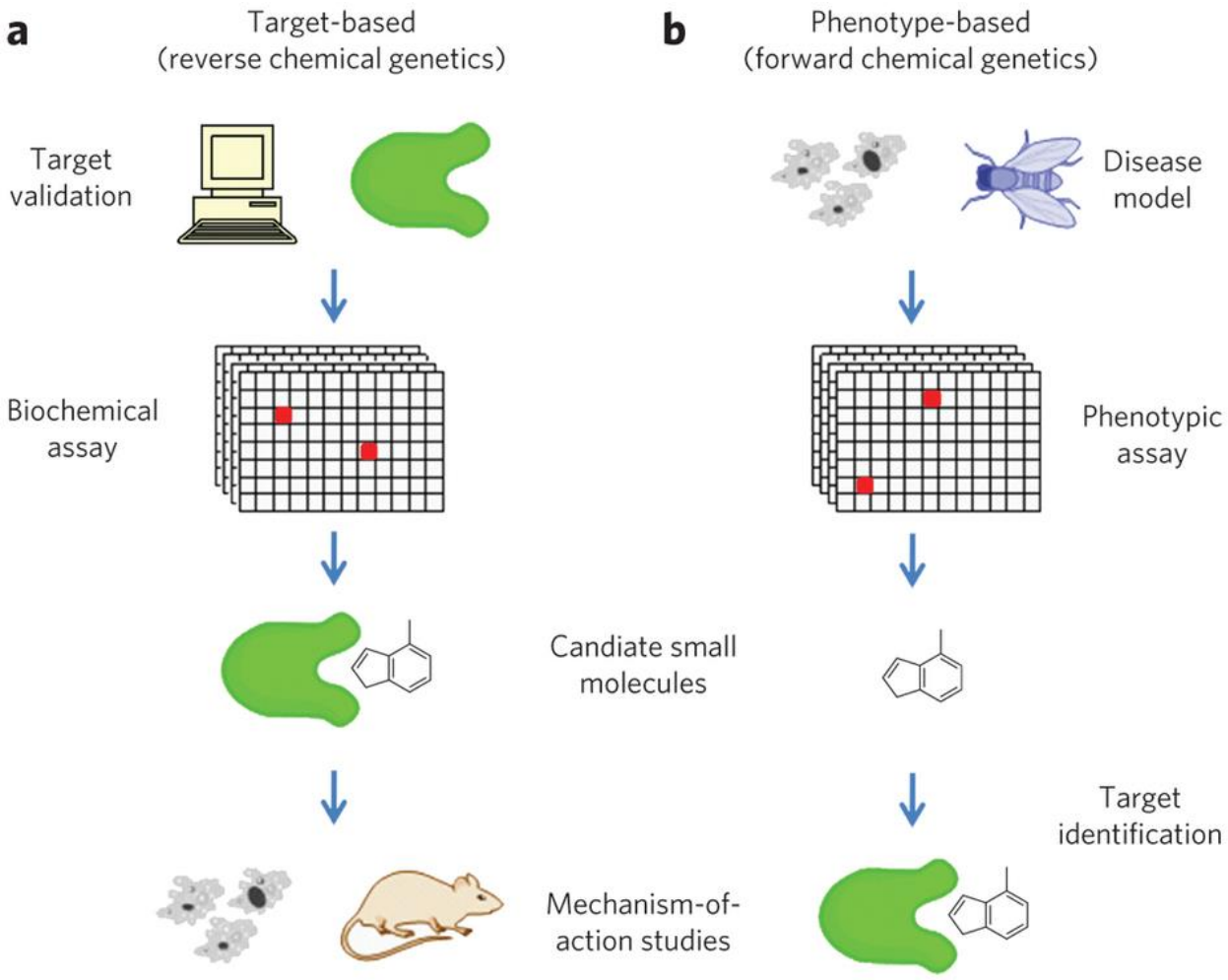


Figure 3. High-throughput screening approaches.
Target-based screen starts with target validation followed by assays to identify candidate compounds. Phenotypic screen starts with the desired phenotype induced by compounds on a system then proceed to pinpoint their target. This figure is adapted from (Schenone et al. 2013)

Target identification approaches in phenotypic screening

In the past decade, a number of technologies have been developed to identify targets from phenotypic screens. In particular, transcriptional (Butte 2002; Yang and Speed 2002), proteomic (Stockwell, Haggarty, and Schreiber 1999; H Zhu et al. 2001), and cell-based

(Iorio et al. 2010; Perlman et al. 2004; Tanaka et al. 2005) approaches have achieved successful target detection.

Gene expression profiling, using a cDNA microarray, measures the mRNA transcript level of hundred or even thousands of genes in an experiment (Butte 2002; Yang and Speed 2002). The technology was designed more than ten years ago and is now widely used (Butte 2002; Yang and Speed 2002). One of the advantages of this method is that it has demonstrated moderate success in identifying the mode of action of a drug using microarray data (Gunther et al. 2003; Hughes et al. 2000). However, apart from the inability to apply this technique in a high-throughput setting, the gene expression profiling approach suffers from two main drawbacks: high cost and the lack of standardization among experimental data sets thus making the comparison a challenge (Bugelski 2002; Butcher and Schreiber 2005).

Protein level and its modifications are crucial indicators of cell signaling and activities (Eipper 2008). Their precise measurement using accurate probes can offer a detailed evaluation of the biological activities and state of a cell (Eipper 2008). Development in the proteomic analysis has produced many new detection approaches. Antibody-based assays, immunoblots (Western blots) and ELISAs (Enzyme-Linked Immunosorbent Assays) are used to detect a protein-state change in HTS (Stockwell, Haggarty, and Schreiber 1999). A great number of multiplexed chip-based protein assays rely on the selective antibodies to be available for detecting the relevant protein modification (Heng Zhu and Snyder 2003). However, the development of selective

antibodies to accurately detect protein modifications remains lacking (Heng Zhu and Snyder 2003).

The application of cell-based phenotypic screening to identify drugs has been demonstrated by a number of studies (Iorio et al. 2010; Perlman et al. 2004; Tanaka et al. 2005) using cutting-edge technologies in high content imaging to measure simultaneously many cellular features while developing cytological profiles that alter according to the drug exposure. Across these studies, cells are drug-treated, fixed, stained with dyes or fluorescent conjugated antibodies to demonstrate cytological features, and measured using high content imaging. Some drawbacks of this method include small library size, cell-loss and systemic artifacts due to cellular fixation, and the high cost and some lot-to-lot variation of immunofluorescent antibodies especially in the case of high-throughput screening.

Hypotheses and Research objectives

The research objectives of this thesis were to 1) investigate the relationship between TP53 status and chemotherapy response in ER+ human breast cancer. Given that mutant TP53 status is associated with chemotherapy sensitivity and TP53 status is a predictor of patient response to chemotherapy but limited to ER+ tumors, it is hypothesized that among ER+ breast tumors, mutant TP53 confers an elevated chemotherapy sensitivity compared to wild-type TP53 and 2) demonstrate a streamlined high-content cell-based phenotypic profiling assay that elucidates the mode of action of unknown compounds. Given the underlying molecular heterogeneity of breast cancer, the predominantly used anthracycline chemotherapy produces an overall clinical response in only 60% of the treated BC patients

(Evans et al. 2005; Mansi et al. 2010). Also, BC patients with ER+ p53 wild-type tumors are shown to be associated with chemotherapy resistance (Hallett 2015; Hallett and Huang unpublished). Taken together, an approach that enables the discovery of anticancer agents tailored to a specific group of patients who share a common response to a therapeutic agent is required. A phenotypic drug discovery approach is highly suitable for this purpose. In order to deduce MoA from drug response phenotype, their relationship must be determined first. It is hypothesized that compounds with related mode of action (MoA) induce similar cellular phenotypes.

The specific aims of this research were two-fold:

First, investigate the role of p53 status in ER+ breast cancer and response to chemotherapy

1) Engineer isogenic p53 mutant ER+ breast cancer cell lines (MCF7, BT474, ZR751)
2) Compared cell cycle kinetics and chemosensitivity of isogenic BC cell lines expression WT and MUT p53
3) Determine the effect of p53 mutations in isogenic cell lines on chemotherapy response in vivo

Next, demonstrate a high-content and high-throughput live-cell phenotypic assay, which streamlines the process of cytological profiling and provides a consistent platform for empirically evaluating drug action and drug discovery.

1) Assess the relationship between compounds' MoA and the induced cellular phenotype
2) Employ structural clustering and phenotypic profiling to elucidate SAR

3) Use the phenotypic profiling platform to identify a novel group of potentially anti-cancer agents derived from natural products

MATERIALS AND METHODS

Cell culture

MCF-7, BT474, ZR-75-1 and T-47D breast cancer cell lines were obtained from the ATCC. MCF-7 cell lines were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS), 1% GlutaMAX (Life Technology), 1% non-essential amino acid (NEAA, GIBCO). BT474, ZR-75-1 and T-47D cell lines were grown in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% FBS, 1% GlutaMAX, 1% NEAA. All cell lines were grown at 37°C and 5% carbon dioxide.

Table 2: ER+ p53 wild-type and mutant breast cancer cell lines

Cell line	p53 status (Wasielewski et al. 2006; Neve et al. 2006)	ER status (Neve et al. 2006)	Culture medium (Neve et al. 2006)
MCF7	wild-type	ER^+	DMEM + 10% FBS + 1% GlutaMax + 1% NEAA
BT-474	wild-type	ER^+	RPMI + 10% FBS + 1% GlutaMax + 1% NEAA
ZR-75-1	wild-type	ER^+	RPMI + 10% FBS + 1% GlutaMax + 1% NEAA
T-47D	mutant	ER^+	RPMI + 10% FBS + 1% GlutaMax + 1% NEAA

TP53 targeting vector construction

Construction of the CRISPR-Cas9 short guide against TP53 was previously described (Malina et al. 2013). The single guide RNA sequences were depicted below along with orientation and genomic coordinates within the human genome. pX459 TP53 was created

by ligating the TP53 insert into a BbsI digested pSpCas9(BB)-2A-Puro plasmid (pX459) (Addgene #48139), and validated by sequencing (MOBIX; McMaster University).

Table 3: Sequences of sgRNA targeting TP53

sgRNA	Genomic coordinate	Target	Sequence	
TP53 exon 7	Chr 17: 7585011..7585030	3' splice acceptor	Forward	5′–3′ CACCGgcctgtgttatctcctaggt
			Reverse	3′–5′ ccggacacaatagaggatccaCAAA

Generation of isogenic p53 mutant breast cancer cell lines

Isogenic cell lines (MCF7 p53 mutant, BT-474 p53 mutant, ZR-75-1 p53 mutant) were produced by transfection with 2.5 µg of pX459-TP53 plasmid DNA. 48 hours post-transfection, the transfected cells were passaged as single cells and plated at low density with media containing 10µM of Nutlin-3a, which causes cells with wild-type p53 to cease proliferation. After 1-2 weeks of incubation in the presence of Nutlin-3a, proliferating cells formed colonies, which were picked and gradually expanded for subsequent experiments. TP53 mutations were validated by sequencing (MOBIX; McMaster University). Primer sequences used as follows: p53-seq1-S, ACCATCCTGGCTAACGGTGAAACCCCGTC (sense strand), p53-seq1-AS, TCCAGTGTGATGATGGTGAGGATGGGCCT (antisense), p53-ex5seq1-F, ACTCCCCTGCCCTCAACAAGATGTTTTGCC (sense strand), p53-ex8seq1-R, GGCTCCCCTTTCTTGCGGAGATTCTCTTCC (antisense).

Immunofluorescence staining and analysis

Breast cancer cells were cultured in 96 well plates, washed with PBS, fixed with 4% PFA, washed with PBS, permeabilized with 100% ice-cold methanol and washed with PBS.

Cells were stained with antibodies to Ki67 (mouse monoclonal, Cell Signaling 8D5; 1:100 dilution) or p21 (rabbit monoclonal, Cell Signaling 12D1; 1:400 dilution) in 1% BSA in PBS at 4°C overnight, washed with PBS and stained with secondary antibodies donkey anti-mouse AlexaFluor 647 (Life Technology A31571; 1:500 dilution), donkey anti-rabbit AlexaFluor 647 (Life Technology A31573; 1:500 dilution). Nuclei were co-stained with Hoechst 33342. Plates were imaged on an Operetta High Content Screening System (Perkin Elmer) and images uploaded to a Columbus database (Perkin Elmer) and image analysis of immunofluorescence and reporter fluorescence was performed using Acapella high content and analysis software (Perkin Elmer). Cell nuclei were identified by Hoechst 33342 staining and the fluorescence intensity of the same nuclei in the GFP, Cy3 and Cy5 channels measured. Custom scripts were then used to quantify the fluorescent intensity of each nucleus in all channels and output statistics.

Immunohistochemical staining of explanted tumors

IHC staining for human mitochondria (hMito), α-smooth muscle actin (αSMA), Ki67, p21, cleaved caspase 3 (CC3), CK5, CK8, Sox2 and Sox9 expression was performed on explanted tumors and lungs of MCF7 p53 wild-type or mutant mice. Excised tumors were cut into 2-3 mm sections, fixed in 10% neutral buffered formalin for a minimum of 48 h and then paraffin embedded. A set of 5 µm thick sections were dewaxed in xylene and rehydrated prior to antigen retrieval. The primary antibodies used were mouse anti-hMito (1:500 dilution, Sigma), rabbit anti-αSMA (1:200 dilution, Sigma), mouse anti-Ki67 (1:400 dilution, Cell Signaling 8D5), rabbit anti-p21 (1:50 dilution, Cell Signaling 12D1), mouse

anti-cleaved caspase 3 (1:1000 dilution, TBD), rabbit anti-CK5 (1:500 dilution, TBD), rat anti-CK8 (1:3 dilution, Troma hybridoma supernatant), mouse anti-sox2 (1:400 dilution, BD 561469), rabbit anti-sox9 (1:400 dilution, abcam ab76997). Secondary antibodies used were donkey anti-mouse AlexaFluor 488 (Life Technology A21202), donkey anti-rabbit AlexaFluor 647 (Life Technology A31573), goat anti-rat AlexaFluor 488 (Life Technology A21212), and Hoechst 33342.

Flow cytometric analysis of apoptosis

To quantify the apoptotic cell death, breast cancer cells were stained with Annexin V-AF647 and the results were analyzed using flow cytometry according to the manufacturer's specifications. Briefly, cells were harvested following treatment with chemotherapy agents (Doxorubicin, 100 nM, L.C. Laboratories D-4000-200MG; Etoposide, 1 μM, L.C. Laboratories E-4488-2G; Docetaxel, 10 μM, L.C. Laboratories D-1000-500MG; 5-Fluorouracil, 10 μM, Sigma F6627-1G) and washed twice with PBS. Cells treated with the DMSO (as a vehicle) were considered as a control. Cell pellets were resuspended in 400 μl of 1× annexin V binding buffer, following gentle vortex, 1 μl of annexin V-AF647 and 20μl of 7-AAD were added to each sample. Cells were incubated 15 min at room temperature in the dark and further analyzed by LSRII flow cytometer (BD). Analysis was performed using FlowJo software.

Transplantation assay

Female NOD/SCID mice, six weeks old, were transplanted subcutaneously with $1x10^7$ MCF7 H2GFOIP (Calder et al. 2013) cells into the second thoracic mammary fat pad. Half of the mice received MCF7 WTp53 cells while the other half received MCF7 Mutp53 cells. Mice were monitored for tumor development and tumor volume was measured with a digital caliper using the formula Tumor volume = (length × $width^2$)/2. Seven weeks post transplantation, the mice were injected with 5 mg/kg doxorubicin (L.C. Laboratories, D-4099-100MG) in PBS intraperitoneally once a week for 4 consecutive weeks. Once the mice reached endpoint, tumors and potential organ for metastasis (lungs, liver, and brain) were harvested, and portions were fixed in 10% formalin for 48 hours and paraffin embedded.

Cell culture and compound screening

The generation and culture of the MCF7 cells containing the H2BGFP-FUCCI reporter were described elsewhere (Hallett 2015). Prior to the screen, MCF7 cells were established in a passage routine of 1.5 x 10^6 cells seeded per 60 cm^2 every 2 days to yield exponential growth, as gauged by FUCCI-G1 expression. For the screen, 384 well plates (Perkin Elmer cat #6007550) were seeded with 3,000 H2BGFP-FUCCI MCF7 cells per well and compounds added 24 hours later. Compounds were sourced from the Canadian Compound Collection bioactive subset (fhs.mcmaster.ca/cmcb/hts_small_molecule_libraries.html) at 1mM and screened in triplicate at a final concentration of 1μM. 48 hours after compound addition, plates were scanned using an Operetta High Content Screening System (Perkin

Elmer) to image the H2BGFP-FUCCI reporter. A minimum of 300 cells was counted per well.

Image analysis

All images acquired on the Operetta were uploaded into Columbus Image Data Storage and Analysis System (Perkin Elmer). Individual cells were identified via H2B-GFP and 12 distinct parameters, including percentage of cells in G1-phase (via FUCCI), nuclear roundness, nuclear shape (normal, fragmented, and condensed), texture features (SER (http://www.perkinelmer.com/Content/ApplicationNotes/APP_PKCActivation_textureanalysis.pdf), Haralick (Haralick, Shanmugam, and Dinstein 1973), Gabor (Grigorescu, Petkov, and Kruizinga 2002)), were measured. Image analysis was completed autonomously using custom scripts providing a consistent and accurate measurement of requested phenotypic parameters.

Compound-activity clustering

Activity clustering was performed using Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm). Activity was calculated by filtering for compounds that displayed at least 1 observation with an absolute value change of >=2.5. Hierarchical clustering of compounds and phenotypic scores was performed using the Absolute Correlation (uncentered) similarity metric with average linkage.

Chemical structure network generation

For each dataset, 1,024-bit circular fingerprints were generated using the ECFP algorithm with a radius of 6 (Rogers and Hahn, 2010). A Tanimoto coefficient matrix was generated by calculating the Tanimoto coefficient of each fingerprint pair. The Chemistry Development Kit (version 1.5.9) (Steinbeck et al. 2006) was used to generate chemical fingerprints and calculate Tanimoto coefficients. Fingerprint pairs with a Tanimoto coefficient greater than 0.45 were used to construct a tabular network file with a single interaction type and the Tanimoto coefficient corresponding to the edge weight. Chemical structure networks were rendered using Cytoscape (version 3.2.1) using the yFiles organic layout algorithm. Attribute data was imported as node tables and used to stylize the networks.

RESULTS

p53 status is a significant predictor of breast cancer patient response to neoadjuvant chemotherapy

In a previous study (Hallett 2015) using detailed genomic analyses in large cohorts of breast cancer patients, we found that p53 status was both a predictor and functionally involved in tumor cell responses to neoadjuvant chemotherapy. To investigate further, we proposed to identify transcriptional predictors of p53 mutations in breast cancers. We identified 40 previously reported signatures of p53 status from the literature as well as using the GSEA database (Fig. 4A). We assessed each signature's capacity to identify breast tumors with mutant TP53 from the TCGA cohort of breast cancer patients (n~1,000) using the Wilcoxon Rank Sum test, and then ranked them based on their predictive accuracy. Ranking based on p-value score (-log[p-value]) revealed a set of 10 p53 status signatures that were robust predictors of breast tumor p53 status. For instance, the p53 signature described by Gatza et al. 2014 (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4300117/), that predicts WTp53, was nominally the best predictor of p53 status as assessed by t-test and receiver-operator characteristic curve analysis (Fig. 4C&D).

To assess the relationship between p53 status and chemotherapy response, we tested the capacity of the 10 best p53 signatures to predict breast cancer patient response to neoadjuvant chemotherapy. We obtained gene expression profiling data derived from approximately 500 breast tumors for which response to neoadjuvant chemotherapy was also available (GSE25066). Based on the Wilcoxon Rank Sum test, we observed that each of the 10 best p53 status signatures was a robust predictor of patient response to

neoadjuvant chemotherapy (Fig. 4A). Using the P53 signature as an example, we found that p53 signature scores were significantly elevated in non-responders (Fig. 4B), and was associated with a response based on ROC analysis (Fig. 4C AUC: 0.68, p<0.0001). Hence, these data suggested that p53 status is a significant predictor of breast cancer patient response to neoadjuvant chemotherapy.

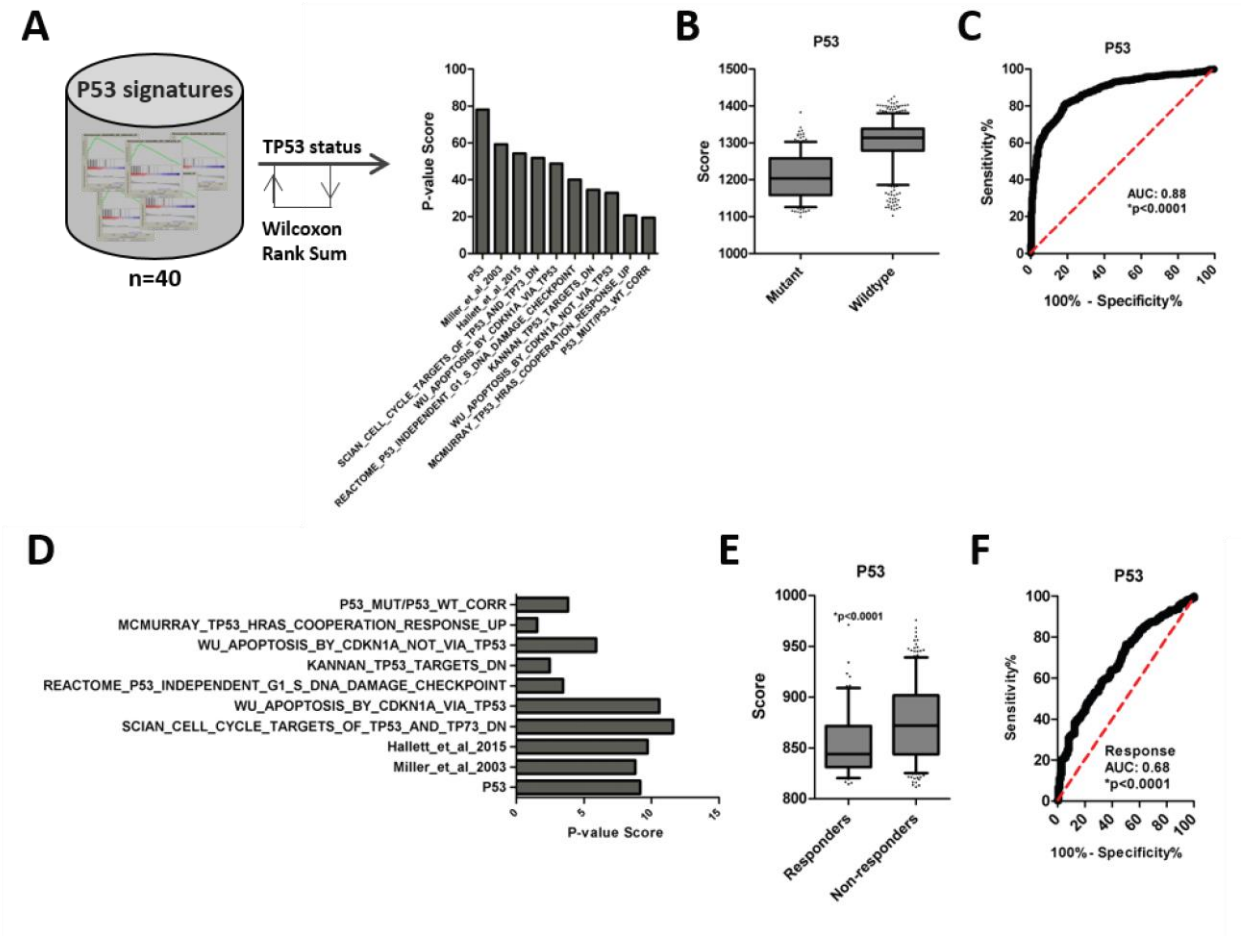


Figure 4. p53 status is a significant predictor of breast cancer patient response to neoadjuvant chemotherapy.
(A, B, C) Top 10 p53 signatures are selected from 40 published p53 signatures based on their capacity to identify p53 mutations in the TCGA breast cancer cohort (>1,000) patients. (D, E, F) The top p53 signatures are also significant at predicting response to neoadjuvant chemotherapy. Analysis and figures generated by Dr. R. Hallett.

p53 status is associated with breast cancer patient response to neoadjuvant chemotherapy only in ER+ tumors

Given differences in response to chemotherapy and the prevalence of p53 mutations in ER+/ER- breast tumors or among the molecular subtypes of breast cancer, we tested whether the relationship between the p53 status signatures and chemotherapy response signatures might be confounded by ER expression. Hierarchical clustering of GSE25066 samples revealed a significant relationship between p53 signature score, molecular subtype and ER status (Fig. 5 D). ER+ tumors were clearly enriched in tumors with high scoring tumors based on the P53 signature, and generally showed low scores for signatures associated with MUTp53 (Remaining 9). Indeed, P53 signature scores were significantly higher in ER+ tumors relative to ER– tumors (Fig. 5E). Based on this data we concluded that TP53 status signatures are associated with patient response to chemotherapy, however, these observations are potentially confounded by ER status. To confirm that that the relationship between TP53 status and chemotherapy response was not completely confounded by ER status, we examined the capacity of TP53 status signatures to predict chemotherapy response in stratified subsets of only ER+ or ER– breast cancer patients. Based on the Wilcoxon Rank Sum test we observed that most of 8/10 of the best TP53 status signatures were significantly associated with response in ER+ patients (Fig. 5F), whereas only 2/10 were associated with response in ER– patients. Overall, these data suggest that in pan-breast cancer TP53 status is a predictor of patient response to chemotherapy, however in stratified breast cancer on the basis of ER status, TP53 predicts outcome in patients with ER+ tumors but not those with ER– tumors.

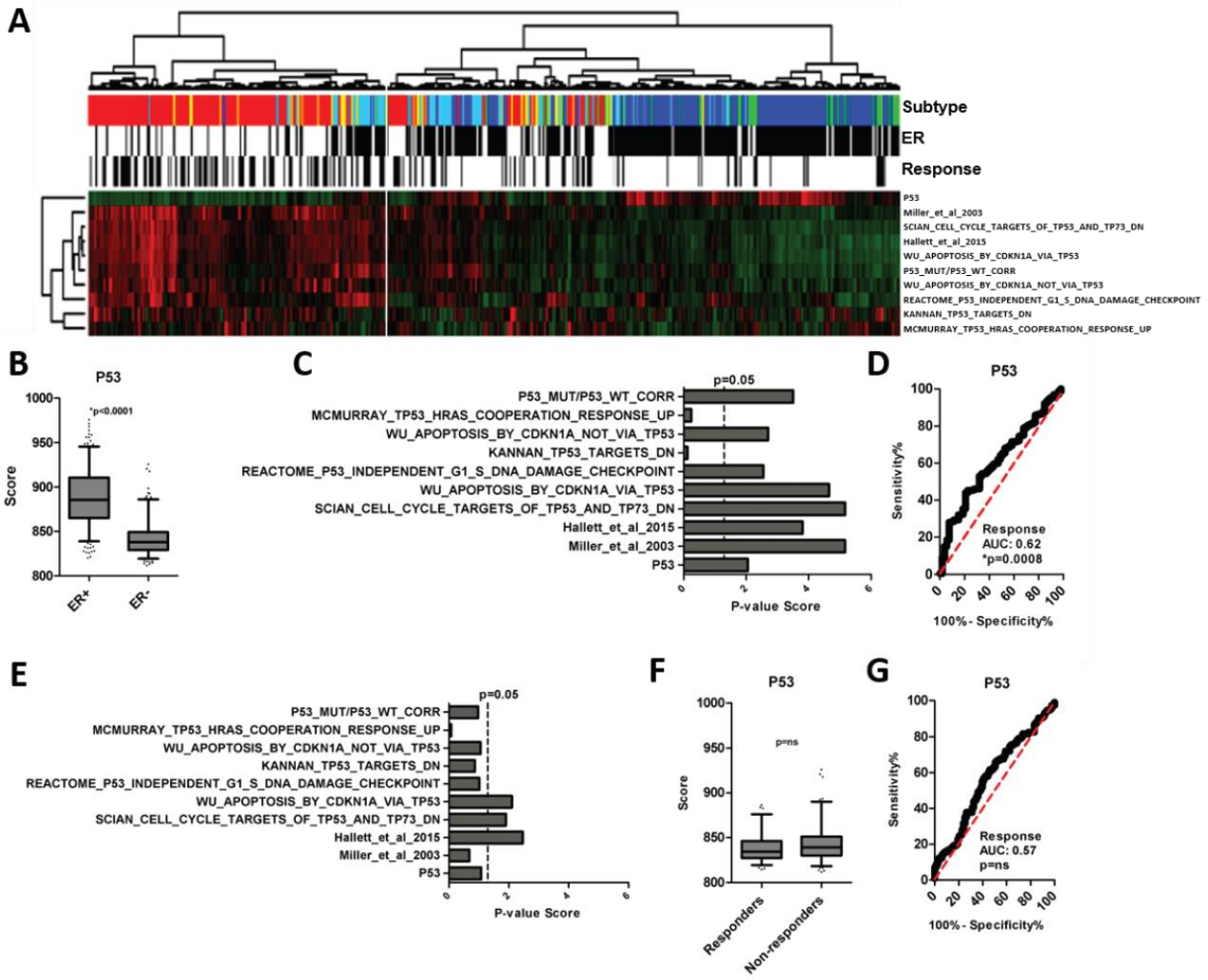


Figure 5. p53 status is associated with breast cancer patient response to neoadjuvant chemotherapy only in ER+ tumors.

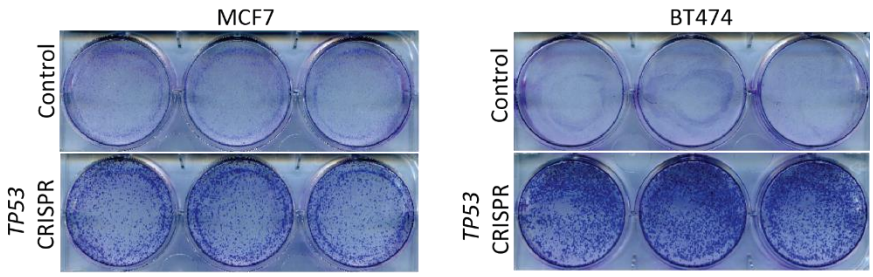
(A) Top p53 signatures are significantly associated with breast cancer subtype, especially ER status. (B) The signatures are significant at identifying ER+/ER– tumors. (C, D) In a stratified cohort of only ER+ tumors, the signatures predict patient response. (E, F, G) In a stratified cohort of the only ER– tumors, the signatures do not predict patient response. Analysis and figures generated by Dr. R. Hallett.

Isogenic p53 mutant BC lines generated from TP53 targeting CRISPR/Cas9 show resistance to Nutlin-3A

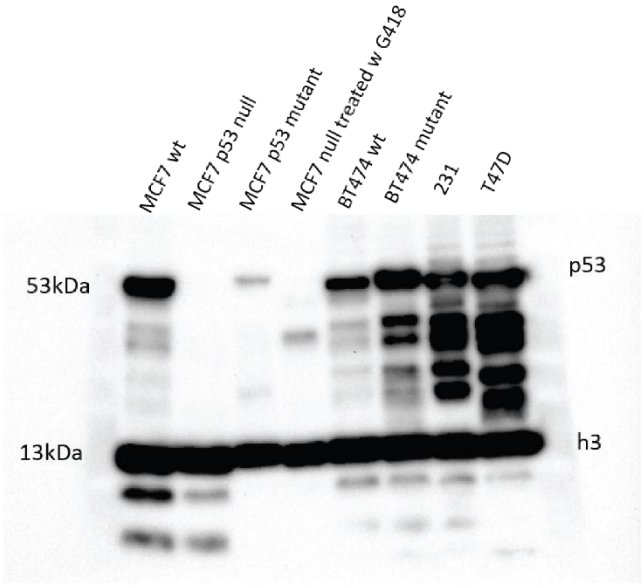
In order to address the relationship between TP53 status and chemotherapy response in ER+ breast cancer, we assayed ER+ isogenic p53 wild-type and mutant BC cell line pairs' response to chemotherapy. Three isogenic p53 mutant cell lines (MCF7, BT474, ZR751) were engineered from natively p53 wild-type human BC cell lines with CRISPR/Cas9-mediated genome editing (Ran et al. 2013; Drost et al. 2015) by introducing a mutation in the 3' splice-acceptor site of exon 7 of TP53. Cells from the three ER+ p53 wild-type BC cell lines were transfected with a Cas9-TP53 targeting vector. Nutlin-3a (Vassilev et al. 2004) was used to select for cells with a functionally inactive TP53 pathway (Fig. 6A). Nutlin-3 inhibits p53-MDM2 interaction and activates p53-dependent p21-mediated cell cycle arrest, and as such Nutlin 3A addition acts as a functional test of p53 activity (Vassilev et al. 2004). In the presence of Nutlin-3a, cells transfected with an empty control vector (PX459) underwent p53-dependent cell cycle arrest (Fig. 6A), whereas cells transfected with Cas9-TP53 targeting vector produced cells that proliferate continuously into colonies and visualized by Giemsa staining (Fig. 6A). p53 mutant stable cell lines were generated after picking colonies and weeks of clonal expansion. We assayed the isogenic mutant cell lines for p53 expression and observed it in all but one of the MCF7 isogenic p53 cell lines (Fig. 6B, lane 2). We postulated that it is p53 null. Mutations introduced by CRISPR/Cas9 can cause pre-mature stop codons, thereby trigger nonsense mediated decay (NMD) of the mRNA transcript. To confirm NMD is responsible for the absence of p53, we treated the cells with an NMD inhibitor, G418 (gentamicin), and it revealed the

truncated p53 protein (Fig. 6B, lane 4). We further examined the isogenic mutant cell lines (n=4) for TP53 mutations by sequence analysis of exon 5 to exon 8 of their genomic DNA. Sequencing analysis showed that the MCF7 p53 mutant and null lines carry different mutations. The MCF7 and BT474 p53 mutant cell lines had a single base-pair deletion at the 3' splice acceptor site located at the intron 6/exon 7 junction. The disruption of the 3' splice acceptor site resulted in alternative splicing via the use of a cryptic splice acceptor located in intron 6. Use of the cryptic splice acceptor led to the in-frame retention of part of intron 6 in the p53 ORF, and incorporation of this intronic sequence into the p53 DNA binding domain (Fig. 6C&D). The MCF7 p53 null cell line, however, had a five base-pair deletion at the 3' splice acceptor site, and use of the cryptic splice acceptor here led to a frame-shift which introduced a pre-mature stop codon. For subsequent assays, we chose to only use the p53 mutant MCF7 and BT474 cell lines because most cancers that carry p53 mutations are p53 mutant rather than p53 null (Hashimoto et al. 1999). It is explained by the fact that some p53 mutations (gain-of-function mutations) confer a selective advantage to cells that carry it (Doyle et al. 2010).

A


MCF7
BT474
Control
TP53 CRISPR
Control
TP53 CRISPR

B


MCF7 wt
MCF7 p53 null
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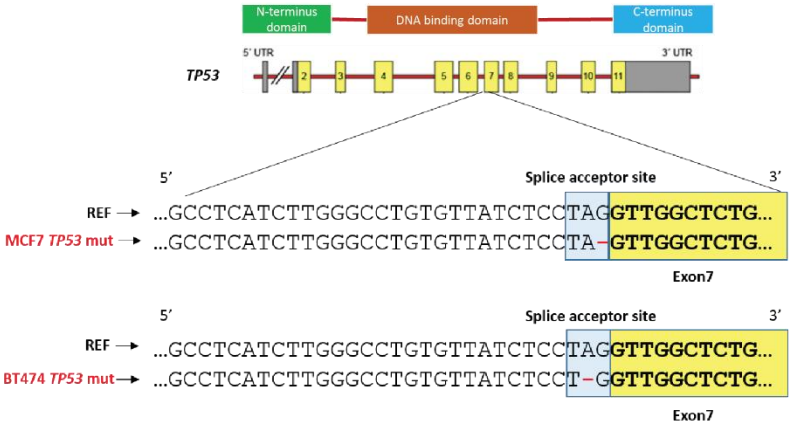
MCF7 null treated w G418
BT474 wt
BT474 mutant
231
T47D
53kDa
p53
13kDa
h3

C


N-terminus domain
DNA binding domain
C-terminus domain
5' UTR
3' UTR
TP53
5'
Splice acceptor site
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MCF7 TP53 mut
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Exon7
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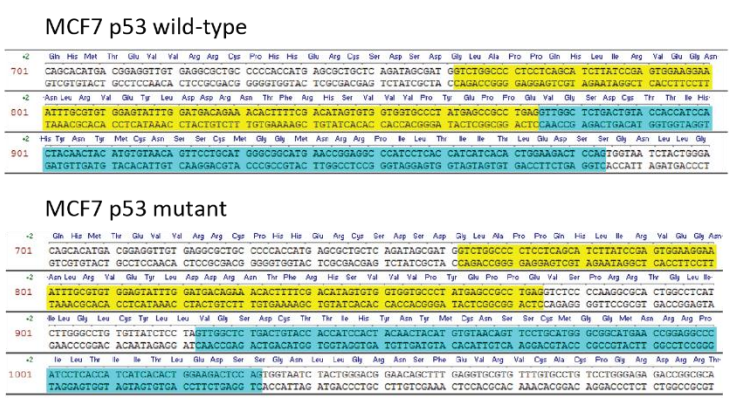
MCF7 p53 wild-type
MCF7 p53 mutant

Figure 6: Isogenic p53 mutant BC lines generated from TP53 targeting CRISPR/Cas9 show resistance to Nutlin-3A.
(A) Colony forming assay of MCF7 and BT474 transfected with TP53 CRISPR. Ten-thousand cells were seeded, exposed to 10 μM of Nutlin-3A. The cells were stained with Giemsa. (B) p53 protein expression levels of p53 wild-type, mutant and null cells, as evaluated by western blotting. (C) Schematic diagram of the TP53 locus and various protein domains. Target site in the splice acceptor site upstream of exon 7 is shown. Sanger sequencing of confirmed a single based deletion in the TP53 exon 7 splice acceptor in Nutlin 3A resistant clones. Clonal colonies were isolated and expanded to establish stable p53 mutant cell lines. (D) Sequencing alignment shows an additional 16 amino acids are translated in the mutant p53 due to intron retention (the non-highlighted region between exon 6 (yellow) and exon 7 (blue)).

Isogenic resembles native p53 mutant breast cancer cell lines in cell cycle response to chemotherapy

To investigate the cell cycle kinetics following chemotherapy, we treated p53 wild-type (WTp53 MCF7 and BT474), isogenic (MUTp53 MCF7 and BT474) and native (T47D) p53 mutant breast cancer cell lines with an anthracycline chemotherapeutic, doxorubicin (DOX), at 100 nM for 2 days. We then measured the cell cycle response using the H2BGFP-FUCCI cell cycle reporter (Calder et al. 2013; Hallett 2015), the mKO2-Cdt1 expression (FUCCI-G1) is restricted to G1/G0. Cell imaging showed that all the p53 wild-type cell lines increased expression of the FUCCI-G1 reporter after treatment, whereas the isogenic and native p53 mutant cell lines remained constant or decreased (Fig. 7A&D). We also stained DOX-treated breast cancer cells for markers of cell proliferation, Ki67 (Scholzen and Gerdes 2000; Hallett 2015), and cell cycle arrest, p21 (Gartel and Radhakrishnan 2005). The p53 wild-type cell lines, MCF7 WTp53 and BT474 WTp53, showed a marked decrease in Ki67 expression (97.88±0.35 to 16.22±3.29, $p = 0.0006$ and 89.47±1.82 to 12.30±1.05, $p < 0.0001$ %Ki67+ve, respectively) while the isogenic and

native p53 mutant cell lines, MCF7 MUTp53, BT474 MUTp53 and T47D, remained constant (98.79±0.43 to 98.28±0.62, p = ns; 96.24±0.45 to 95.08±1.06, p = ns, and 55.50±10.45 to 55.53±9.56, p = ns %Ki67+ve, respectively) (Fig. 7B). The p21 expression demonstrated the converse trend (MCF7 WTp53 12.50±3.20 to 95.07±1.74, p = 0.0012; BT474 WTp53 32.12±0.84 to 87.67±1.88, p = 0.001 %p21+ve, and MCF7 MUTp53 0.40±0.28 to 4.87±0.54, p = 0.0095; BT474 MUTp53 4.82±0.73 to 12.04±2.35, p = 0.026; T47D 26.72±30.22 to 22.70±20.82, p = ns %p21+ve) (Fig. 7C). Taken together these findings suggest that the isogenic resembles native p53 mutant breast cancer cell lines in cell cycle response to chemotherapy.

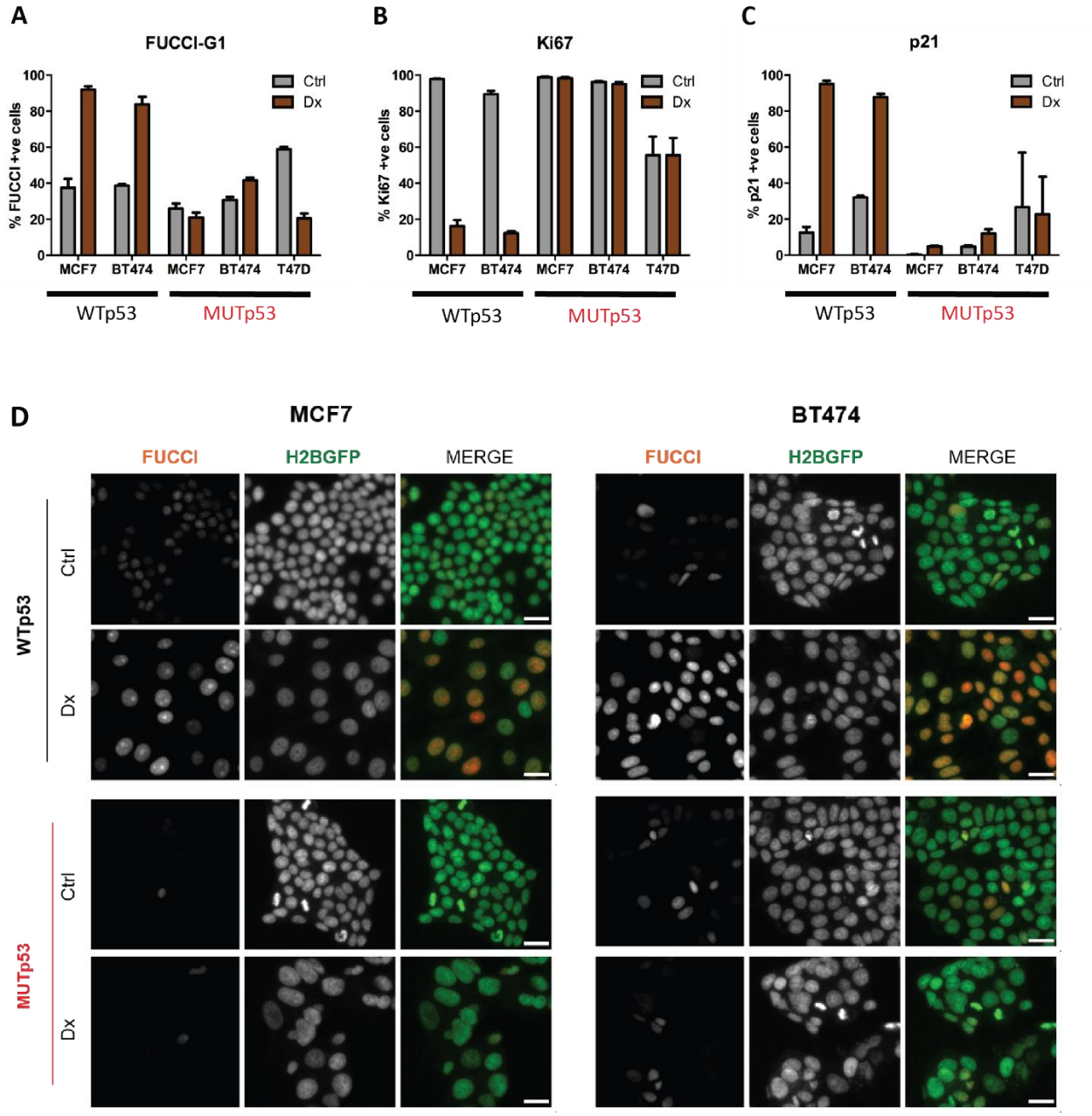

Figure 7. Isogenic resembles native p53 mutant breast cancer cell lines in cell cycle response to chemotherapy.

(A) FUCCI-G1 expression is consistent among isogenic and mutant p53 mutant breast cancer cell lines in response to DOX (100 nM). T47D is an endogenously mutant p53-expressing cell lines. (B) Ki67 expression is maintained in isogenic and mutant p53 mutant breast cancer cell lines following treatment with DOX (100 nM). (C) p21 expression not up-regulated in isogenic and mutant p53 mutant breast cancer cell lines following treatment with DOX (100 nM). (D) Representative micrographs of WTp53 and MUTp53 MCF7 & BT474 cell response to DOX (100 nM). Scale bar = 20 um.

Isogenic p53 mutant breast cancer cells directly confirmed functional p53 signaling induced chemotherapy resistance

We previously showed that activation of functional p53 signaling and G1/G0 arrest protects cells during exposure to the cytotoxic effects of docetaxel (TAX) (Hallett 2015), but the comparison was made between different breast cancer cell lines expressing either wild-type or mutant p53. To minimize any cell line specific response variation between wild-type and mutant p53, we examined cell nuclear morphology in response to TAX in isogenic cell line pairs that differed only in TP53 integrity. p53 wild-type cell lines that were pre-treated with Nutlin3A underwent G1/G0 arrest and subsequent exposure to TAX did not induce nuclear fragmentation (Fig. 8A). However, the isogenic versions of these lines that contained mutant p53 underwent nuclear fragmentation in ~60% of nuclei similar to TAX treatment alone (Fig. 8B). This data shows that functional p53 signaling alone triggers a cell cycle arrest that can protect cells from the chemotherapy-induced mitotic catastrophe.

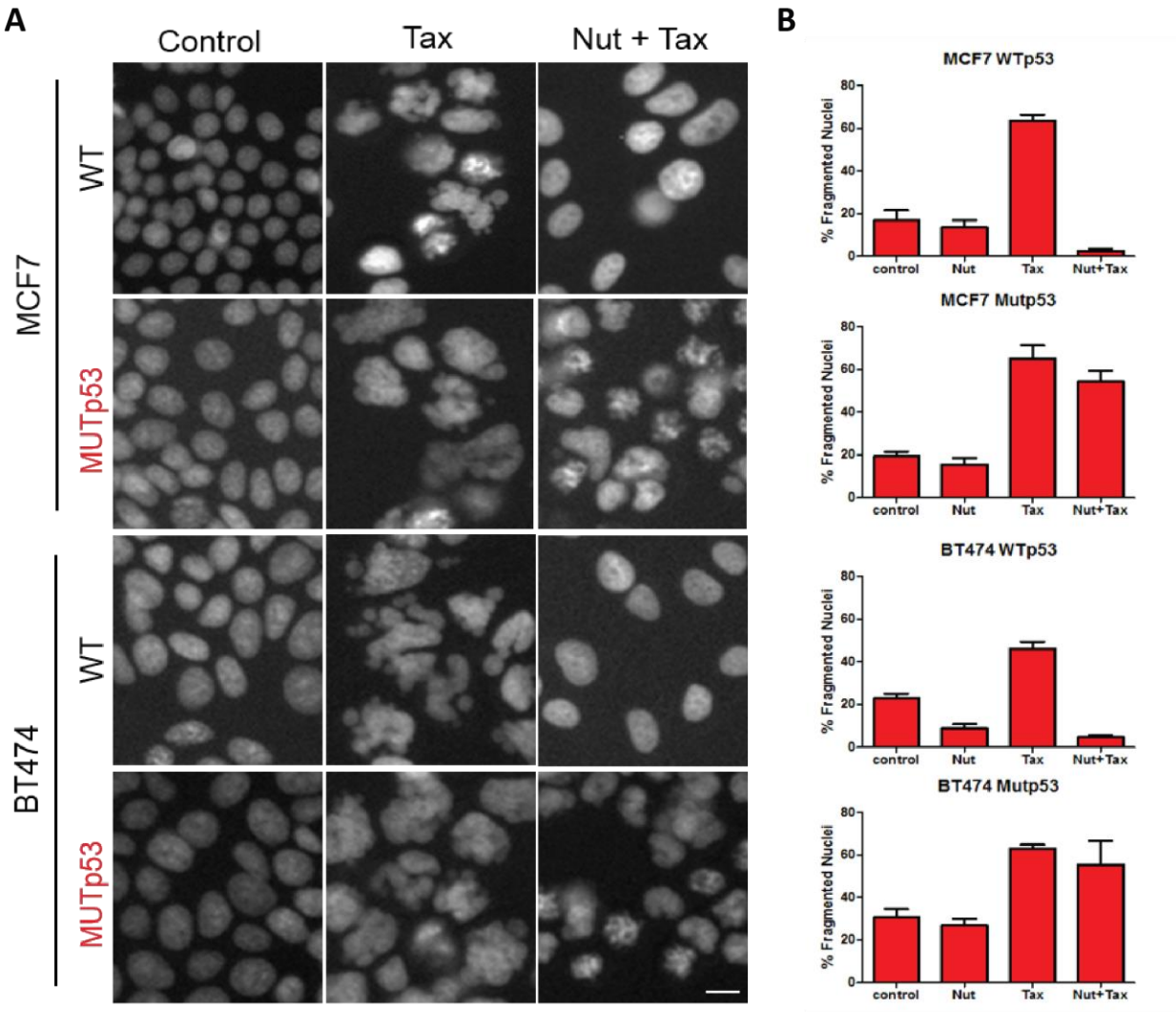

Figure 8. Isogenic p53 mutant breast cancer cells provided definitive evidence that functional p53 signaling confers chemotherapy resistance in ER+ BC cell lines.
(A) Representative micrographs of wild-type and mutant p53 MCF7 and BT474 cell response to TAX only or combined Nutlin 3A and TAX treatment. Isogenic p53 mutant breast cancer cell lines show mitotic catastrophe following treatment with Nutlin 3A and TAX indicating the loss of wild-type p53 mediated chemotherapy resistance. Scale bar = 20 um. (B) Graphs comparing the percentage of TAX-induced nuclear fragmentation at day 3 in the absence and presence of Nutlin 3A. (C) Colony forming efficiency of wild-type p53 and isogenic mutant p53 cell lines following 24, 36, 48 and 72 hr of DOX (100 nM) treatment.

p53 mutant breast tumors, mirroring the response profile of adherent cells, show improved sensitivity to chemotherapy

To examine the relationship between p53 status and chemotherapy response in vivo, we chose an orthotopic human tumor xenograft model (Fig. 9A). We injected p53 wild-type or mutant breast cancer cells orthotopically into the second thoracic mammary fat pads of NOD/SCID mice (Clarke 1996; Fleming et al. 2010). After the development of primary xenograft tumors, mice were treated with DOX and the size of the tumors was monitored (Fig. 9B).

To delineate the significance of p53 status in chemotherapy response, we analyzed the correlations between p53 status and overall survival. The survival curves of DOX-treated mice with p53 wild-type and mutant tumors are indistinguishable (p = ns, log rank test) (Fig. 9C). However, the mice with p53 mutant tumors died earlier than p53 wild-type tumor mice with a median survival of 18 and 20 days, respectively (Fig. 9C).

Next, we analyzed the correlations between p53 status and tumor volume. We found that, regardless of p53 status, tumors of vehicle-treated mice underwent rapid and continuous growth with an average 10.67-fold increase in tumor volume, whereas tumors from DOX-treated mice remained relatively stagnant with an average 2.21-fold increase (Fig. 9D). We did not find a significant difference in tumor volume in p53 wild-type versus mutant tumors. One possible explanation for the lack of a significant difference is that the 5mg/kg of DOX used in the study may be too toxic for the mice, thereby killing them before a difference be observed. However, the most likely explanation is that the difference in response may have been masked. Untreated p53 wild-type MMTV-*Wnt1* tumors were

reported to be highly positive for Ki67, a marker of cells outside G0 of the cell cycle (Jackson et al. 2012). However, following DOX treatment, p53 wild-type MMTV-Wnt1 tumors were Ki67 negative (sparsely positive), demonstrating cell-cycle exit (Jackson et al. 2012). This corresponds with our *in vitro* data showing that the p53 wild-type cells undergo chemotherapy-induced p53-mediated cell cycle arrest, which explains why the DOX-treat p53 wild-type tumors were on average 1/5 of the volume of the same tumors but treated with a vehicle. Conversely, p53 mutant MMTV-*Wnt1* tumors were shown to be Ki67 positive following DOX treatment (Jackson et al. 2012). They also showed a significant increase in both cleaved caspase-3 and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells (two established methods for quantifying apoptosis) after DOX treatment (Jackson et al. 2012). Following DOX treatment, tumors lacking wild-type p53 unable to exit cell cycle resulted in aberrant mitosis and apoptosis (Jackson et al. 2012). We, therefore, postulated that an increase in apoptosis contributed to the reduced volume in the DOX-treated p53 mutant tumors.

We examined the cell cycle kinetic response to chemotherapy in wild-type and mutant p53 tumors by immunohistochemically staining tumor tissues for Ki67 and p21. Following treatment, only p53 wild-type MCF7 tumors had large areas that were Ki67 negative, demonstrating cell cycle exit, while p53 mutant tumors remained positive for Ki67 (Fig. 9E). p21 expression demonstrated the opposite trend. Quantification reveals a significantly higher percentage of DOX-treated p53 mutant breast tumor cells undergoing cellular proliferation compared to p53 wild-type tumor cells (45.29 ± 20.62 to 30.26 ± 16.96 %Ki67+ve; p=0.0289) (Fig. 9F). Conversely, it shows a lower percentage of DOX-

treated p53 mutant breast tumor cells in cell cycle arrest compared to p53 wild-type tumor cells (4.19 ± 3.41 to 7.24 ± 6.85 %p21+ve; p=ns).

The p53 mutant MCF7 tumors also showed a significant increase in cleaved caspase 3 positive cells after DOX treatment (Fig. 9G). p53 wild-type tumors, however, underwent apoptosis only marginally following treatment (Fig. 9G). Quantification reveals a significantly higher percentage of DOX-treated p53 mutant breast tumor cells undergoing apoptosis compared to p53 wild-type tumor cells (11.25% ± 6.06% to 1.67% ± 1.29% cleaved caspase 3+ve; p=0.0157) (Fig. 9H).

Taken together, our data show that the presence of functional p53 induced growth arrest, not apoptosis, in p53 wild-type tumors following DOX treatment, whereas the lack of functional p53 in mutant tumors resulted in continued cell cycle progression, aberrant mitoses, cell death and, ultimately, a superior clinical response in the short term.

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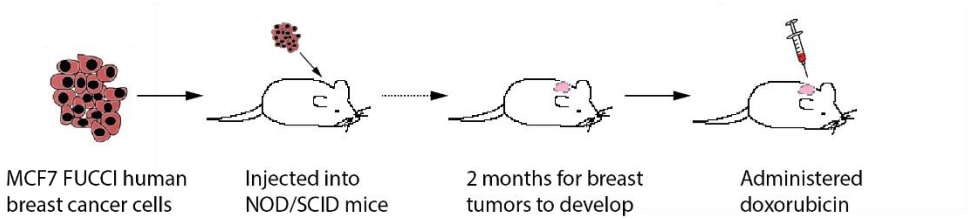

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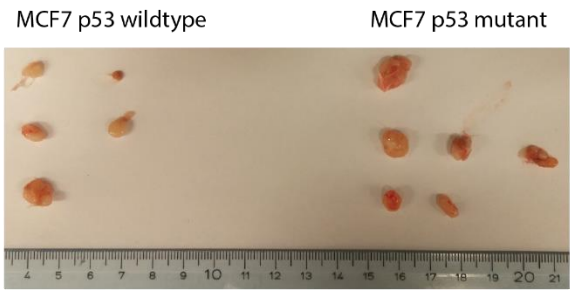

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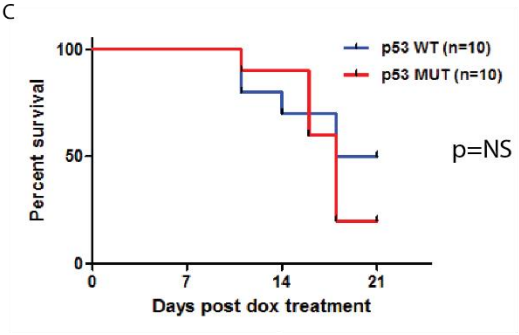

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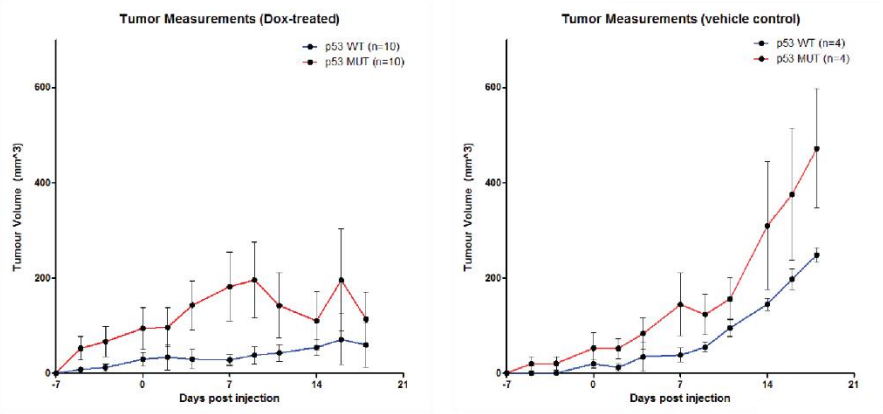

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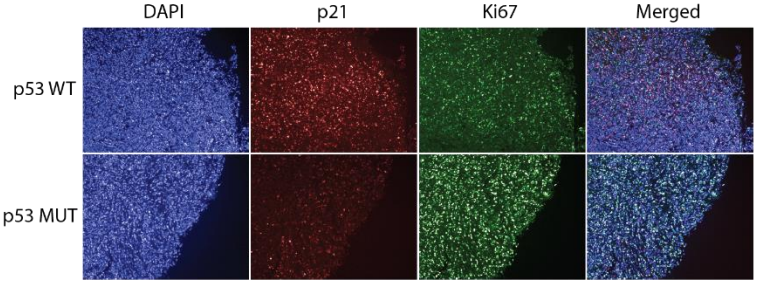

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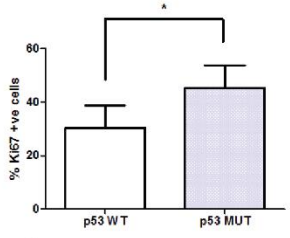
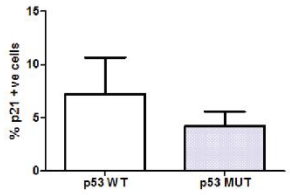
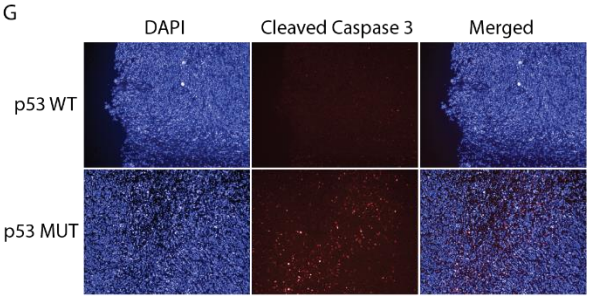


p=0.0289

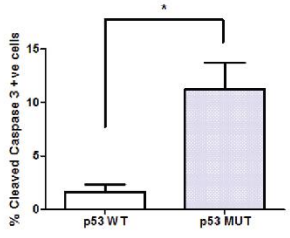


p=NS

G



H



p=0.0157

Figure 9: p53 mutant breast tumors, mirroring the response profile of adherent cells, show improved sensitivity to chemotherapy.
(A) Schematic illustrating the experimental approach of our orthotopic human tumor xenograft model. (B) Representative image of tumors generated in mice that were injected with MCF7 p53 wild-type or mutant BC cells. (C) Kaplan-Meier survival curve for DOX-treated p53 wild-type (n=10) and p53 mutant (n=10) mice are shown. p53 mutant mice did not show a survival advantage in response to DOX treatment (p = ns). (D) Mean tumor volume for DOX-treated p53 wild-type (n=10), p53 mutant (n=10) mice, and p53 wild-type (n=4), p53 mutant (n=4) vehicle-treated control mice are shown. Mean tumor volume was plotted in mm^3 ± SEM. Each mouse was injected with $1x10^7$ MCF7 p53 wild-type or mutant BC cells and treated with the vehicle or 5 mg/kg of DOX via i.p. injection once a week for three weeks. The tumor volume was measured three times a week. (E) Representative images of DOX-treated p53 wild-type or mutant MCF7 breast tumor slides stained with DAPI (blue), p21 (red) and Ki67 (green) antibodies. (F) The DOX-treated mutant p53 xenografts demonstrated a 1.5-fold increase in proliferation in response to chemotherapy. Quantification showed that the DOX-treated p53 mutant tumors have a lower %p21+ve cells (4.19±3.41 to 7.24±6.85; p=NS) while a significantly higher % Ki67+ve cells (45.29±20.62 to 30.26±16.96; p=0.0289). The converse trend was observed in the p53 wild-type tumors. (G) Representative images of DOX-treated p53 wild-type or mutant MCF7 breast tumor slides stained with DAPI (blue) and cleaved caspase 3 (red) antibody. (H) The DOX-treated mutant p53 xenografts demonstrated a 10-fold increase in apoptosis in response to chemotherapy. Quantification showed that the DOX-treated p53 mutant tumors had a significantly higher %cleaved caspase 3+ve cells (11.25±6.06 to 1.67±1.29; p=0.0157).

A streamlined cell-based phenotypic screening platform

In order to address the problem of breast cancer, an improved understanding of chemotherapy resistance is insufficient, we also need new anticancer agents. Phenotypic screening, which focuses on the perturbation of cellular phenotype by chemical substances rather than specific molecular targets, is an unbiased and suitable method to discover active compounds in a complex biological system. Previously reported cell-based phenotypic assays typically require fixation and staining for phenotypic markers to reveal the cytological status, which reduce screen throughput and introduce the potential for reagent-based variation between assays (Iorio et al. 2010; Perlman et al. 2004; Tanaka et al. 2005).

To overcome some of the limitations of existing cell-based phenotypic assays, we developed a high-content and high-throughput live-cell phenotypic assay, which streamlines the process of cytological profiling and provides a consistent platform for empirically evaluating drug action.

We chose the MCF7 FUCCI cell line for the assay because it is equipped with an H2BGFP-FUCCI cell cycle reporter (Sakaue-Sawano et al. 2008; Calder et al. 2013; Hallett 2015) that encodes a fusion of H2B-GFP, which decorates chromatin, and mKO2-Cdt1 (Calder et al. 2013; Hallett 2015) (FUCCI-G1), the expression of which is restricted to G1/G0. The H2BGFP provided a means for the nuclear identification in high-content imaging while the FUCCI-G1 permitted the live measurement of cells in G1/G0 phase. Moreover, the FUCCI cell cycle reporter enabled a streamlined phenotypic profiling assay that dispensed with the need to fix and stain cells to obtain cytological profiles (Fig. 10A).

We screened 3,923 bioactive compounds (of which 3,580 were unique) at a concentration of 1μM in triplicate. All compounds were drawn from the Canadian Compound Collection, which is curated by The Centre for Microbial Chemical Biology at McMaster University, and includes bioactive compounds sourced from several libraries such as Lopac-1280, Prestwick Chemical Library, BIOMOL Natural Products Library and Microsource Spectrum Collection. The compound libraries are composed of FDA-approved drugs, bioactive molecules, and natural products. A bioactive compound enriched library was chosen to increase the potential of identifying hits when contrasted to similar sized non-enriched chemical library.

Forty-eight hours after compound addition, high content imaging was performed on the live cultures. Using automated image analysis, measurements for cell cycle position (FUCCI-G1), nuclear morphology (nuclear roundness, nuclear integrity (normal, fragmented and condensed)) and nuclear texture (SER (Spot, Hole, Valley, Ridge), Haralick (Homogeneity, Correlation) and Gabor) were calculated (Fig. 10B). Haralick Homogeneity or Correlation, which are not shown in Fig. 10B, measure as their namesake the texture homogeneity or correlation of an image. The data of 12 distinct parameters for each compound were aggregated and standardized. Hierarchical clustering was performed and the resultant heat map is herein referred to as the General Cluster (Fig. 10C). The General cluster then underwent a Z-score transformation with a threshold of 2.5 and produced a list of 340 'active' compounds that elicited a phenotypic change. Clustering of these compounds produced an Active Cluster (Fig. 10D). Within the Active Cluster, 14 subclusters contained four or more compounds and reached a clustering coefficient ≥ 0.85 were considered for subsequent investigation.

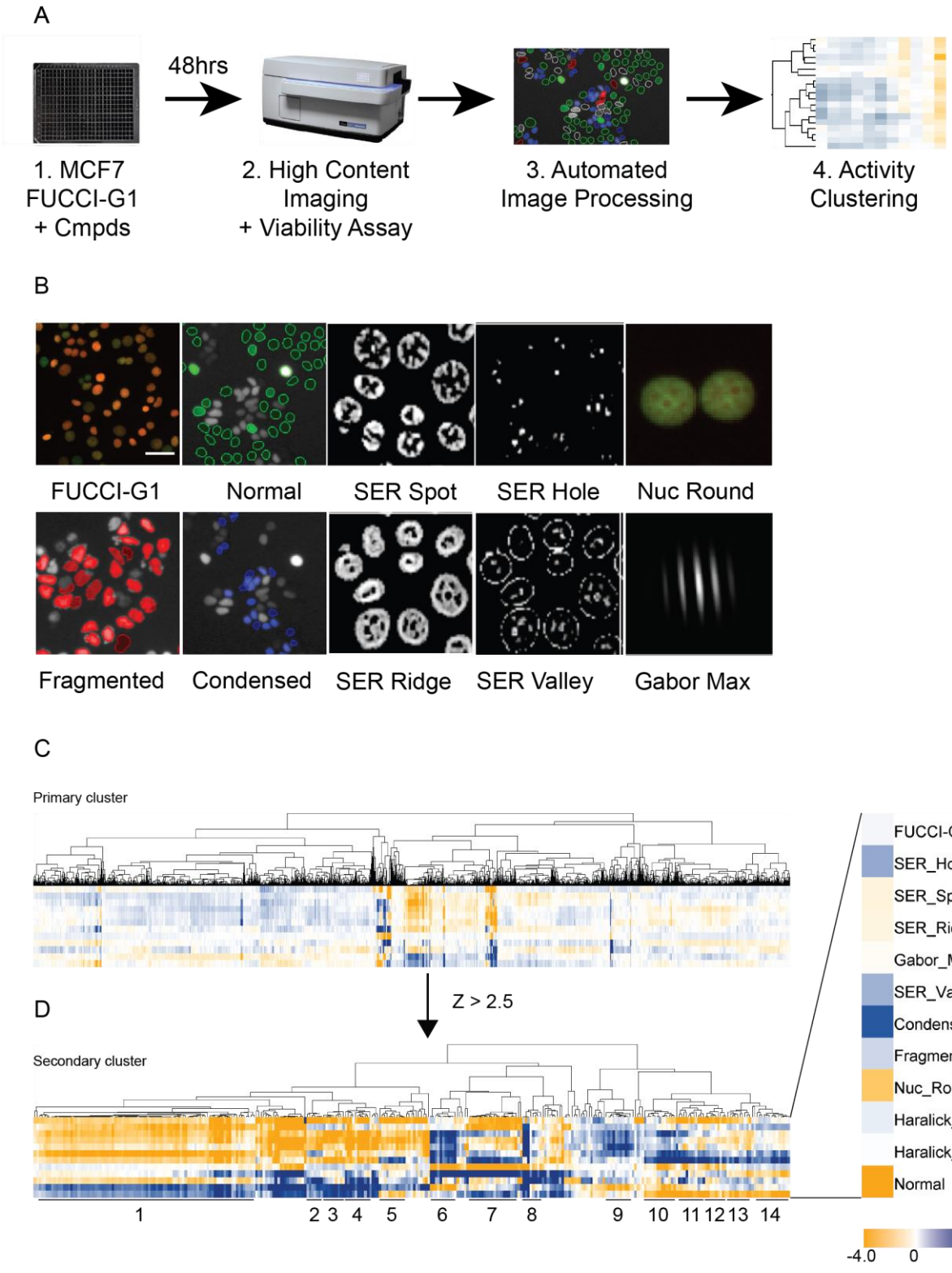

A
48hrs
1. MCF7 FUCCI-G1 + Cmpds
2. High Content Imaging + Viability Assay
3. Automated Image Processing
4. Activity Clustering
B
FUCCI-G1
Normal
SER Spot
SER Hole
Nuc Round
Fragmented
Condensed
SER Ridge
SER Valley
Gabor Max
C
Primary cluster
Z > 2.5
D
Secondary cluster
1
2 3 4
5
6
7
8
9
10
11 12 13
14
FUCCI-G1
SER_Hole
SER_Spot
SER_Ridge
Gabor_Max
SER_Valley
Condensed
Fragmented
Nuc_Roundness
Haralick_Corre.
Haralick_Homog.
Normal
-4.0
0
4.0

Figure 10: Phenotypic screen setup and compound-activity profiling
(A) Strategy for a cell-based multiparametric phenotypic profiling of compounds (B) Individual cells were evaluated on 12 distinct parameters, including the percentage of cells in G1-phase (via FUCCI), nuclear roundness, nuclear shape (normal, fragmented, and condensed) and texture features (SER, Haralick, Gabor). (C) The General cluster was established through a hierarchical clustering of 3921 compounds based on their phenotypic parameters' scores. Increased scores were represented in yellow and decreased in blue, with intensity encoding magnitude. (D) Passing the General cluster through a Z-score transformation with a threshold greater than 2.5 distilled the list down to 340 'active' compounds. Clustering of these compounds generated the Active cluster. A group of four or more compounds with a clustering coefficient greater than 0.85 was considered a subcluster. There were 14 subclusters within the Active cluster.

A common drug-induced phenotypic profile is associated with a similar MoA

Before investigating the relationship between drug-induced phenotypic alteration and MoA, compounds within each subcluster were validated to share a common phenotype (Fig. 11A). To examine the relationship between drug-induced phenotypic alteration and MoA, the MoA information for each compound of was obtained from published databases (DrugBank, ChemBank, PubChem, and ChemSpider). The mechanism of actions indicated by the databases is based on the U.S. Food and Drug Administration's (FDA) Pharmacologic Classes. According to the FDA, "pharmacologic class is a group of active moieties that share scientifically documented properties" (FDA 2013). A pharmacologic class is defined by three attributes of the active moiety: mechanism of action (MOA), physiologic effect (PE), and chemical structure (CS) (FDA 2013). As part of the drug approval process, each new molecular entity must provide empiric evidence showing that its active moiety's pharmacologic class is known, relevant and specific to its indication (FDA 2013). MoA annotation of each compound in the sub-clusters revealed that ~80% of compounds within each subcluster have the same MoA (Fig. 11B). The molecular

similarity concept suggests structural similarity and functional similarity are associated (Bender and Glen 2004), so we next investigated if the clustered phenotypic profiles could provide insights into the structural relationships of the screened compounds. Tanimoto coefficients (Godden, Xue, and Bajorath 2000) were used to calculate the structural similarity relationships for compounds using extended connectivity fingerprint 6 (ECFP_6), a 2D fingerprint for molecular characterization. Tanimoto coefficients were calculated for the compounds, and used to graph chemical structural similarity networks. The structural similarity networks showed a significant (0.45) structural correlation among compounds in each subcluster (Fig. 11C). However, not all compounds that induced a similar phenotype were structurally related, and likely reflects that multiple different targets can converge on a common phenotypic outcome.

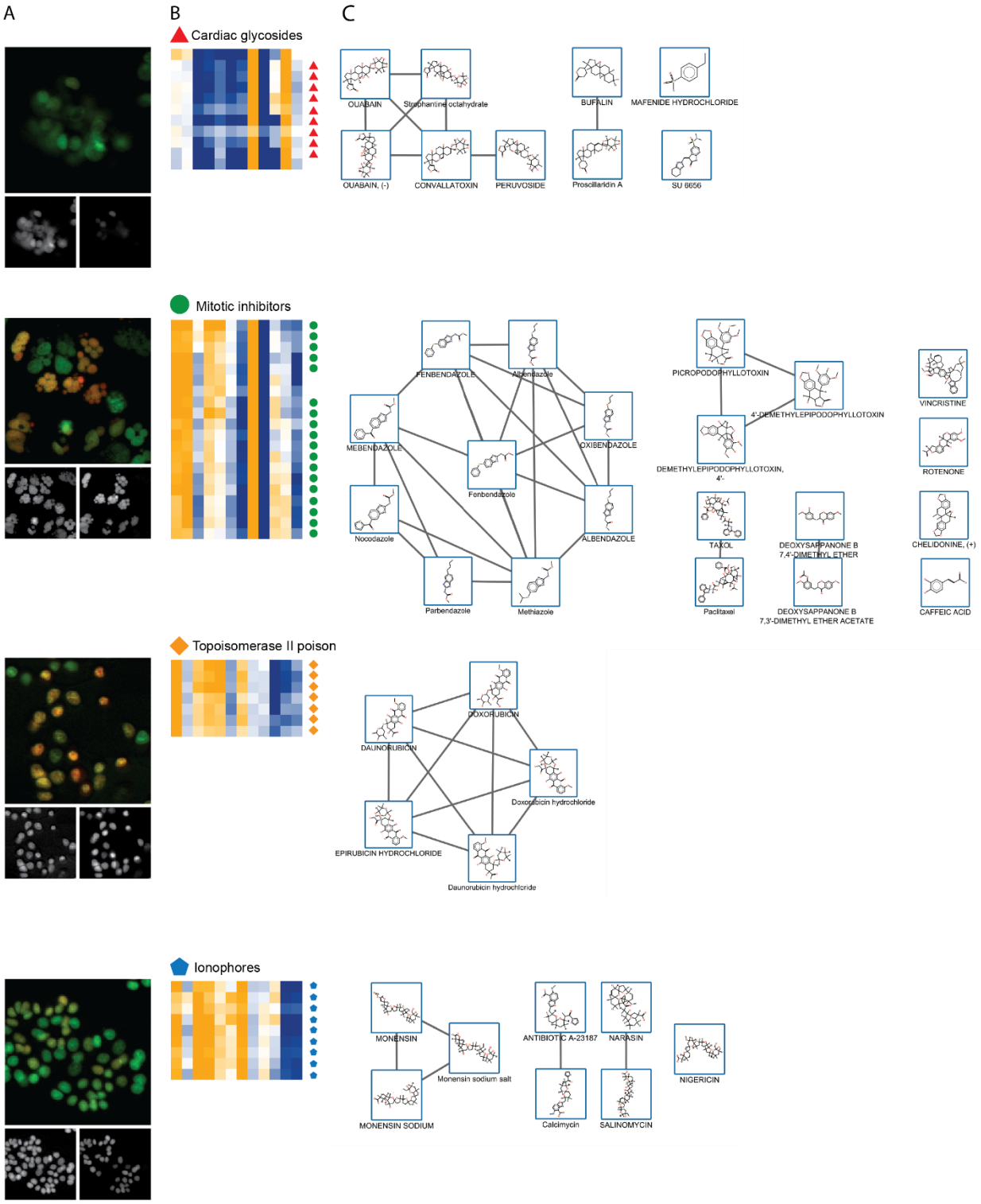

A
B
C
Cardiac glycosides
OUABAIN
Strophanthine octahydrate
BUFALIN
MAFENIDE HYDROCHLORIDE
OUABAIN, (-)
CONVALLATOXIN
PERUVOSIDE
Proscillaridin A
SU 6656
Mitotic inhibitors
FENBENDAZOLE
Albendazole
MEBENDAZOLE
OXIBENDAZOLE
Fenbendazole
Nocodazole
ALBENDAZOLE
Parbendazole
Methiazole
PICROPODOPHYLLOTOXIN
4'-DEMETHYLEPIPODOPHYLLOTOXIN
DEMETHYLEPIPODOPHYLLOTOXIN, 4'-
VINCRISTINE
ROTENONE
TAXOL
DEOXYSAPPANONE B 7,4'-DIMETHYL ETHER
CHELIDONINE, (+)
Paclitaxel
DEOXYSAPPANONE B 7,3'-DIMETHYL ETHER ACETATE
CAFFEIC ACID
Topoisomerase II poison
DOXORUBICIN
DAUNORUBICIN
Doxorubicin hydrochloride
EPIRUBICIN HYDROCHLORIDE
Daunorubicin hydrochloride
Ionophores
MONENSIN
Monensin sodium salt
ANTIBIOTIC A-23187
NARASIN
NIGERICIN
MONENSIN SODIUM
Calcimycin
SALINOMYCIN

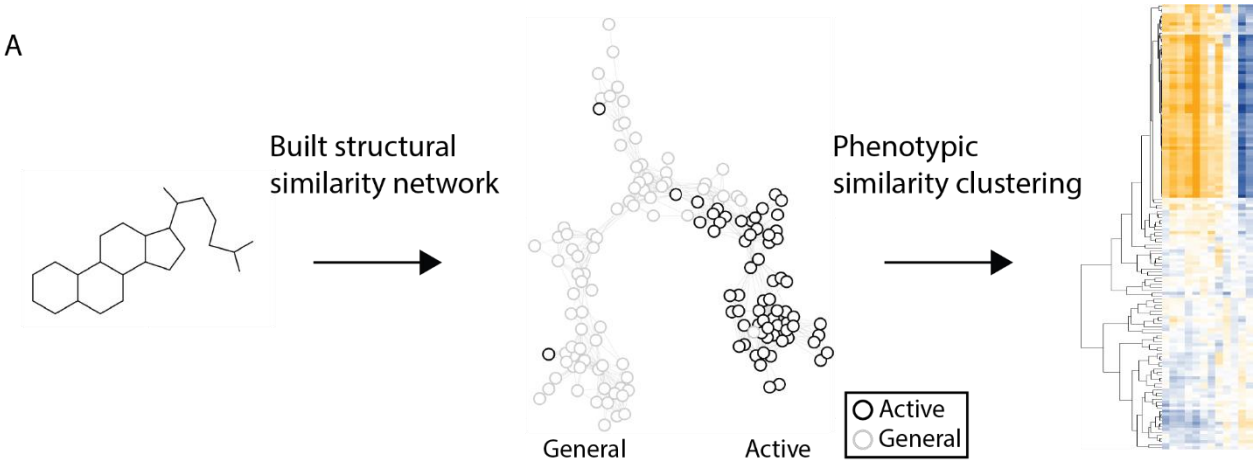
Figure 11: Compounds within a sub-cluster showed a common phenotype and MoA, and might belong to the same chemical class.
(A) Compounds in each individual subclusters shared a common phenotype as shown in the fluorescence micrographs. (B) Clustering of compounds by phenotype showed that compounds within each subcluster also shared a common mode of action. (C) Chemical structure similarity networks presented compounds in each subcluster. Each compound was a node in the network, and an edge was drawn between two nodes if the Tanimoto coefficient was greater than 0.45.

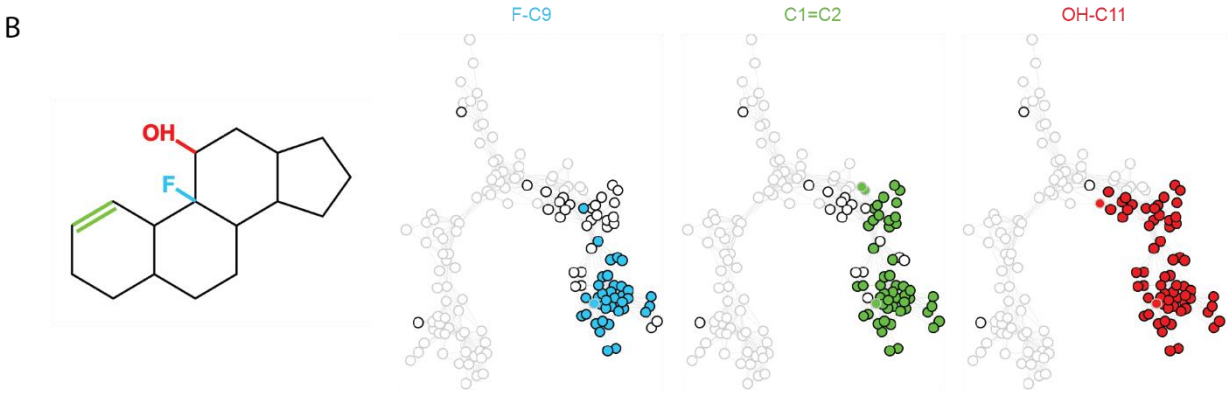
Drug-induced phenotypic profile with chemical structural network can provide inference to SAR

To examine the connection between phenotype and structure-function relationship, a chemical structural similarity network was generated from 147 steroids in the screen (Fig. 12A). The network graph showed the steroids are separated into two different groups. Overlaying the information of cluster status (Active vs. General) revealed that almost all of the compounds that appear in the Active Cluster localized on one branch of the network, whereby compounds that appear only in the General Cluster occupied mostly the other branch. Chemical moiety analysis showed that steroids with a three specific moieties were confined to the Active Cluster, and absent from the General Cluster. These moieties including a double bond between C1 and C2, a fluorine group attached to C9 and a hydroxyl group attached to C11 (Fig. 12B), all of which have previously been described to be important in the steroid receptor activation (He et al. 2014; Ponec et al. 1986a). Receptor binding preference for all of the steroids in the screen was obtained from the literature (Derendorf and Hochhaus 1995), and revealed that nearly all Active steroids bind to the glucocorticoid receptor, whereas steroid members of the General Cluster bound either to

one of the remaining four types of steroid hormone receptors or are steroid hormone precursors that do not bind (Fig. 12C).

From evaluating the screening platform, it has become apparent that the phenotypic screen platform we have developed can be used to determine the MoA of novel compounds. We hypothesized that the primary screen data could be used as a training set for new compounds with unknown MoA. Co-clustering of compounds with unknown MoA with our dataset would then reveal insights into MoA for these new compounds, which could be inferred known MoA for cluster neighbors.


A
Built structural similarity network
Phenotypic similarity clustering
General
Active
Active
General


B
OH
F
F-C9
C1=C2
OH-C11

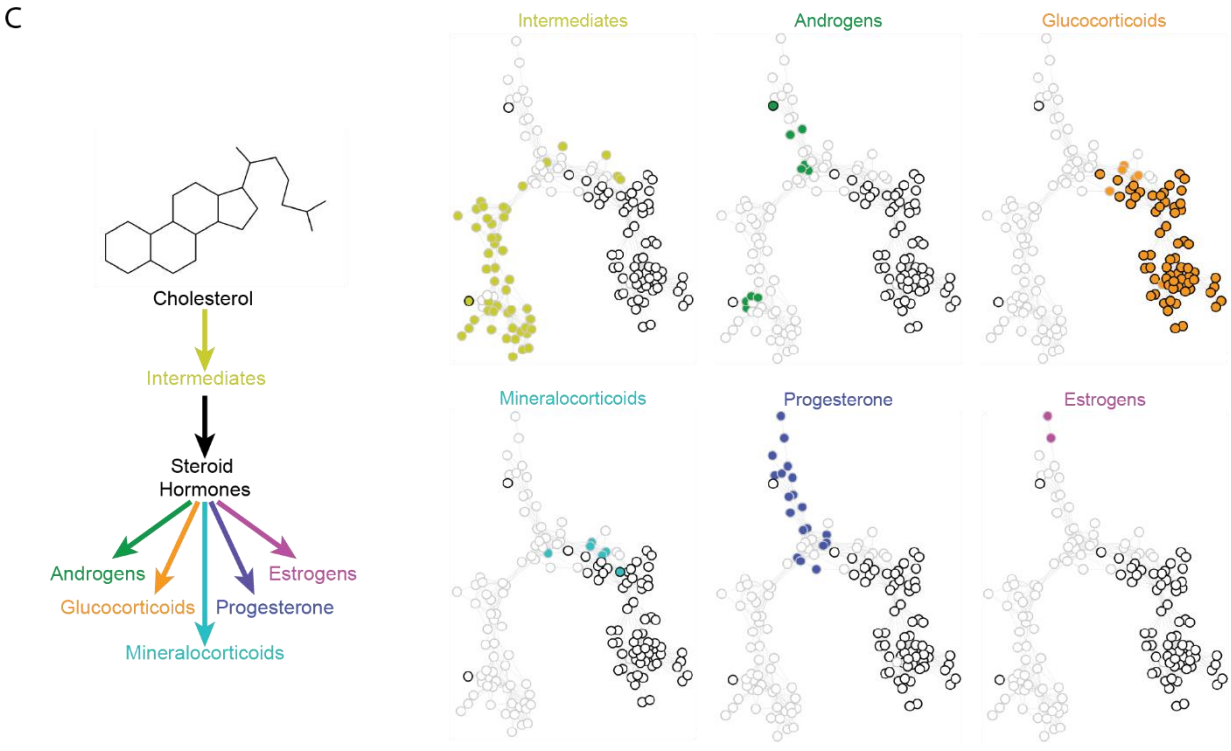

C
Cholesterol
Intermediates
Steroid Hormones
Androgens
Glucocorticoids
Progesterone
Estrogens
Mineralocorticoids
Intermediates
Androgens
Glucocorticoids
Mineralocorticoids
Progesterone
Estrogens

Figure 12: Phenotypic profiling based on compound-activity in conjunction with chemical structural similarity network revealed SAR.
(A) A chemical structural similarity network was generated from 147 steroids in the screen. Steroids from the General and Active cluster are represented by gray and black circles, respectively. A steroid cluster was established through a hierarchical clustering based on their phenotypic parameters' scores. (B) Three moieties that were common to the Active steroids but absent the General, including a double bond between C1 and C2, a fluorine group attached to C9 and a hydroxyl group attached to C11. (C) Receptor binding analysis revealed that nearly all Active steroids bind to the glucocorticoid receptor, whereas the General steroids bound either to one of the remaining four types of steroid hormone receptors or were steroid hormone precursors that do not bind.

Phenotypic profiling is independently reproducible between screens and suitable for screening unknown compounds

To assess the robustness of the screening platform, a short list of compounds, including two compounds from each subcluster and 8 compounds from the General cluster as controls, were screened under the same conditions as the primary screen. After the data was standardized and clustered, the re-screened compounds emerged in the same subcluster they were previously. After the robustness of the screening platform was confirmed, another screen was performed with 34 previously unknown microbial natural products and 8 controls. Among the unknown molecular entities, 5 of them induced a significant phenotypic change. A speckled pattern was observed in the nuclei. Four of the five compounds were found in a significant sub-cluster with a known anticancer, pro-apoptotic drug, SU 9516 (Fig. 13).

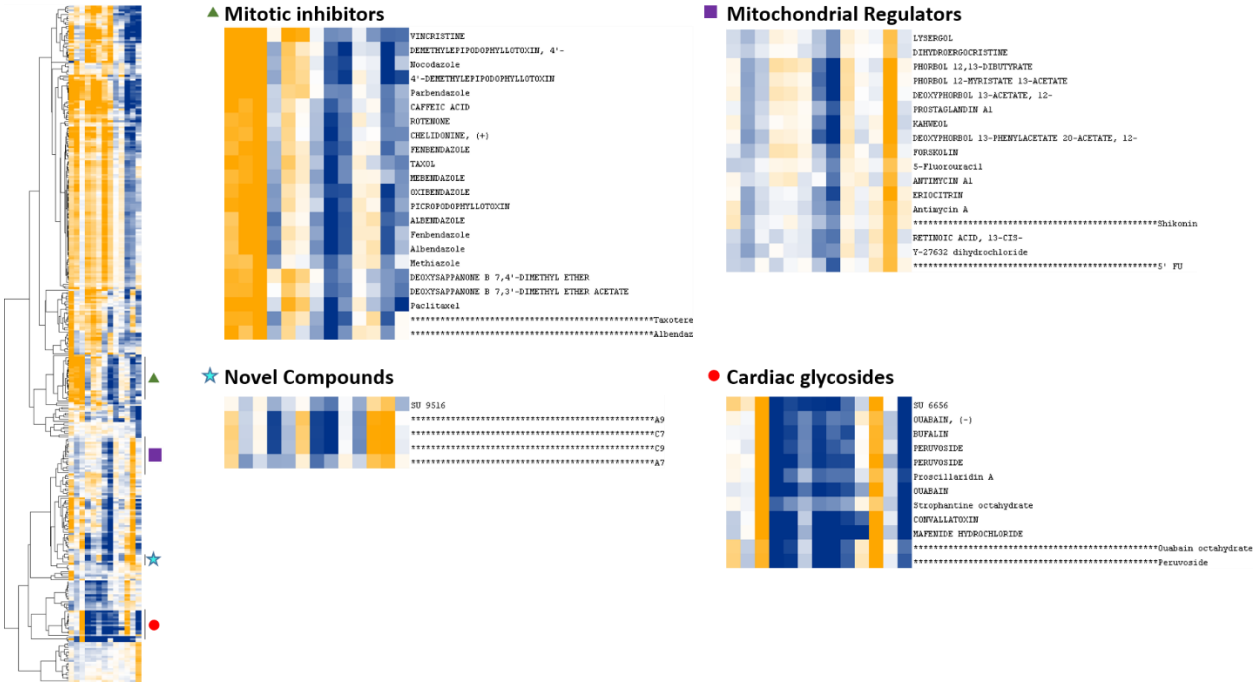


Figure 13: Four unknown compounds clustered significantly with an identified anticancer drug, SU 9516. Reference compounds appeared in their original clusters which indicate reproducible measurement between screens. Reference compounds were denoted by a string of asterisks proceeding their name. Four novel compounds are found in a sub-cluster with a known anticancer drug, SU 9516.

DISCUSSION

Breast cancer is the most commonly diagnosed and the second most deadly cancer among women in Canada. Anthracycline-based chemotherapy is the mainstay regimen in the adjuvant/neoadjuvant setting. Although it gives improved outcomes compared with cyclophosphamide, methotrexate and fluorouracil, only a small percentage of treated patients actually receives a benefit while these agents are associated with significant toxicity (Henderson 2013). The utilization of a predictive marker of patient response to chemotherapy should spare predicted 'poor responders' from the toxicity associated with such treatments.

The TP53 gene is a prime candidate for predicting the response of tumors to classic chemotherapy (Bonnefoi et al. 2011). It is a master gene in the stress response pathway that plays a critical role in cancer development. TP53 is the most frequently mutated gene in human cancer, with mutations occurring in at least 50% of human cancers (Tewari, Krishnamurthy, and Shukla 2008). p53 mediates checkpoint or stress responses to several insults and suppresses tumor formation through several mechanisms, including apoptosis, senescence, and autophagy (Zilfou and Lowe 2009). Experimental evidence suggests a key role for p53 in apoptosis in response to genotoxic agents (Lowe et al. 1993; Fridman and Lowe 2003).

The use of TP53 status as a biological marker to predict the response of breast cancer to neoadjuvant chemotherapy, however, has yielded conflicting results. While some reports show wild-type TP53 activity is beneficial to response (Berns et al. 2000; Kröger et al. 2006), others show that TP53 mutant tumors respond better (P Bertheau et al. 2002;

Anelli et al. 2003; Aas et al. 2003). The relevance of this gene to clinical therapy thus remains unknown.

We described the novel observation that p53 status predicts patient response to neoadjuvant chemotherapy but only in ER+ tumors. To date, many p53 signatures have been reported. We found that these signatures have the capacity to identify p53 mutations and predict response to neoadjuvant chemotherapy, suggesting there is a relationship between p53 status and response. However, our examination of the top p53 signatures revealed that they are also significantly associated with breast cancer subtype and ER status. To confirm that the relationship between TP53 status and chemotherapy is not confounded by ER status, we examined the p53 signatures separately in ER+ or ER– group. The signatures predict response in ER+ tumors but not ER– tumors, which suggests that p53 mutations are only associated with response in the ER+ tumors.

Base on the p53 signature study, we proceed to investigate the relationship between p53 and chemosensitivity among ER+ breast tumor. Using CRISPR-Cas9 mediated gene knockout, we generated p53 mutant lines from three ER+ isogenic breast cell lines, and differing only in the expression of p53. We showed that activation of functional p53 induces G1/G0 arrest only in the p53 wild-type lines, which in turn protected cells from further chemotherapy treatments. However, in the absence of functional p53, the tumors are more susceptible to chemotherapy, as measured by a significantly higher expression of cleaved caspase 3 compared the p53 wild-type tumors. This susceptibility appears to arise from the lack of a chemotherapy-induced wild-type p53-mediated cell cycle arrest. p53

mutant cells proceeding through the cell cycle, which permits genotoxic agents to cause aberrant mitosis events, and cell death.

This is consistent with the results from our previous study where we examined cell cycle response of human breast cancer cell lines to DOX treatment and found that the integrity of cell cycle regulator gene, TP53, is associated with chemotherapy outcome (Hallett 2015). However, data from comparing isogenic cell line pairs provides a more robust evaluation because it minimizes line-line variation, and any confounding mutations that non-isogenic lines may not share. The similar response to chemotherapy between the native and isogenic p53 mutant breast cancer cell lines suggests that the relationship between p53 status and chemosensitivity is independent of any cell line specific variations.

It is a possible, however, that the cell cycle kinetic and chemotherapy response that we observed in the isogenic p53 mutant cells are not because of Cas9 mediated TP53 mutation but off-target events. One way to rule out any off-target effect is by conducting a whole-genome sequencing. However, a more practical way may be to repair the original TP53 mutation that was introduced with the CRISPR/Cas9 using homologous recombination with a donor template in combination with the same CRISPR/Cas9 to see if it recovers the wild-type TP53 phenotype. It is highly improbable that the off-target events would be correctly repaired by this approach.

We further explored chemotherapy response associated with p53 status using an orthotopic human tumor xenograft model. We showed that following DOX treatment, p53 wild-type breast tumors underwent cell cycle arrest whereas p53 mutant tumors failed to arrest resulted in aberrant mitosis and apoptosis. These results are consistent with MMTV-

Wnt1 mouse mammary tumors which showed p53 mutant breast to produce superior clinical response than its p53 wild-type counterpart (Jackson et al. 2012).

To examine the relationship between p53 status and chemotherapy response in vivo, we chose a human tumor xenograft model. After the development of primary xenograft tumors, mice were treated weekly with 5mg/kg of DOX. We found that the dose we administered was too toxic for the mice. They started to become sick after the second treatment and after the third treatment, very few had survived. In a similar study looking at the role of TP53 mutation in breast cancer response to chemotherapy (Jackson et al. 2012), MMTV-*Wnt1* p53 wild-type, heterozygous mutant and null mice were treated with 4 mg/kg doxorubicin once daily for 5 consecutive days. However, the dose was tolerated in the p53 wild-type and heterozygous mutant mice. It is possible that the 20% increase in dose (4 to 5 mg/kg) is intolerable in the NOD/SCID strain. As such the maximum-tolerated dose for the NOD/SCID strain needs to be determined for future experiments, otherwise the dose should be reduced to 3mg/kg.

Overall, we showed that p53 status is a significant predictor of breast cancer patient response to neoadjuvant chemotherapy for patients with ER+ tumors. We also demonstrated that p53 mutant breast tumors are more susceptible to neoadjuvant chemotherapy. One important implication of finding would be its adoption in the clinic to distinguish patients likely to receive benefit from those who are not, sparing predicted 'poor responders' from chemotherapy-associated toxicity.

A streamlined cell-based phenotypic screening platform

Although there has been an enormous increase in our knowledge concerning the molecular pathogenesis of cancer and mechanisms associated with chemotherapy resistance in the past two decades, our ability to control, much less cure, cancer has been disappointing (Ruddon 2010). Anthracycline chemotherapy is a prefer treatment yet produces clinical response in only 60% of the treated BC patients (Evans et al. 2005; Mansi et al. 2010). Latest information indicates that wild-type p53 is associated with chemotherapy resistance in BC patients with ER+ tumors (Hallett 2015; Hallett and Huang unpublished). As such, new anticancer agents effective for the group of BC patients with ER+ p53 wild-type tumors are urgently needed. This is further exacerbated by the fact that oncology has one of the poorest records in terms of novel drugs in clinical development (Kamb, Wee, and Lengauer 2007). To address this issue, we designed a streamlined high-content cell-based phenotypic profiling assay that facilitated the identification of MoA of four natural products which are potential anticancer agents. A cell-based phenotypic screen is not a novel concept (Jones et al. 2009; Perlman et al. 2004; Woehrmann et al. 2013; Young et al. 2008), and the reports thus far required cells to be treated with drugs, fixed and then cytological features, such as nuclear DNA, organelle morphology or protein expression, are highlighted by stains/dyes or antibody-based immunofluorescence and then measured by high content imaging. However, stain- and immunofluorescence-based visualization introduces additional steps that limit throughput by significantly elongating the screening workflow, and can induce other unintended consequences: fixation/staining require wash steps that can cause loosely attached cells to slough off, resulting lost phenotypes, while reagent batch-to-batch variation can compromise screen-to-screen consistency. Here we

demonstrated a live multi-parameter cellular phenotypic screening assay that reduces the workflow for acquiring compound cytological profiles. In this approach, multivariate clusters were generated from quantitative phenotypic measurements of cellular responses in live cells and used to classify and provide MoA insights for synthetic compounds, as well as novel natural product derivatives.

A common drug-induced phenotypic profile is associated with a similar mode of action

Compound-clustering based on phenotypic activity enabled us to demonstrate that a common drug-induced phenotype is a reflection of a similar MoA. Furthermore, phenotypic resemblance also suggested closely relation in chemical structure. For example, a sub-cluster of 10 compounds all displayed nuclei with a 'halo' appearance (Fig. 11A-C). Their MoAs revealed that 8 of them are cardiac glycosides with the exception of SU 6656 and Mafenide hydrochloride which were not reported as cardiac glycosides. The structural similarity network of this sub-cluster showed a small network of five compounds that are structurally related to ouabain, a pair of compounds associated with bufalin and two independent compounds, mafenide hydrochloride and SU 6656. Not surprisingly, the two independent compounds that were not reported as cardiac glycosides are also structurally distinct from the others in the cluster. However, a question arises: why the other seven compounds, all of which are cardiac glycosides, did not cluster together? Further investigation revealed that chemically there are two classes of cardiac glycosides: cardenolides and bufadienolides (Allmaier and Schmid 1986; Kumar et al. 2013). They are

separated by the R group attachment at the C-17 position (Kumar et al. 2013). The small network of five compounds that were structurally related to ouabain belong to cardenolides class, whereas the pair of compounds associated with bufalin is a member of the bufadienolides class (Kumar et al. 2013).

Another sub-cluster of 20 compounds shared a distinct feature – fragmented nuclei (Fig. 11A-C). Their MoAs revealed that 18 of them are mitotic inhibitors with the exception of caffeic acid and chelidonine (+). The structural similarity network of this sub-cluster showed that 9 compounds are benzimidazole derivatives (MacDonald et al. 2004). They were known to function as 'mitotic poison' by binding to tubulin and inhibiting microtubule polymerization (MacDonald et al. 2004). Another group of three compounds is podophyllotoxin glucosides which also inhibits microtubule assembly (Qi et al. 2005). Taxol (Paclitaxel) is a cytotoxic chemotherapy drug (Xiao et al. 2006), and unlike benzimidazoles and podophyllotoxin glucosides, taxol stabilizes microtubules and reduces their dynamicity, promoting mitotic arrest and cell death (Xiao et al. 2006). It is worth noting that caffeic acid and chelidonine (+), two compounds that did not share the MoA of its sub-cluster, also did not share a similar structure with the others. Although caffeic acid and chelidonine are not recognized as mitotic inhibitors, a reasonable account is available to explain how these two compounds may induce a comparable phenotype and thus be misidentified. Caffeic acid is an antioxidant which has been shown to induce apoptosis in human leukemic HL-60 cells (Chen, Shiao, and Wang 2001). It causes mitochondrial dysfunction by selectively scavenging hydrogen peroxide (Chen, Shiao, and Wang 2001). Chelidonine, on the other hand, is an alkaloid extract from Chelidonium majus (El-Readi

et al. 2013). It was reported to inhibit the activity of CYP3A4, a key enzyme associated with drug metabolism, and to also induce apoptosis (El-Readi et al. 2013). More importantly, DNA fragmentation and nuclear morphological changes have been a well-documented phenomenon in the later stages of apoptosis (Collins et al. 1997).

A third sub-cluster of 8 compounds readily induced G1-phase cell cycle arrest that was reflected by the high FUCCI expression (Fig. 11A-C). Their MoAs revealed that all of them are DNA intercalators (Frost et al. 2006). These compounds have a common anthracycline backbone and different R group attachments. The structural similarity network of this sub-cluster showed a great level of interconnectedness because of the high level of structural similarity, illustrating the power of our phenotypic screen to identify compounds with like MoA.

In summary, the phenotypic cluster aligned closely with MoA for some compounds. In addition, a similar phenotypic profile was correlated with similar chemical structure. However, it may not be a one-to-one relationship because activating different signaling pathways may ultimately converge onto a common downstream effector and elicit an identical response.

Drug-induced phenotypic profile with chemical structural network provided inference to SAR

Phenotypic profiling enabled us to demonstrate that phenotypic resemblance is a reflection of a similar MoA. Furthermore, our data supported the long-standing notion that activity is related to chemical structure. To test whether phenotypic profiling can demonstrate SAR,

we looked closely at the steroid molecules from our screen, a class of compound that are structurally similar but functionally distinct. All steroid hormones are derived from cholesterol. They share a common 4-rings structure. There are five types of steroid hormones: glucocorticoids, mineralocorticoids, androgens, estrogens and progestogens. Among the 3921 compounds in the General cluster, there are 147 steroids. Intriguingly, nearly half of the steroids induce significant phenotypic changes and belong to the Active cluster, while the rest do not (Fig. 12A).

To ask whether this phenotypic difference is related with their chemical structure, a chemical structural similarity network was graphed on Cytoscape with the Tanimoto coefficients calculated using the ECFP6 algorithm. The resultant network graph displayed a clear bifurcation among the steroids. Overlaying the information of cluster status (compounds appear in the Active cluster vs. those only appear in the General cluster) revealed that compounds appear in the Active cluster all localized on one branch of the network (Fig. 12A). It suggested that they are structurally distinct from the other compounds.

Next, we asked whether the steroids from the General and the Active cluster are functionally distinct as well. To assess the function of steroid hormones, we examined the receptor binding of each compound and overlaid the data onto the structural similar network. Intriguingly, nearly all Active compounds bind exclusively to the glucocorticoids receptor (Fig. 12B). Steroids from the General cluster bind either to the remaining four types of steroid hormone receptor or are steroid hormone precursors that do not bind.

We then asked whether there is any structural-functional relationship between steroid hormones and receptor affinity. Based on their chemical structures, we noticed three moieties that are common to glucocorticoids but absent in other steroid hormones, including a double bond between C1 and C2, a fluorine group attached to C9 and a hydroxyl group attached to C11 (Fig. 12C). The information was overlaid onto the structure similarity network and it revealed that these moieties were found almost exclusively among the Active compounds. In addition, a nearly perfect correlation was observed between compounds with a hydroxyl attachment at C11 and the Active compounds (Fig. 12C).

Previous work has shown that the most important substituents which increase the binding affinity of steroid are an olefinic (double) bond between C-1 and C-2, a fluorine atom at the 9a position, a hydroxyl group at the 11B position, and an ester group at the 17a position (Ponec et al. 1986b). Further evidence from the literature helped to explain the high FUCCI expression and low viability associated with the Active compounds. Studies have shown that glucocorticoids (esp. dexamethasone (Li et al. 2012)) inhibit thymidine incorporation into DNA (Cidlowski and Cidlowski 1981), cause DNA damage and induce glucocorticoid receptor-activated p53-mediated cell cycle arrest (Goya et al. 1993; Rogatsky, Trowbridge, and Garabedian 1997). Taken together, our results showed that the phenotypic profiling can provide the inference of SAR.

Phenotypic profiling was independently reproducible between screens and suitable for screening unknown compounds

Based on the success of our result, we then asked whether we can use our phenotypic screen platform to deduce MoA of unknown compounds. But first we had to ascertain the reproducibility of our phenotypic platform. To test the reproducibility of our phenotypic platform, we re-screened a short list of compounds including two compounds from each subcluster and 8 compounds from the General cluster as controls. After standardizing the data, we found that the re-screened compounds appeared in the same subcluster they were previously. Our data suggested that we established a robust assaying platform and it gave us the confidence to use it to screen novel compounds.

Potential discovery of novel anticancer agents

SU 9516 is a drug that has anti-proliferative and pro-apoptotic activity in tumor cells(Lane et al. 2001). It is a potent, selective inhibitor of cyclin-dependent kinases (CDKs) with selectivity for Cdk2 (Lane et al. 2001). Four unknown microbial nature products, A7, A9, C7, C9, clustered significantly with SU9516 suggesting that they share potentially related MoA (Fig. 13). Chemical structural analysis revealed that the A9 molecule is identical to JBIR-06, a grp78 inhibitor (Ueda et al. 2008). A follow-up experiment using the CRISPR-Cas9 system to knockout grp78 and comparable the phenotypic change to compound addition will elucidate the genetic target of A7, C7 and C9 and their relationship to A9.

An improved cell-based phenotypic screening platform with good potential despite a few shortcomings

Despite the results we have demonstrated, the use of phenotypic profiling to identify compound MoA could use some refinement. For instance, we identified 14 subclusters

denoting 8 unique MoAs. In reality, there are more than 20 MoAs of anticancer drugs alone (Payne and Miles 2008). In theory, we could screen a compound library that is sufficiently large to contain compounds with all known modes of action. Also, due to the nature of the phenotypic clustering, each compound can only be identified by one MoA. For compounds with more than one MoA, they will be identified based on their primary or most prominent phenotypic perturbation. Moreover, in situations where multiple phenotypic changes are manifesting in similar proportion, the mode of action of that compounds could be misidentified. However, we have made progress in phenotypic screening by demonstrating a streamlined approach to identify MoA of unknown compounds – a step closer to target elucidation. We believe this method has considerable potential. Further integration of a CRISPR genomic knockout screen could lead to target identification.

FUTURE DIRECTIONS

To confirm that the cell cycle kinetics and chemotherapy response in the isogenic p53 mutant cells were due to Cas9-mediated TP53 mutation and not any off-target events, a donor template can be used to repair the TP53 mutation that was introduced with the CRISPR/Cas9 via homologous recombination to see if the wild-type TP53 phenotype is restored. So far, we introduced a p53 mutation in natively p53 wild-type BC cell lines and showed an increase in chemotherapy sensitivity. However, we can also reverse the process by repairing the p53 mutation in a natively p53 mutant cell line, T47D, and assess if it confers chemotherapy resistance.

We examined the growth kinetics and apoptosis in tumors explanted from doxorubicin-treated mice and found that the p53 mutant tumors had a 1.5-fold increase in proliferation and a 10-fold increase in apoptosis. An analogues experiment can be followed up in the p53 wild-type and isogenic p53 mutant cell lines. Following doxorubicin treatment, we can stain the isogenic p53 cell line pairs with annexin V and quantify the cells in apoptosis via flow cytometry.

So far we used our streamlined high-content cell-based phenotypic profiling assay to identify the mode of action of four novel natural products which are potential proapoptotic and anticancer agents. However, to understand the drug action we must first identify the drug targets, which can be accomplished using a CRISPR-Cas9 genetic KO screen. The purpose of the genetic knockout screen is to determine whether the loss of a gene can recapitulate the phenotypic response to a compound. Identical cellular imaging process to the compound screening is implemented to quantify any targeted gene knockout-

induced perturbations on cellular phenotype. A phenotypic readout is be based on morphological and texture-based parameters. Clustering the readout from the genetic KO screen with the compound treatment screen gives an unbiased comparison of phenotypic resemblance. Significant correlation in phenotype clustering between the genetic KO screen and the compound treatment screen indicate the genetic target is associated with the observed phenotype. 8 genetics targets are chosen for the screen (6 targets, 1 positive control, and 1 negative control).

GRP78

GRP78 is a chaperone located in the lumen of ER that binds newly synthesized proteins as they are translocated into the ER (A. S. Lee 2005). It maintains the protein in a state ready for subsequent folding. When there is an accumulation of unfolded polypeptides in the ER, the unfold protein response (UPR) triggers an increase expression of GRP78 (A. S. Lee 2005). One of the novel compound A9 is known in the literature as JBIR-06. JBIR-06 is reported to inhibit the expression of GRP78 (Ueda et al. 2008). I hypothesize that knocking out GRP78 in MCF7s may result in comparable phenotypic change as adding one of the novel compounds A7, A9, C7, C9.

PERK

PERK, an ER transmembrane kinase, is activated by ER stress and functions as an effector of UPR (Brewer and Diehl 2000). PERK links stress in the ER to the regulation of cell-cycle progression(Brewer and Diehl 2000). One study demonstrated that the activation of PERK is sufficient to mediate loss of cyclin D1 and promote cell-cycle arrest (Brewer and Diehl 2000). Based on the screen, compounds A7, A9, C7, and C9 induced a significantly elevated FUCCI-G1 expression. I hypothesize that after knocking out PERK in MCF7s, the addition of a novel compound (A7, A9, C7, and C9) may no longer induce a significantly elevated FUCCI-G1 expression.

TOP2A

DNA topoisomerase 2-alpha is an enzyme that catalyzes the transient breaking and rejoining of the double-stranded DNA during mitosis. Anthracyclines, such as Doxorubicin and Daunorubicin, are known topoisomerase II poisons (Bodley et al. 1989; Tewey et al. 1984). They intercalate into DNA and prevent its re-ligation after breaking (Bodley et al. 1989; Tewey et al. 1984). I hypothesize that knocking out TOP2A in MCF7s may result in comparable phenotypic change as adding a topoisomerase IIa poison.

KATNA1 and STMN1

Paclitaxel (Taxol) binds to beta-tubulin and inhibits the depolymerisation of the microtubules(Amos and Löwe 1999). Exposure to paclitaxel is known to cause cell death through mitotic catastrophe (Merlin et al. 2000). Both Katanin and Op18/Stathmin are considered microtubule destabilizers and facilitate microtubule disassembly (Hung et al. 2004). Katanin (KATNA1) is a microtubule destabilizing protein that functions as a severing factor. Op18/Stathmin (STMN1) increases the catastrophe rate of microtubules, sequestering tubulin dimers and promoting GTP hydrolysis. I hypothesize that knocking out either KATNA1 or STMN1 in MCF7s may result in comparable phenotypic change (nuclear fragmentation) as adding a mitotic inhibitor.

ATP1A1

Na^+/K^+-ATPase is an enzyme found in the plasma membrane of all animal cells that pumps sodium out of cells while pumping potassium into cells, both against their concentration gradients. Cardiac glycosides inhibit the pumps by stabilizing it in the E2-P transition state (Weiford 2005). It results in an elevated intracellular sodium concentration. I hypothesize that knocking out Na^+/K^+-ATPase (ATP1A1) in MCF7s may result in comparable phenotypic change as adding a cardiac glycoside.

MDM2 - Positive control

MDM2 is a protein that in humans is encoded by the MDM2 gene. Mdm2 is an important negative regulator of the p53 tumor suppressor. Increase expression of p53 will lead to cellular senescence and cell cycle arrest in the G1/G0 phase. This can be measured from an increase in FUCCI-G1 expression. I hypothesize that knocking out MDM2 in MCF7s may result in comparable phenotypic change as adding a p53 activator.

HPRT - Negative control

HPRT is a housekeeping gene and will be used a negative control.

CONCLUSIONS

First, we investigated the relationship between TP53 status and chemotherapy response in ER+ human breast cancer. We used isogenic p53 mutant ER+ BC cell lines and human BC cancer cell line xenografts to demonstrate that p53 mutations confer an elevated susceptibility to anthracycline-based chemotherapy compared to p53 wild-type in ER+ breast cancer. By circumventing p53-mediated cell cycle arrest, p53 mutant ER+ cancer cells become more sensitive to cytotoxic assault that lead to increased apoptosis and improved response. The relationship between TP53 status and treatment response remains controversial. While some reported that wild-type p53 activity is beneficial in breast cancer response, others have shown the opposite. In this study, we provided some compelling evidence that TP53 mutation in human breast tumors leads to an improved response to anthracycline-based chemotherapy which is consistent with previous studies (Jackson et al. 2012; P Bertheau et al. 2002). TP53 status is not used clinically to manage breast cancer (Harris et al. 2007). However, given that TP53 mutation occurs in over 50% of cancers and 30% of breast cancer (Varna et al. 2011), it would be ideal if TP53 is a useful biomarker for the management of breast cancer. In this study, we also provided the evidence supporting the notion that TP53 gene signatures can be a clinically meaningful predictive and prognostic marker of response to chemotherapy for patients with ER+ breast cancer.

Next, we described a streamlined high-content cell-based phenotypic profiling assay that elucidate the mode of action of novel natural products. Our streamlined pipeline harnessed the benefit of high content screening, which combines the efficiency of high-throughput techniques with the capability of cellular imaging to quantify any compound-

induced perturbations on cellular phenotype via a series of morphological and texture-based parameters, while eliminating the need for fixation and staining, which affects existing assays in reducing screen throughput and introducing the potential variations and artifacts. To further couple it with a CRISPR-Cas9 knockout screen would enable a faster identification of drug targets. Ultimately, using differentiated cells derived from patients for the phenotypic screening assay can contribute the development of cell-based disease models and personalized medicine.

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