Validation of novel secondary therapies in relapsed/refractory cancers using Pharmacogenomics and Single-Cell transcriptomics

by

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ABSTRACT

Drug resistance has remained the Achilles' heel in cancer chemotherapy which serves as the principal limiting factor in achieving favorable treatment outcomes in cancer patients. Drug resistance that exists even before drug exposure (intrinsic/innate resistance) or resistance that develops in the course of treatment (acquired) are responsible for the majority of therapy failures and clinical progression (relapse or recurrence). Intra-patient and inter-patient tumoral heterogeneity also play a significant role in therapy resistance and failure as they govern the treatment response. The heterogeneity in drug response is governed by the underlying sub-cellular molecular characteristics of the tumor. Thus, the development of drug resistance and disease relapse in cancer are largely attributed to the treatment-refractory subpopulations of tumor cells. The presence of treatment-refractory subpopulations of tumor cells or cancer stem-like cells (CSCs) with potential self-renewal and differentiation capacities are also believed to drive drug resistance and disease relapse in various cancers. Due to their quiescent nature, which allows them to escape conventional therapeutics, standard agents fail to significantly improve long-term clinical outcomes. Moreover, a major limitation of cancer drug discovery is the low predictive value of the pre-clinical studies as they mostly ignore the cellular heterogeneity and complexity, which resulted in extensive inter-individual variation in response, drug resistance, and dose-limiting toxicities. So, the goal of my graduate research was to validate novel predicted secondary drugs against the most aggressive forms (relapsed/resistant) cancers, taking into account the key features underlying tumor subclonal heterogeneity and personalized sensitivity to chemotherapy.

ACKNOWLEDGEMENTS

*I would like to dedicate the thesis to my parents, my husband, and the blessing of my life, my daughter***.**

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Review of Literature

Cancer

When healthy cells become untamed, escaping cellular death, evading restrictions of cellular homeostasis, tricking immunity due to genetic aberrations within various growth-stimulating receptors, they keep clustering distinctly from one organ to another, thus diminishing regular cellular routine, leading to cancer [1,2].

Cancer has the second highest death occurrence, after cardiovascular disease. According to WHO and the American Cancer Society, the average rate of cancer occurrence is 20.2%. Cancer causes severe socio-economic pressure upon individuals. Lungs, stomach, and breast cancers have the lowest survival rate, while blood cancers (including leukemia and Myeloma) are the most common. The deadliness of cancer has made it the most concerning disease [3]. According to SEER statistics, approximately 1,918,030 new cancer cases were found all over the U.S. in 2022, with an estimated 609,360 deaths. The highest occurring cancers include prostate (268,490 cases), lungs (254,850 cases), and stomach (343,040 cases). The odd of cancer is slightly higher in men (40.2%) than women (38.5%), and person older than 60 are at high risk. Fortunately, with improved targeted therapies, early detection, and better awareness, the survival rate has increased from 49% to 68%. [4]

More than 100 different cancers exist. Typically, cancer types are based on the organs or tissue sites in which they first appear. For instance, according to histological types, these are a few major cancers (https://training.seer.cancer.gov/disease/categories/classification.html):

1. Carcinoma (originated from epithelial cells that line the organs)
    a. Adenocarcinoma (a subtype of carcinomas that originates from glands lining organs)
    b. Squamous cell carcinomas (originates from squamous epithelium and subtype of carcinoma)
2. Sarcoma (originates from both supportive and connective tissues)
3. Leukemia (originates from bone marrow)
4. Lymphoma (originates from lymphatic systems)
5. Multiple Myeloma (originates from plasma cells and subtype of lymphoma)
6. Melanoma (originates from melanocytes which produce melanin)[5,6].

Cancer treatment is a global issue, and researchers are working constantly for its management, improving survival rate, and providing a better quality of life[5]. Some common treatments include surgery, chemotherapy, radiation therapy, immune therapy, bone marrow transplants, and hormone therapy.[6] (https://www.cancer.net/navigating-cancer-care/cancer-basics/cancer-terms-treatment). Among all these treatments, chemotherapy using a combination of multiple drugs is very prevalent with the possibility for long-term treatment [7]. [10] Furthermore, several ongoing studies are engaged in the development of better therapeutic strategies using targeted therapies against signaling pathways blocking cancer cell proliferation, initiating apoptosis, terminating angiogenesis, and disturbing cancer environment. Current target`eted therapies include alkylating agents, antimetabolites, antimicrotubular agents, and proteasome inhibitors.[7]

Cancer drug resistance

Despite these innumerable targeted drugs, several cancers are still incurable, primarily because of relapse/recurrence. One of the major causes of relapse/refractory cancer is drug resistance.

or differential response to standard-of-care chemotherapy. Drug resistance can thus be intrinsic before drug exposure or acquired secondary resistance after drug exposure. Relapsed/refractory cancer types are the most aggressive forms of cancer and the hardest to treat [8-10].

Understanding the drug resistance mechanisms is therefore vital to designing novel cancer chemotherapeutics for better treatment outcomes [8,10]. Several studies have shown that drug resistance may occur when a variety of molecularly and histologically different sub-populations are present within the tumors [11-13]. For example, Innate resistance may result from the presence of small population of pre-existing cells resistant to conventional therapies due to some genetic aberrations [8,14,15]. On the other hand, acquired resistance may occur in selective subpopulations after the drug exposure and lessens the efficacy with time[16]. A genetic mutation that alters the drug target or its level of activation, secondary proto-oncogene activation, driver mutation expression, contact with the tumor microenvironment, or other circumstances may activate alternative pathways[17,18]. This may also result in resistant subclones and immune cells[19]. Differential drug sensitivity may then occur when the target molecule has different expression levels in these subclones, resulting in therapy failure, resistance, poor prognosis, and survival [20-22].

Another plausible cause of drug resistance is that the tumor microenvironment may involve the influence of stromal cells, immune cells, and extracellular matrix, which may communicate with the tumors for their growth, survival, and escape apoptosis [23-28]. Cancer stem cells are another potential cause of drug resistance where tumor-initiating cells undergo self-renewal, causing drug resistance, therapy failure, and disease prognosis. It happens when ATP binding pockets (ABC) transporters are highly expressed operating drug efflux pumps, negating drug action and other tumorigenic potentials [29,30]. Other drug efflux transporters (P-glycoprotein MDR, non-Pgp MDR) also cause multidrug resistance by pumping out the drug constantly and preventing drug binding inside cells [31].

A deeper understanding of these drug resistance mechanisms will be very helpful in the development of better chemotherapeutic therapeutic strategies to combat drug resistance.

Multiple Myeloma

Multiple Myeloma (MM) is the second most common hematologic malignancy. MM occurs in B-cells of bone marrow; Unchecked over-proliferation of monoclonal plasma cells produces aberrant proteins and immunoglobulins and makes the cancer fatal [32]. The median age of diagnosis is 69 years [33]. Major symptoms of Myeloma (CRAB Criteria) include hypercalcemia, renal insufficiency, anemia, and bone lesions[34]. Multiple Myeloma is detected through blood count test, blood chemistry test (to check immunoglobulin, LDH, creatinine, albumin, and calcium levels), electrophoresis to test abnormal antibodies, and bone marrow biopsy[32].

According to SEER statistics, estimated new cases in 2023 are 35730 and estimated deaths are 12590; approximately 1.8% of all new cancer cases death is 2.1%. From 2013-2019, the 5-year survival rate is 59.8%. The chance of getting Myeloma in a lifetime is 1 in 132 (0.76%). (https://seer.cancer.gov/statfacts/html/mulmy.html).

Based on the symptoms and level of M protein production by abnormal plasma cells, Myeloma is divided into the following stages (Table 1) [35-37].

Table 1. Multiple Myeloma Stages

Asymptomatic Myeloma	**Symptomatic Myeloma**
Monoclonal gammopathy of undetermined significance (MGUS)- low M protein level, no end organ	Active (symptomatic) multiple Myeloma- has symptoms related to disease, M protein in the blood, urine, 10% or more blood cells in bone marrow, plasmacytoma in bone or tissue, anemia,

damage/CRAB criteria, 1% chance to develop Myeloma.	kidney failure, hypercalcemia, osteolytic lesions, etc.
Smoldering multiple Myeloma (SMM)- High M protein level (> 30g/L), higher plasma cells in bone marrow (10% to 60%), no end-organ damage, might have a low density of bone minerals, serum free light chain ratio is >100,	Solitary plasmacytoma of the bone- single tumor found in one bone, pain in bone, treated with radiation, 1/3rd develop to myeloma[38].
	Extramedullary plasmacytoma- tumor outside the bone marrow, treated with radiation or surgery
	Light chain myeloma- make only light chain or immunoglobulin, not heavy chain
	Non-secretory Myeloma- no secretion of M protein or light chains in blood or urine; only x-ray shows osteolytic lesions [39].
	Rare Myeloma (IgD, IgE)- IgD effects at younger ages; IgE type is more aggressive and spreads faster

According to the International Staging System (ISS), multiple Myeloma the severity of Myeloma is denoted by the following stages (**Table 2**)[32,36,37].

Table 2. International Staging System (ISS) of Multiple Myeloma

Stage	**Frequency (% of patient)**	**%-year survival rate**
ISS stage I (serum albumin > 3.5 g/dL, serum beta-2-microglobulin < 3.5 mg/L) and no high-risk cytogenetics, Normal LDH (lactate dehydrogenase)	28	82
Stage II (serum albumin < 3.5 g/dL, serum beta-2-microglobulin 3.5 to 5.5 mg/L). Neither stage I nor III	62	62
ISS stage III (serum beta-2-microglobulin > 5.5 mg/L) and High risk cytogenetics [t(4;14), t(14;16), del (17p)] or elevated LDH	10	40

Multiple Myeloma: Treatment

Patients with localized plasmacytoma are treated with radiation therapy and often surgery. But if the tumor spreads or metastasizes, then chemotherapy bone marrow transplant or stem-cell

Transplantation are the options based on cytogenetic risk groups. The top conventional combinations of chemotherapy are proteasome inhibitors and immunomodulatory agents., Etoposide, Doxorubicin, and Melphalan[40]. Treatment strategies for Myeloma include proteosome inhibitors (Bortezomib, Carfilzomib) and immunomodulatory drugs (Thalidomide, Lenalidomide)[41].

Standard of care for Myeloma is Proteasome inhibitors (P.I.s) that Inhibiting tumor metastasis and angiogenesis by speeding up the unfolded protein response (UPR) or the ubiquitindependent proteolysis of critical regulatory proteins involved in importa nt physiological and pathophysiological cellular processes in cancer cells and by inhibiting the NF-B-enabled regulation of drug resistance caused by cell adhesion. In 2003, the U.S. Food and Drug Administration (FDA) approved the first P.I. for use in clinical settings for the treatment of relapsed and refractory Myeloma: bortezomib (Bz/Velcade).[42-44] Ixazomib (Ix /Ninlaro/MLN9708) and Carfilzomib (Cz/Kyprolis), two more examples, are second-generation PIs.[43-45] P.I.s are effective anti-MM drugs when used alone or in combination with other anti-cancer agents like immunomodulatory drugs (IMiDs), alkylating agents, topoisomerase inhibitors, corticosteroids, and histone deacetylase inhibitors (HDACi).[42,43]

Proteasome inhibitor induces unfolded protein response (UPR) by blocking the degradation of the misfolded proteins and activating the E.R. stress.[46,47] Prolong E.R. stress leads to programmed cell death by activating PERK/eIF2α/CHOP signaling, IRE1α signaling and caspase pathway[45].

Immunomodulators (IMiDs) target the immune system by turning down or turning up some proteins. In multiple Myeloma, IMiDs have multiple mechanisms of eliminating myeloma cells, stimulating the immune system, preventing cellular adhesion, decreasing angiogenesis, and disrupting cancer-stimulating cytokines. Thus, stops myeloma growth and controls the bone marrow microenvironment[48].

FDA-approved IMiDs for myeloma therapy include pomalidomide, Lenalidomide, and thalidomide, and they are given in combination with monoclonal antibody steroids with dramatically improved outcomes.IMiD works by inducing cyclin-dependent-kinase (CDK) inhibitors: p21, p27, p15 and thus, it inhibits CDK activity, causing cell cycle arrest at the G0/G1 phase. They also cause changes in the expression of the early growth response gene (Erg)-1,2 and SPARC (Secreted protein acidic and cysteine-rich). They downregulate NFkB by lowering expressions of anti-apoptotic proteins (cIAP2 and FLIP). They also diminish caspase 3,8 and 9 activities.[75] Patients with IMiD resistance show low levels of Cereblon (CRBN), which is the target protein for IMiD. Thus, inadequate response to IMiDs with poor survival rate. IMiDs bind to CRBN to induce protein degradation[48,49]. Also, there is a mutation in IRF4 IKZF1 genes causing CRBN-IKZF1-IRF4 inactivation. IRF4 harbors a truncating mutation that renders it resistant to Lenalidomide-mediated downregulation[50].

Corticosteroids such as Dexamethasone and prednisone reduce inflammation by inhibiting pro-inflammatory cytokines (IL-6) and NF-kB. These drugs also stimulate immune cells to fight against cancer cells[51].

Immunotherapy is a common and effective treatment strategy. It stimulates the body's own immune cells to attack malignant cells[52]. One of these is the Bispecific T-cell engager (BiTE), which is attached to T-cells and the antibody of the BCMA protein. When attached to the BCMA protein of myeloma cells, it stimulates T-cells to attack myeloma cells[53].

Another type of immunotherapy is monoclonal antibodies. For example, daratumumab binds to CD38 (highly expressed cell surface marker in Myeloma), inducing apoptosis via antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC)[54].

Relapse/Refractory Multiple Myeloma: Drug-resistant Myeloma

However, despite these therapies, some patients undergo relapse/refractory disease and progress toward more aggressiveness[38]. Myeloma is still a difficult-to-cure illness with dose-limiting toxicities and treatment resistance, with a median survival rate of just about 7 years, despite these and other recent advancements in therapies.[55,56] Not all patients react to treatment equally well, and those who do frequently develop resistance during the course of care. As a result, there are two types of drug resistance: (1) innate resistance, which is already present in drug-naive patients who never respond to treatment, and (2) emerging/acquired resistance, which occurs when a patient's tumor relapses or "acquires" the ability to resist therapy during treatment, despite having responded well to initial therapy.[42,56] Thus, Relapsed/refractory Myeloma (RRMM) has increased M-protein level one or more CRAB symptoms[51,57]. Drug resistance in Myeloma causes myeloma relapse, which has a poor prognosis aggressiveness. Various mutations in somatic regions, chromosomal aberrations, and epigenetic and micro-environment transformation lead Myeloma towards aggressiveness (asymptomatic form to high-risk Myeloma)[46,58]. Further, due to heterogeneity at clonal and sub-clonal levels, myeloma patients respond differently. Thus, precision medicine approaches have huge potential for better efficacy and lowering side effects, which are customized for individual patients based on their genetic profile[47,59].

MM with resistance to proteasome inhibitors (Bortezomib) shows PSMB5 gene elevated expression with a point mutation in the B5 subunit at the S1 pocket, reducing its hydrophobic interactions[60,61].

In Len-resistant cells, IL-6 and STAT3 expression are upregulated than in Len-sensitive cells, which upregulate MYC and activate MAPK and PI3K pathways[60].

Thus, currently, the major problem in myeloma chemotherapy is drug resistance to standard care treatments and a lack of novel and affordable therapies.

Prostate Cancer

Another common and life-threatening cancer is prostate cancer (PCa). According to SEER statistics, the estimated new cases is approximately 288300 in 2023 or 14.7% of all new cancer cases; estimated deaths were 34700 or 5.7% of all cancer deaths (https://seer.cancer.gov/statfacts/html/prost.html).

The prostate is a gland in males beside the genital area that produces seminal fluids for transporting and nourishing sperm. But when there is uncontrolled growth of prostate gland cells that hampers its homeostasis, it often results in prostate cancer[62].

The major symptoms of PCa are erectile dysfunction, prostate enlargement causing pain, discomfort, blood, and frequent urination[63].

Screening tests performed to detect PCa involve detecting PSA (Prostate-specific antigen) level; PSA >4 ng/ml indicates a higher risk of prostate cancer[64,65.] Physical examination of prostate gland abnormalities is done via digital rectal exam (DRE)[64]. Other diagnostic tests include prostate biopsy examination collecting prostate tissue, transrectal ultrasound (TRUS) of the prostate gland, and magnetic resonance imaging (MRI) to locate target areas of prostate cancer and its metastasis[62].

According to the type of cells in prostate cancer, the major forms include adenocarcinoma of the prostate (originates from glandular epithelial cells that make the lining of the prostate gland). It has 2 subtypes: acinar adenocarcinoma (originates from acinar cells making the lining of the fluid-secreting gland) and ductal adenocarcinoma (it originates from cells lining the ducts of the prostate gland being the most aggressive and rare form)[66,67]. Other prostate cancer types

are transitional cell carcinoma/urothelial cancer (originating from the renal pelvis and ureter) and neuroendocrine prostate cancer, whose subtypes are small cell prostate cancer and squamous cell carcinoma[67].

Prostate Cancer: Treatment Options

Initially, prostate cancer is treated with surgical removal (castration) radiation therapy, and later, if it keeps progressing, then the standard therapy regimens include androgen deprivation therapy (ADT), cryosurgery, chemotherapy, immunotherapy, CART therapy, monoclonal antibody(**Table 3**)[68,69]. Androgen hormone induces prostate growth, so androgen receptor blockers are used to reduce the action of androgen (anti-androgen therapy) when Androgen Deprivation Therapy (ADT) fails. They diminish the activity of androgens for promoting cancer growth. FDA-approved androgen receptor blockers are Enzalutamide, apalutamide, and darolutamide [70-73].

Table 3: Current treatment modalities for different stages of prostate cancer[68,69]

Stage	**Standard Treatment Options**	**In Clinical Trials**
Stage I	Radical prostatectomy, External-beam radiation therapy (EBRT) with or without adjuvant hormonal therapy, Interstitial implantation of radioisotopes.	High intensity focused ultrasound therapy, Photodynamic therapy.
	Radical prostatectomy, External-beam radiation therapy (EBRT) with or without adjuvant	Ultrasound-guided percutaneous cryosurgery, Proton-beam

Stage II	hormonal therapy, Interstitial implantation of radioisotopes	radiation therapy, Photodynamic therapy, neoadjuvant hormonal therapy followed by radical prostatectomy
Stage III	External-beam radiation therapy (EBRT) with or without adjuvant hormonal therapy, Hormonal manipulations (with or without radiation therapy), Radical prostatectomy with or without EBRT	
Stage IV	Hormonal manipulations with or without chemotherapy, Bisphosphonates, External-beam radiation therapy, Palliative radiation therapy (EBRT) with or without adjuvant hormonal therapy, Palliative radiation therapy, Palliative surgery with transurethral resection of the prostate (TURP)	

Various classes of drugs are approved for the treatment of different stages of prostate cancer. These include (**Table 4**)

Table 4: FDA-approved drugs for different stages of prostate cancer [69,74]

Class	**Drugs**	**Comments**
Cancer vaccine	Sipuleucel-T	Used to treat advanced prostate cancer that's no longer

		responding to hormone therapy but is causing few or no symptoms
Immune Checkpoint inhibitor	PD-1 inhibitor	
Chemotherapy	Docetaxel, Cabazitaxel Mitoxantrone Estramustine	It is used when cancer spreads outside the prostate gland, and hormone therapy isn't working.
Hormone therapy: Treatment to lower testicular androgen levels	Orchiectomy (surgical castration)	Removal of the testicles, where most of the androgens (testosterone and DHT) are made
	LHRH agonists: Leuprolide, Goserelin,	Lower the amount of testosterone made by the testicles
	LHRH antagonists: Degarelix	Used to treat advanced prostate cancer
Hormone		Blocks an enzyme (protein) called CYP17, which helps

therapy: Treatment to lower androgen levels from the adrenal Glands	Abiraterone	stop these cells from making androgens. It can be used in men with advanced prostate cancer that is either High-risk or Castration-resistant.
	Ketoconazole	Used to treat men just diagnosed with advanced prostate cancer
Hormone Therapy: Drugs that stop androgens from working	*Anti-androgens*: Flutamide Bicalutamide Nilutamide	If orchiectomy or an LHRH agonist or antagonist is no longer working by itself,
Hormone Therapy: Newer anti-androgen	Enzalutamide Darolutamide Apalutamide	Men with cancer that has not spread but is no longer responding to other forms of hormone therapy (known as non-metastatic castrate-resistant prostate cancer (CRPC)
	Abiraterone-acetate	It's approved for men with advanced prostate cancer who have tried other hormone therapies.

Lethal variants of Prostate cancer: mCRPC and Drug-resistant PCa

Prostate cancer slowly moves from localized cancer and eventually metastasizes to other organs with increasing PSA levels. Metastatic prostate cancer is of two different types: androgen-dependent/androgen-independent. Androgen-dependent prostate cancer may be a hormone naïve or sensitive and is thus treated with ADT-coupled androgen-pathway directed therapies (Enzalutamide, Abiraterone, taxanes or radiotherapy). However, most patients undergo relapse after responding to initial treatment. These are very aggressive forms called metastatic or non-metastatic castration-resistant prostate cancer [73,75-77].

Eventually, all the therapy options fail, and the cancer turns into a more aggressive form where it becomes unresponsive to hormone deprivation therapy. These prostate cancers thus become androgen-independent, and they progress irrespective of androgen support. They are, therefore, called metastatic castrate-resistant prostate cancer (mCRPC). These are most lethal and have a poor prognosis, with a median survival of <3 Years and 5-year survival rate is 30%[70-72].

The current first line of therapy for mCRPC is taxanes (Docetaxel. Cabazitaxel). Taxanes target microtubules and disrupt their normal functions in mCRPC patients.[94] As prostate cancer cells rapidly proliferate, the effect on microtubule causes cell-cycle arrest in metaphase and, ultimately, apoptosis[75,76]. Taxanes work by inhibiting microtubular depolymerization by binding to the B-tubulin of the microtubule, promoting and stabilizing its assembly in the absence of GTP. It leads to disruption of microtubulin dynamics and cell cycle arrest followed by activating apoptotic pathways in cells[73,77]. It also inhibits Androgen Receptor translocation and facilitates FOXO1-mediated repression of A.R. transcriptional activity[77]. It also attenuates the effect of Bcl-2 and Bcl-xl gene expression by DTX-mediated microtubule stabilization, inducing Bcl-2 phosphorylation, leading to the loss of Bcl-2 anti-apoptotic function. It occurs by decreasing its binding with pro-apoptotic Bax protein followed by apoptosis[78].

However, despite successful taxane therapy for mCRPC patients, eventual resistance is universal. Firstly, there is upregulation of efflux transporters, causing reduced binding affinity due to mutated microtubule binding sites. Secondly, they have higher stemness and a higher risk of mutations. Thirdly, the upregulation of phosphorylated AKT in CRPC is also associated with resistance[79].

Furthermore, the prostate cancer cells undergo de-differentiation (lineage plasticity), extensive transcriptional reprogramming (SOX2, SOX11), loss of p53 & phosphatase and tensin homolog (PTEN), and chromatin structure rewiring. These are rapidly progressing with poor prognosis, median survival of 1-3 years, and higher tumor burden[80-84]. Androgen Deprivation Therapy (ADT), taxane, and radiation resistance lead to Neuroendocrine Prostate Cancer (NEPC). It is very rare and in <2% of cases, poor survival of <2 years, upregulated Bcl-2. Almost 40% of patients with primary therapy resistance led to NEPC[84-86].

Moreover, stemness is a major issue where it is seen as upregulated and more prone to mutations. This stemness is associated with epithelial to mesenchymal signaling (EMT) promoting stem cell signaling pathways (https://seer.cancer.gov/statfacts/html/prost.html).

GAP in Literature

Most of the advanced state cancers (like relapsed/ refractory Myeloma and drug-resistant mCRPC) are difficult to cure, highly heterogeneous, with high recurrence rates and poor long-term prognosis[87]. There are very limited or no therapeutic options if patients undergo continuous relapse/refractory state[88].

Therefore, there is an unmet need to discover novel drugs against these lethal forms of cancers for disease management, improved survival rate, and better quality of life.

Hypothesis

We hypothesize that we will identify novel strategies for circumventing resistance to primary drugs in the lethal forms of the following cancers: RRMM and drug-resistant mCRPC,

Objective

Our lab has compiled a panel of >70 human myeloma cell lines (HMCLs) and >10 Prostate Cancer Cell Lines representing innate and acquired P.I. resistance and the broad spectrum of biological and genetic heterogeneity of myeloma patients.

My **goal** is to use our cell line models to i) identify novel secondary drugs to treat these lethal forms of cancer and ii) create strategies to functionally validate drug-related genes/pathways as novel therapeutic targets. Our ultimate aim is to improve cancer patients using pharmacogenomics-guided strategies (precision medicine).

CHAPTER 2

Validation of novel secondary drug candidates in drug-resistant Multiple Myeloma

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BACKGROUND/RATIONALE

Multiple Myeloma ranks 2nd among all the hematopoietic cancers with substantial molecular complexity and heterogeneity[42,88,89]. Despite novel and improved therapies, Myeloma recurs in several patients after the initial response to treatments and becomes unresponsive or shows clinical progression while on treatment or within 60 days of the most recent treatment where the patient showed at least minimal response.[51,57]

Finding novel secondary therapy options, in which novel chemicals could be paired with well-established medicines to have synergistic benefits, is crucial for treating drug resistance in Myeloma.

In order to forecast the effectiveness of anti-cancer treatments and avoid delaying the choice of more effective alternative medicines, it is crucial to comprehend the significant elements of underlying tumor heterogeneity and personalized sensitivity to chemotherapy.[36,42,55,56,89,90] To evaluate the survival endpoints in clinical applications, these drugs must be administered to a number of patients over a period of months or years. As a result, creating response prediction algorithms can be a tedious operation. One alternative is to use *In vitro* drug response modeling as an approach that employs collections of human cancer cell lines from patient tumors that represent a wide spectrum of the biology and genetic heterogeneity of cancer. A panel of more than 70 human myeloma cell lines (HMCLs) has been assembled by us, representing the wide range of biological and genetic variability of myeloma patients. Because of this, developing response prediction algorithms can be time-consuming. Our strategy, called *in vitro* drug response modeling, uses groups of cancer cell lines derived from patient tumors that cover a

broad range of cancer biology and genetic heterogeneity. We have put together a panel of more than 70 human myeloma cell lines (HMCLs), which represent the broad spectrum of biological

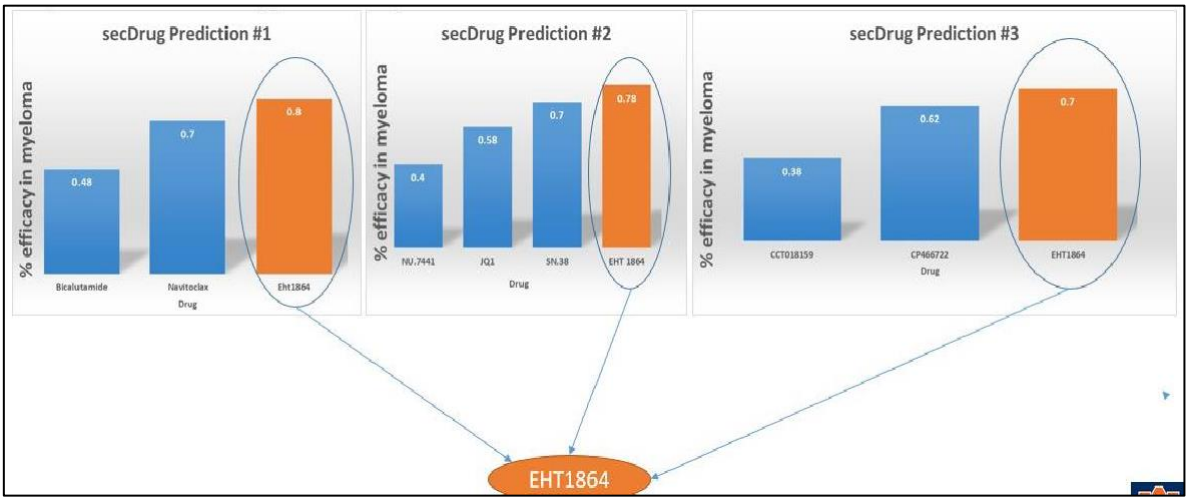


Figure 1 Top Combination Therapy candidates predicted by secDrug Algorithm.

and genetic heterogeneity of myeloma patients.[90]

Furthermore, we have created a computational technique called secDrug for finding novel synergistic secondary medication combinations that may successfully reverse resistance as combination regimens and enable lower dosages and less toxic FDA-approved myeloma medicines[91]. The design and development of the secDrug pipeline is based on a non-trivial and mathematically involved algorithm[91]. Briefly, a novel, data-driven modified greedy algorithm/minimal set-cover/computational optimization method was used, followed by regularization to seek all secondary drugs that could kill the maximum number of cell lines in the Genomics of Drug Sensitivity in Cancer (GDSC version GDSC1000) database belonging to the test disease (B-cell cancers including Myeloma) resistant to the test drug (the Proteasome inhibitor/P.I. drug Bortezomib/Bz/Velcade) in a sequential manner ordered by the number of cell lines killed[12]. The Genomics of Drug Sensitivity in Cancer (GDSC1000) resource is the largest public collection of information on drug sensitivity in human cancer cells (contains drug-sensitivity data on >550 drugs covering a wide range of targets and processes involved in cancer biology on more than 1000 human tumor cell lines, representing a wide spectrum of human cancers, along with a wide array of genetic information including gene expression

analysis data) [26,27]. Among the predicted top secondary drug combinations in PI-resistant + PI-neutral myeloma with a P.I. backbone was the Rac-1 inhibitor (EHT-1864) as a common secDrug predicted in each algorithm showing possible potential therapy against

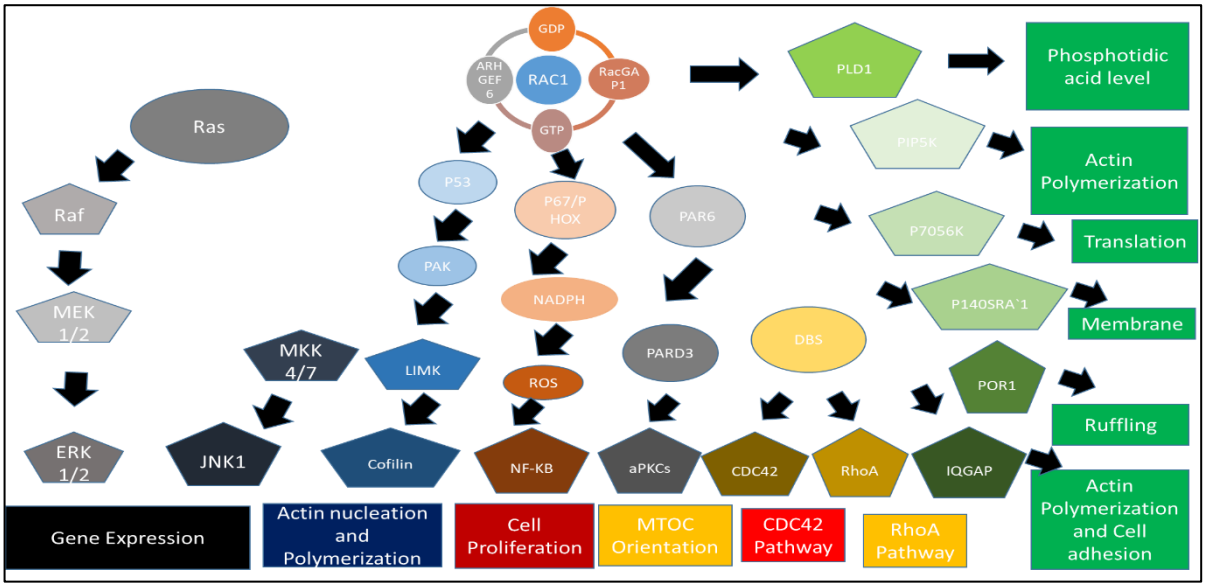


Figure 2 Rac-1 Associated Genes/Pathways

relapsed/refractory Myeloma (**Figure 1**).

Ras-related C3 botulinum toxin substrate 1 or Rac1 proteins are key members of the Rho family GTPases[92,93]. These molecular switches play important roles in the modulation of a wide range of cellular processes, including cell migration, cell polarization, membrane trafficking, cytoskeleton rearrangements, proliferation, apoptosis, and transcriptional regulation (**Figure 2**)[94-96]. Aberrant activation of Rho family small GTPases (Rac1) promotes the uncontrolled proliferation, invasion, and metastatic properties of human cancer cells[94,96]. EHT-1864 (Exon Hit Therapeutics) is a thioquinoline-type compound that serves as a selective small molecule Rac inhibitor, potentially targeting downstream protein kinase effectors[97,98]. It binds to Rac1 tightly, locking the Rho GTPase in an inert and inactive state[97,98]. Furthermore, EHT1864 can inhibit the association of Rac with its effector Pak, as well as a variety of downstream Rac signaling pathways[97,98].

We hypothesize that the novel secDrug, EHT1864 (a Rac1 inhibitor), will circumvent resistance to primary drugs in relapsed and refractory Myeloma and generate better treatment outcomes.

Therefore, our goal was to create a multi-pronged approach/pipeline to discover, validate and characterize EHT1864 as a potential secondary choice for circumventing resistance to primary drugs in Myeloma and generate better treatment outcomes.

First, we showed that the EHT1864+Ixazomib combination was likely to be very effective when we presented single-cell transcriptomics as a unique screening approach for prioritizing secDrug combinations based on the sub-clonal expression of the drug targets. Next, we employed our HMCL panel as an in vitro model system exhibiting inter-individual variation in drug response/resistance to validate our prediction results and demonstrate that EHMT1864 was effective as a single agent and in combination against P.I.- and IMiD resistance. Additionally, we added functional assays and next-generation RNA sequencing following EHT1864 (a Rac1 inhibitor) treatment to discover novel pathways and differentially expressed (D.E.) genes linked to effective medication combinations.

We conclude that EHT1864/Rac1 inhibitor may be considered as a secondary option for avoiding primary drug resistance in Myeloma and to produce better treatment outcomes. The combination regimens may also enable lower dosages and less toxicity from FDA-approved myeloma medicines.

MATERIALS AND METHODS

Drugs, reagents, antibodies, and kits

Ixazomib (Ixa) was procured from Takeda (Takeda Pharmaceuticals Inc., Deerfield, IL, USA). All other drugs were purchased from Selleck Chemicals (Houston, TX, USA). Drugs were dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C. Recombinant Human IL-6 was obtained from PeproTech, Inc. (Cranbury, NJ, U.S.)

Cleaved caspase-3/8/9, HSP90, c-Myc, p65, and IRF4 antibodies were purchased from Cell Signaling Technology (Danvers, MA, U.S.). Monoclonal Anti-β-Actin-Peroxidase antibody produced in mouse was purchased from Sigma-Aldrich (St Louis, MO, USA). Goat anti-Mouse/Rabbit IgG (H + L) secondary antibody (HRP conjugated) was obtained from ThermoFisher Scientific (Waltham, MA, USA). Caspase-Glo 3/7 Assay System and CellTiter-Glo 2.0 Assay were purchased from Promega (Madison, WI, USA).

Human myeloma cell lines (HMCLs)

HMCLs generated through the immortalization of primary myeloma cells were used as *in vitro* model systems to screen top secDrugs against sensitive, innate resistant, and acquired (Parental/P vs. clonally derived resistant/R pairs generated using dose escalation over a period of time) myeloma[90]. We have also generated *in vitro* drug response profiles for the four P.I.s: Bz, Cz, Oprozomib (Opz), and Ixa as single agents in all the HMCLs included in panel [90]. PI-sensitivity in these cell lines was highly correlated, which suggests that any of these four P.I.s could be used as surrogates[99]. Therefore, we used Ixazomib as the representative PI in this study. Further, we have used machine learning-based computational approaches to derive a gene expression signature predictive of baseline PI-response in myeloma[90]. For *in vitro validation*, we used the myeloma cell lines from our HMCLs representing drug-sensitive

(FLAM76), innate/refractory resistance (LP1), and acquired/relapsed resistance (parental and clonally derived PI-resistant and IMiD-resistant HMCL pairs U266 P/VR, RPMI P/VR, MM1S P/LenR). The IMiD-resistant cell line, MM1S LenR, was obtained as a gift from Dr. Keith Stewart, Mayo Clinic, AZ. The FLAM76 K-Ras (FLAM76 K12) and FLAM76 N-ras (FLAM76 N61) cell lines were generated from the FLAM76 WT cell line using Adeno-associated viral (AAV) vector-mediated delivery of CRISPR-Cas9 for genome editing in humans. Ras mutations were validated using the Sanger DNA sequencing method. All cell lines were authenticated at source and tested randomly at regular intervals at the A.U. Center for Pharmacogenomics and Single-Cell Omics (AUPharmGx) using Gene-Print 24 System from Promega (Madison, WI, USA). All cell lines are mycoplasma negative. HMCLs were maintained in HMCL media supplemented with IL-6.

***In vitro* chemosensitivity assays and drug synergy analysis.**

Cells were treated with increasing concentrations of secDrugs and P.I.s (represented by Ixazomib) or IMiDs (represented by Lenalidomide) as single agents or in combination for 48 h, and cytotoxicity assays were performed using CellTiter-Glo® Luminescent cell viability assay (Promega Madison, WI) which measures viability using ATP produced by living cells. Luminescence was recorded in a Neo2 Microplate Reader (Biotek), and half-maximal inhibitory concentration (IC50) values were determined using GraphPad Prism software by calculating the nonlinear regression using sigmoidal dose-response equation (variable slope). Drug synergy was calculated using Calcusyn software based on Chou–Talalay's combination index (CI) method and the isobologram algorithm (Biosoft, US).

Apoptosis assays

Caspase-3/7 activity assay was performed on the HMCLs using Caspase-Glo 3/7 luminescent assay kit according to the manufacturer's instructions (Promega Madison, WI) using Synergy 2 Microplate Reader (BioTek; Winooski, VT, U.S.). Cell death by apoptosis was also measured by immunoblotting analysis.

Single-cell RNA sequencing (scRNAseq)

Automated single-cell capture, and cDNA synthesis were performed at ~1500 tumor cells/sample using 10× Genomics Chromium platform from 10X Genomics (Pleasanton, CA, USA) that uses droplet-sequencing-based chemistry. Single-cell RNA sequencing was performed on Illumina HiSeq 2500 NGS platform (Paired-end. 2 × 125 bp, 100 cycles. v3 chemistry) from Illumina (San Diego, CA, USA) at >50 million reads per sample.

scRNAseq data analysis

scRNAseq datasets were obtained as matrices in the Hierarchical Data Format (HDF5 or H5). A combination of Seurat and Partek Flow software packages was used to pre-process the data and perform single-cell transcriptomics analysis. Highly variable genes for clustering analysis were selected based on a graph-based clustering approach. The visualization of cell populations was performed by t-SNE.

Next-generation RNA sequencing (NGS)

HMCLs were plated at a density of 4×10^5 cells per mL, and 20 μM of EHT-1864 was added as a single agent or in combination with 15 nM of Ixazomib. Baseline (untreated) and post-

treatment (treated) cells were collected 24 h post-treatment. High-quality RNA was extracted using QIAshredder and RNeasy kit (Qiagen). RNA concentration and integrity were assessed using a Nanodrop-8000 spectrophotometer (Thermo-Fisher Scientific; Waltham, MA, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA, USA) and stored at −80. °C. An RNA integrity number threshold of eight was applied, and RNA-seq libraries were constructed using Illumina TruSeq RNA Sample Preparation kit v2 from Illumina (San Diego, CA, USA). NGS Libraries were size-selected, and RNA sequencing (RNAseq) was performed on Illumina's NovaSeq platform using a 150 bp paired-end protocol with a depth of > 20 million reads per sample.

RNAseq data analysis

Gene expression data were pre-processed, log2-transformed, and analyzed using a combination of command-line-based analysis pipeline (DEseq2 and edgeR) and Partek Flow software to identify differential gene expression profiling (GEP) signatures. Genes with mean counts<10 were removed, and CPM (counts per million) data was used to perform differential expression testing to identify GEP signatures. Due to the small sample sizes, we used GSA to perform differential gene expression analysis between groups that applies limma, an empirical Bayesian method, to detect the D.E. genes (DEGs). Genes with mean fold-change > |1| and $p < 0.05$ were considered as the threshold for reporting significant differential gene expression. Heatmaps were generated using unsupervised hierarchical clustering (H.C.) analysis based on the top DEGs.

Pathway analysis

Ingenuity pathway analysis (IPA) software (QIAGEN, Hilden, Germany) was used to identify the molecular pathways, and upstream regulators predicted to be activated or inhibited in response to EHT-1864 treatment (single-agent and combination with P.I.s) based on the list of significantly differentially regulated genes[91].

Western Blotting

HMCLs were treated with EHT-1864 alone, Ixa alone, and EHT-1864 + Ixa combination. Cells were harvested, washed, and lysed using radioimmunoprecipitation assay (RIPA) lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP40, 5 mM EDTA, 1 mM DTT, phosphatase, and protease inhibitors cocktail (Sigma) and incubated on ice for 15 min. Samples were then centrifuged at 14,000 rpm at 4 °C for 30 min. The supernatant was then aspirated and quantified using Pierce™ BCA Protein Assay Kit (Thermo-Fisher Scientific; Waltham, MA, USA). Samples were solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, and equal amounts of protein were loaded per lane of 10% sodium dodecyl sulfate-polyacrylamide gels and transferred onto PVDF membranes (Millipore; Billerica, MA, USA). Membranes were blocked in TBS with SuperBlock™ blocking buffer (Thermo-Fisher Scientific; Waltham, MA, USA) incubated with primary antibodies and secondary antibodies in TBS with 0.2% Tween 20 and 2.5% bovine serum albumin. Immunoreactivity was detected by chemiluminescent HRP substrate (BioRad Laboratories; Hercules, CA, USA) and the exposed image was captured using a ChemiDoc™ MP Imaging System (Bio-Rad).

Statistical analysis

All statistical analyses were performed using R (the project for statistical computing and graphics) and GraphPad Prism 9.0 software. All tests were two-sided, and $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Drug Screening: Single-cell transcriptomic analysis predicted EHT1864 as a top secDrug based on enrichment of Rac1 targets in the Myeloma sub clonal population.

First, we used single-cell RNA sequencing (scRNAseq) as a biomarker-based drug screen to identify chemo-resistant, drug-tolerant single-cell sub-clones in Relapsed/Refractory Myeloma (RRMM) cell lines that harbor secDrug target genes.

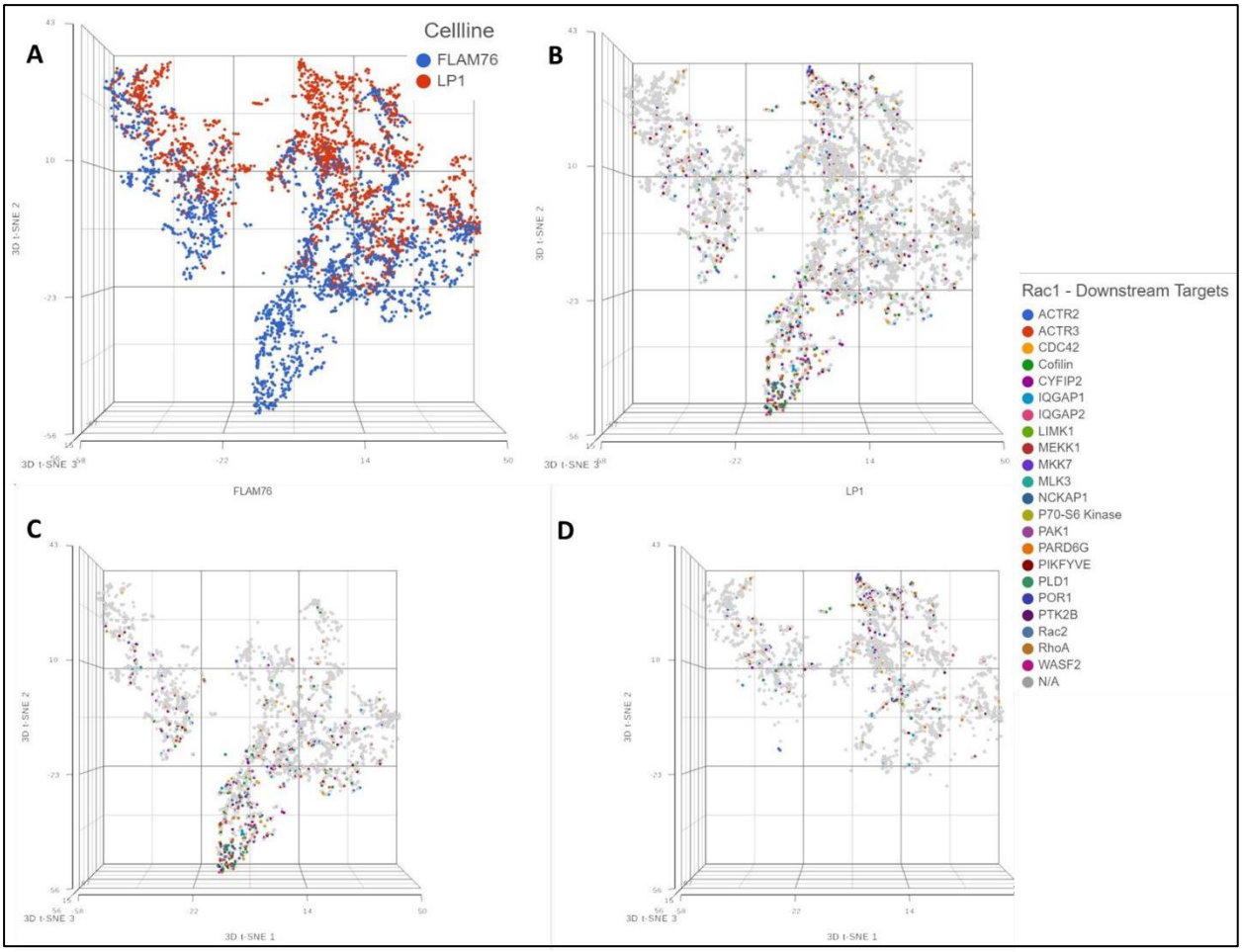


Figure 3 Single cell transcriptomics identifies enrichment of Rac-1 targets in myeloma subclones.

We performed single-cell RNA sequencing using the Droplet sequencing method (10X Genomics) to investigate the enrichment of drug targets and 'screen' for subclones informing potential combinations. The t-distributed stochastic neighbor embedding (tSNE) plots in **Figure 3** show the enrichment of genes, which are primarily downstream targets of Rac1 within the single-cell sub clonal population. Each dot in **Figure 3A** shows single cells of Flam76 and

LP1 cell lines. In **Figure 3B**, the abundance of Rac1-associated genes in each single cell of Flam76 and LP1 is presented. **Figure 3C** and **Figure 3D** show these targeted genes separated into two cells. Interestingly, we could find a high percentage of cells within both the PI-sensitive cell line, FLAM76, and the PI-resistant myeloma line, LP1. This is important since these scRNAseq results suggest that the EHT1864+PI combination may be effective against a broader spectrum of myelomas, irrespective of the sensitivity to primary drugs.

EHT1864 effectively induces loss of viability in HMCLs as single agent treatment.

We determined the cytotoxic effects of the top predicted secondary drug, EHT1864, on a panel of the PI-resistant and Ras mutant cell lines represented by FLAM76, LP1, RPMI, U266 and the clonally derived acquired P.I.- resistant lines U266Vr and RPMIVr and Ras mutant cell lines (NRas and KRas). The single agent survival curves in **Figure 4A** showed that EHT1864 worked effectively against all the cell lines and significantly diminished the viable cell numbers in a dose-dependent manner. The relative IC50 values are

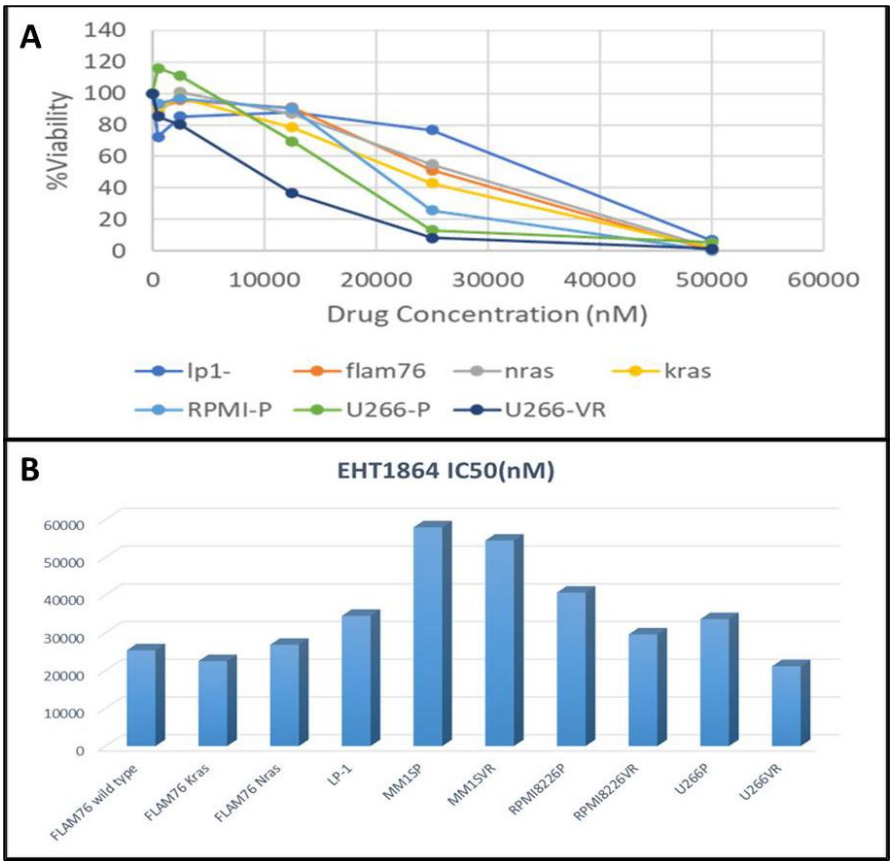


Figure 4 Single-agent *in vitro* cytotoxicity of EHT1864 in HMCLs

provided in **Figure 4B**. Furthermore, we also found significant single-agent cytotoxicity in

HMCLs for the following secDrugs, which were shown as combination options for EHT1864 in Figure 1, CCT018159 (Hsp90 inhibitor) and Navitoclax (Bcl-2 inhibitor) (data not shown). Interestingly, we have earlier shown that another Hsp90 inhibitor, 17-AAG or Tanespimycin, is highly efficacious against P.I.- and IMiD-resistant Myeloma as single agents or as a combination[91].

Drug combination treatment with EHT1864 showed synergy with P.I., IMiD and other secDrugs

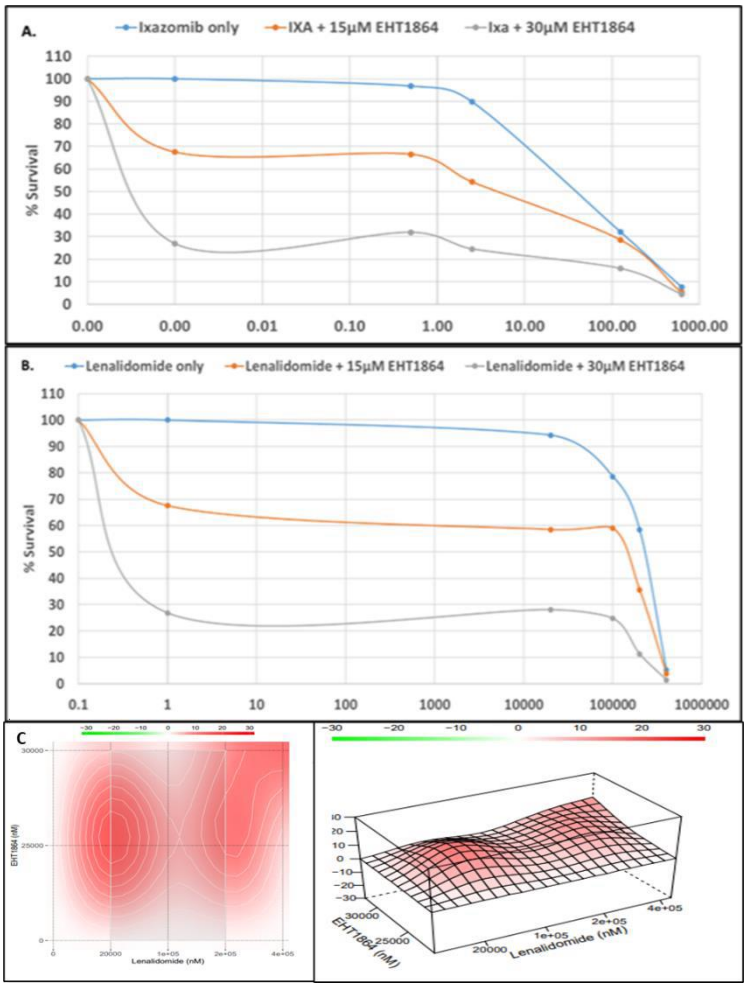


Figure 5 *In vitro* cytotoxicity of EHT1864 drug combination in myeloma cell lines. Representative dose-response curves showing the following drug combinations. A. Ixazomib+ secDrug EHT-1864. B. Lenalidomide+ secDrug EHT-1864. C. Bliss scores.

Next, we evaluated the cytotoxic effect of EHT1864 in combination with either Proteasome Inhibitor drugs (Ixazomib) or Immunomodulatory agent (Lenalidomide). The dose-response curves for the drug combinations and Bliss scores indicated high synergy, which was even more profound (Bliss score >5) in the resistant lines (**Figure 5**). The Bliss model for drug synergy evaluation is based on the Bliss independence

principle to study the combination of two mutually nonexclusive drugs where each drug targets a different signaling pathway[100]. Thus, the Bliss score is derived to identify potential synergistic drug combinations[101]. Furthermore, as shown in **Figure 6**, we also observed synergy between EHT1864 and the secDrugs, CCT018159 and CP466722 (Ataxia-telangiectasia mutated (ATM) inhibitor), that were predicted to work together with EHT1864 in Figure 1.

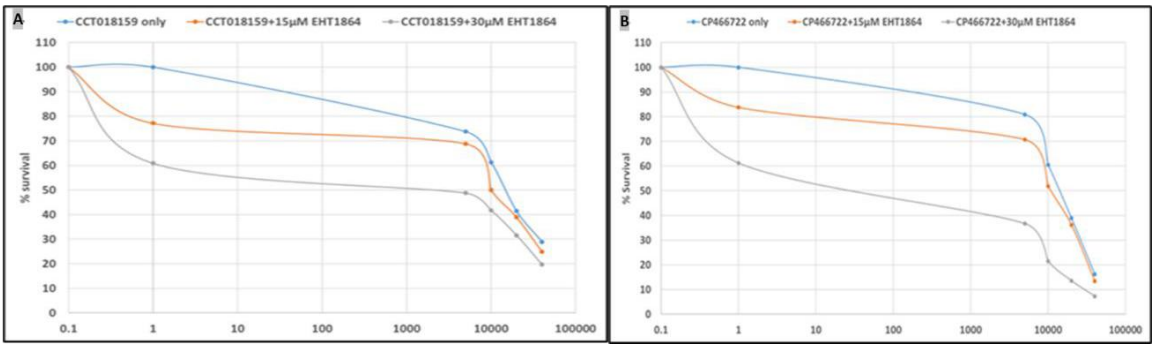


Figure 6 Representative dose-response curves showing the following secDrug-secDrug combinations. A. secDrug CCT018159+ secDrug EHT-1864. B. secDrug CP466722+ secDrug EHT-1864.

EHT1864 single-agent and combination treatment enhances apoptosis in myeloma cell lines.

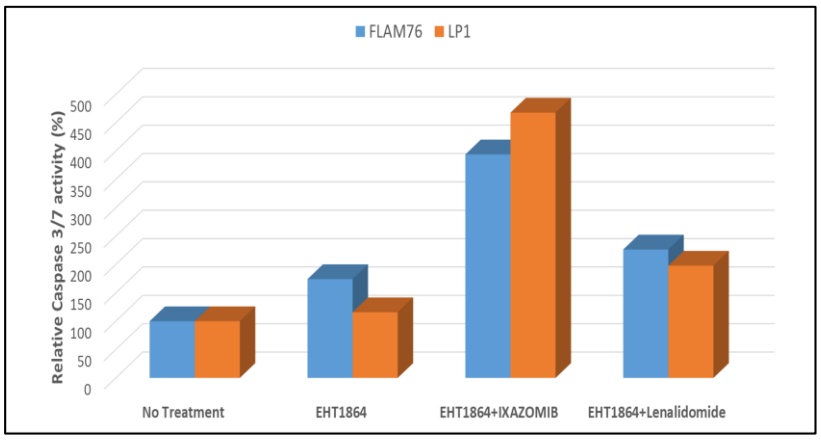


Figure 7 Representative bar plots showing Relative Caspase 3/7 activity following EHT1864 treatment as single-agent or in combination with PI (Ixa) and IMiD (Len).

The impact of EHT1864 on cellular apoptosis as a single agent and in combination with P.I. (Ixazomib) and IMiDs (Lenalidomide) was assessed using Caspase 3/7 Glo Assay. We observed significantly elevated levels of

Caspase 3/7 activity following EHT1864 treatment as a single agent as well as in combination with Proteasome Inhibitor drugs (Ixazomib) or Immunomodulatory agent (Lenalidomide), indicating higher apoptosis (**Figure 7**). The relative Caspase 3/7 activity was more profound when the drugs were used as combination compared to individual single-agent treatments. This was further confirmed by RNA sequencing analysis followed by KEGG pathway enrichment analysis for Apoptosis (**Figure 8**). The data shows a higher level of induction of apoptotic pathway in combination treatment compared to single-agent treatment.

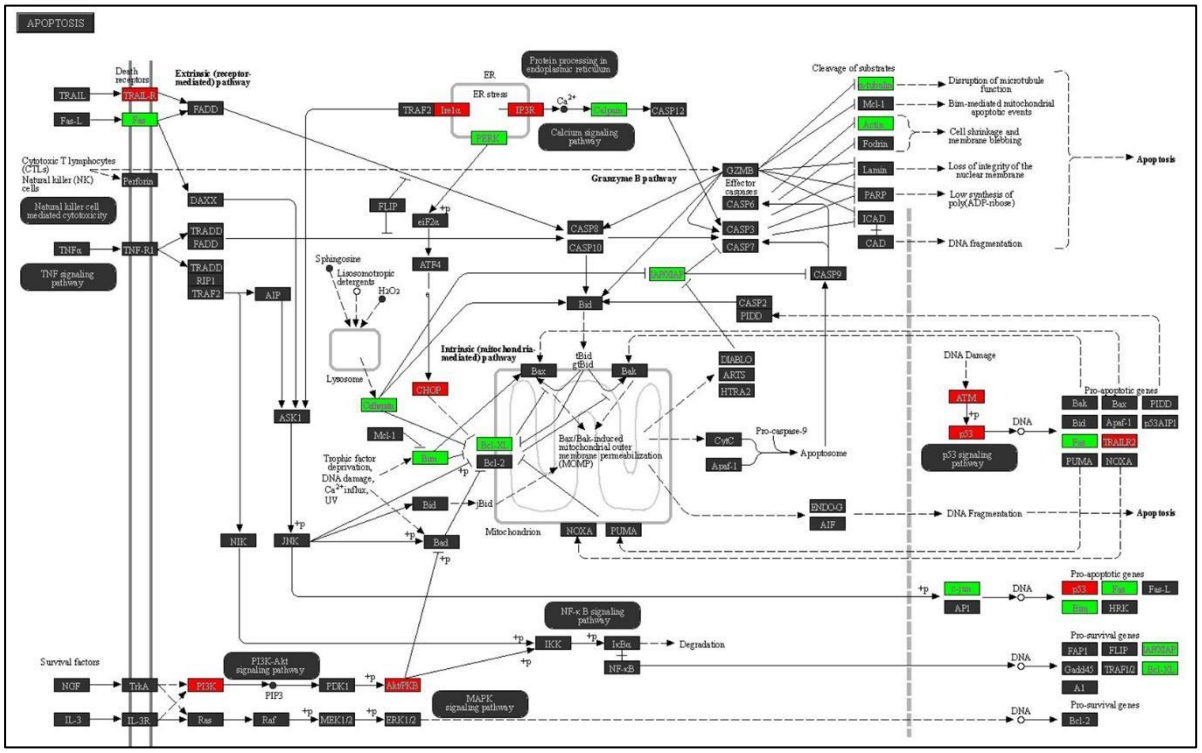


Figure 8 KEGG pathway enrichment analysis following RNA sequencing confirmed induction of apoptosis following EHT1864 treatment. Green represents downregulated genes. Red represents upregulation.

Immunoblotting analysis revealed EHT1864 induced downregulation of key myeloma-specific protein markers.

Next, we performed western blotting to validate the anti-myeloma effect of EHT1864 in HMCLs. We observed that EHT1864 down-regulates the expression. of key myeloma-specific markers, including C-myc, IRF-4 and NFkb in myeloma cell lines (**Figure 9**). This serves as a validation that this drug exerts significant anti-myeloma properties.

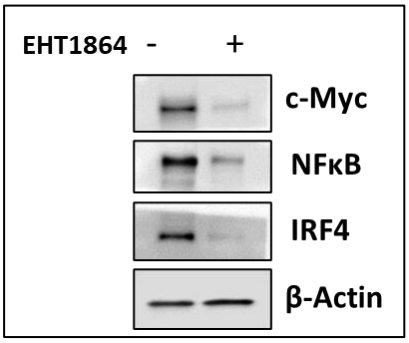


Figure 9 Western Blotting results. Representative results showing EHT1864 significantly down- C-myc, IRF-4, NFkB in myeloma cells.

Gene expression profiling reveals molecular pathways targeted by EHT1864 treatment.

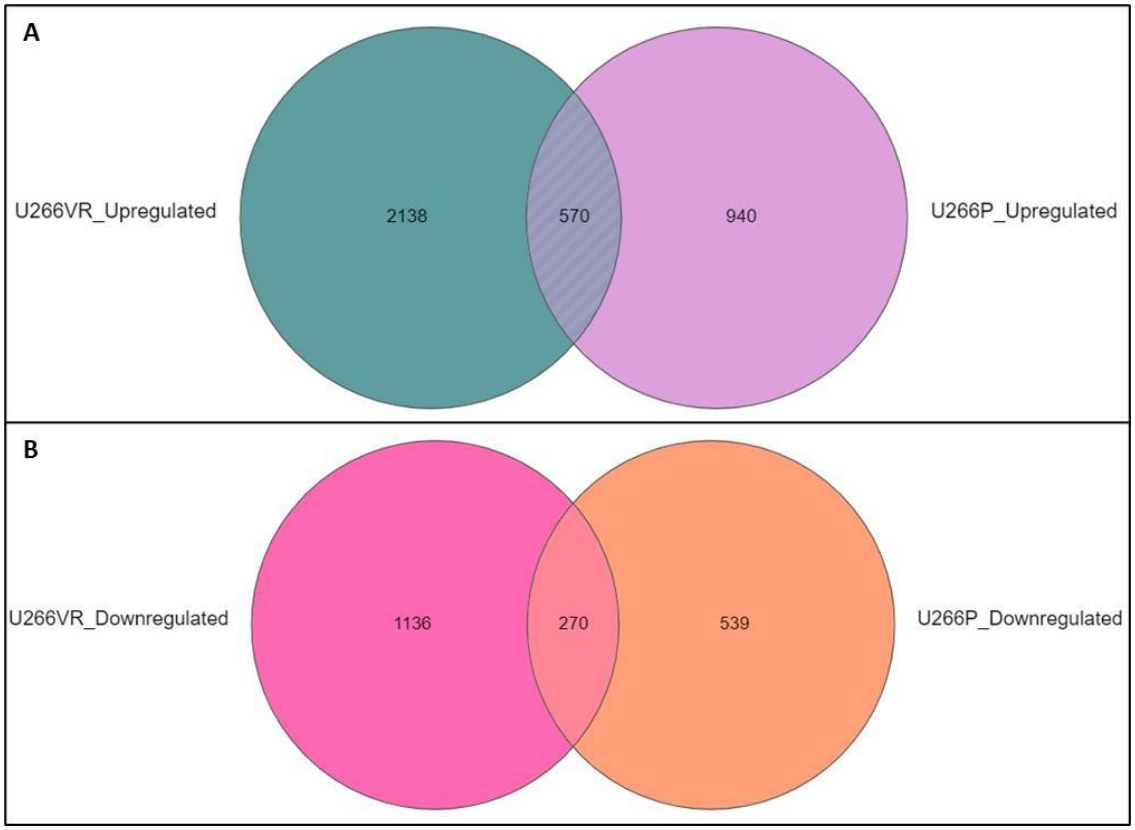


Figure 10 Venn diagrams representing A. Unique and B. Common upregulated/downregulated DEGs following EHT1864 treatment.

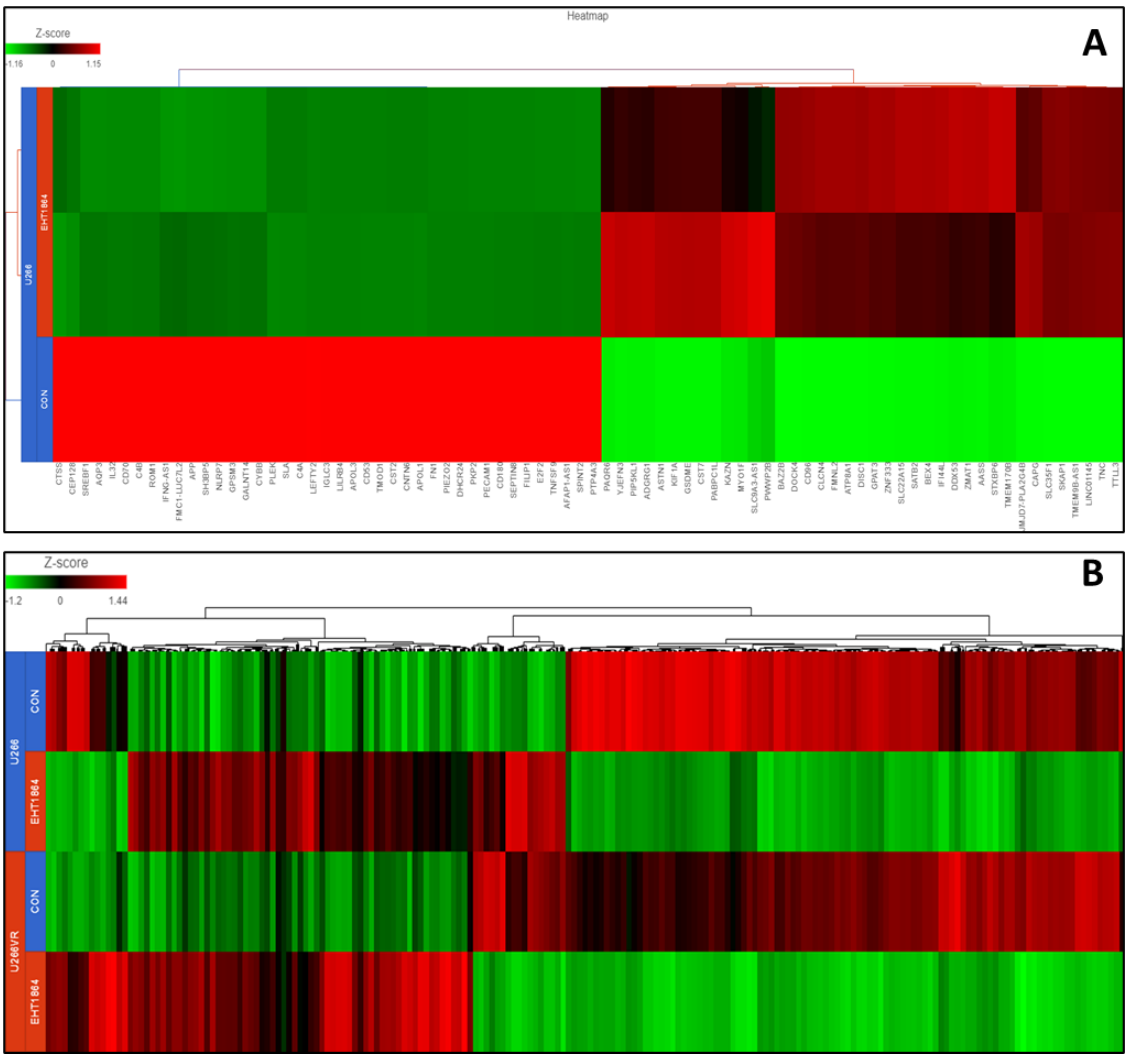
Next, we performed pre-vs-post treatment whole-transcriptome profiling by next-generation RNA sequencing to compare changes in gene expression induced by EHT1864 in myeloma cell lines in order to elucidate the mechanism of drug action and find additional effects of secDrug treatment. GEP data were normalized to baseline (no treatment), and differential gene expression was performed between untreated and EHT1864-treated cell lines. The Venn diagrams in **Figure 10** represent common and uniquely upregulated/downregulated DEGs following EHT1864 treatment. Among the genes that were differentially regulated (F.C.>1; $p<0.05$), a total of 2138 and 940 genes were significantly upregulated in U266VR and U266P, respectively, while 570 genes were common (**Figure 10A)**. 1136 genes were uniquely downregulated in U266VR compared to 539 upregulated genes in U266P and 270 upregulated genes that were common between the two cell lines 24Hr post-EHT1864 single-agent treatment (**Figure 10B)**.

Heatmaps were generated following differential gene expression analysis.

Figure 11A shows the heatmaps with hierarchical clustering of genes representing top differentially expressed genes (DEGs) following EHT1864 treatments in the myeloma cell line U266P, 24h following drug exposure.

Figure 11B represents a comparison between the EHT1864-induced gene expression profiles of the PI-sensitive line U266P with the clonally derived PI-resistant cell line U266VR. Fold Change values are depicted in a color scale where red represents upregulation and green represents downregulation.

Figure 11 Heatmaps generated using unsupervised hierarchical clustering (HC) analysis showing top differentially expressed genes following EHT1864 exposure.



U266P; B. U266P vs U266VR

Columns represent cell lines, and rows represent genes. Prior to hierarchical clustering, gene expression values were filtered (samples with max TPM < 1 were removed), and z-score normalized.

Pathway Analysis revealed top dysregulated molecular networks in response to EHT1864 treatment.

Ingenuity Pathway Analysis (IPA) was performed based on the top DEGs associated with EHT1864 single-agent treatment. Our IPA analysis results revealed several novel networks as the top differentially regulated pathways following exposure to EHT1864 (**Figure 12**). Importantly, we observed several distinct differences among the

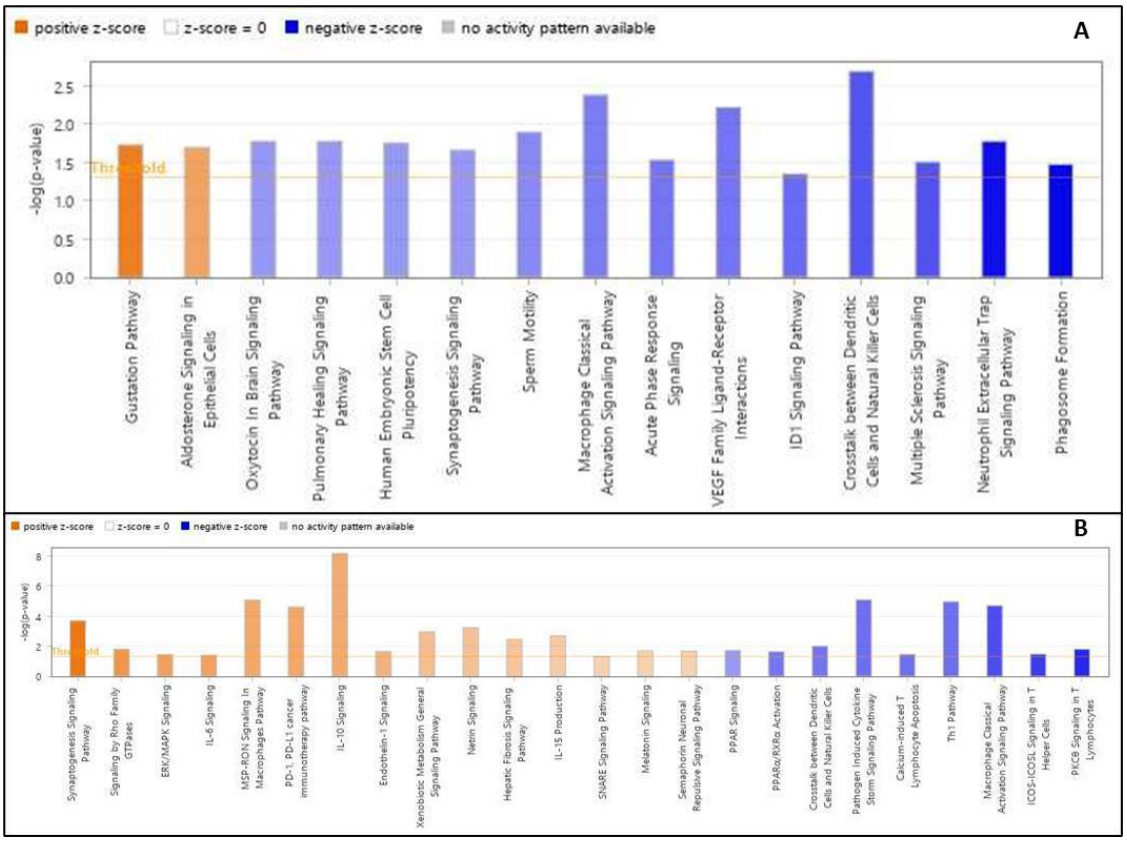


Figure 12 Bar plots representing top EHT1864-induced Canonical Pathways predicted by Ingenuity Pathway Analysis in: A. U266P. B. U266VR

dysregulated pathways between the PI-sensitive (**Figure 12A**) and P-resistant myeloma cell lines (**FIGURE 12B**).

Furthermore, IPA has a novel module called the IPA Upstream Regulator Analysis tool that predicts potential activation or inhibition of upstream regulators based on the observed gene expression changes in our experimental dataset. When we used the IPA Upstream Regulator Analysis tool on our pre-vs post-EHT1864 treatment dataset, we

could predict the inhibition of the myeloma markers NFkB (**Figure 13A**) and MYC (**Figure 13B**) as well as p38 MAPK (**Figure 13C**) and EZH2 (**Figure 13D**). Enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of the polycomb repressive complex

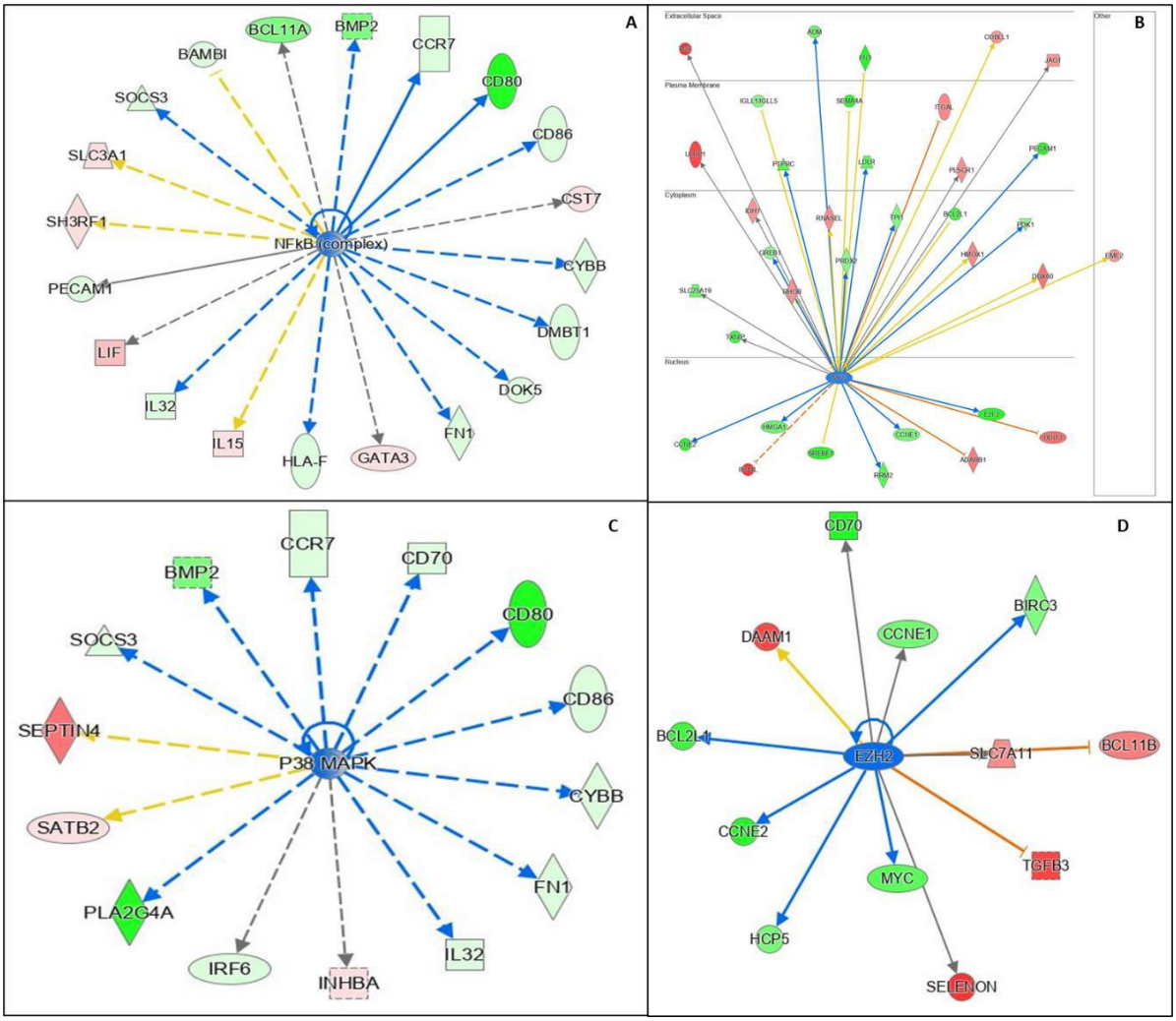


Figure 13 IPA upstream analysis of pre-vs-post EHT1864 treatment dataset. Blue/Green = Inhibition; Red = Activation.

Furthermore, the IPA Upstream Regulator Analysis tool also predicted the downregulation of TNF, Tumor Necrosis Factor (TNF), based on the observed expression of top DEGs (**Figure 14**). TNF is a pro-inflammatory cytokine that can promote the growth and survival of myeloma cells[104,105]. Altered expression of TNF family members has been shown to be associated with pathological conditions such as autoimmune disease and cancer[106].

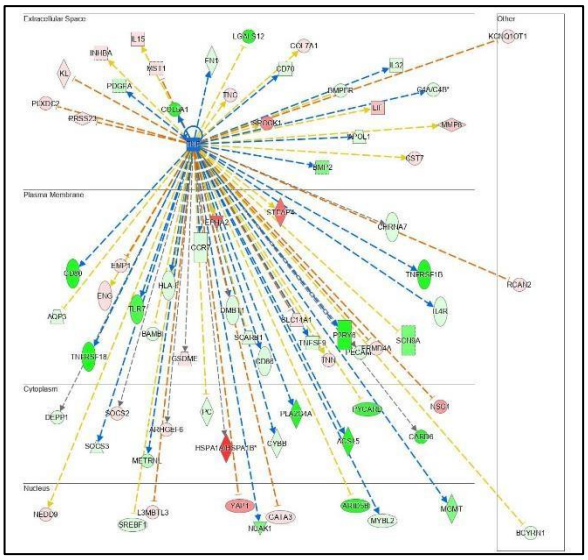


Figure 14 IPA upstream analysis showing predicted inhibiton of TNF following EHT1864 treatment. Blue/Green = Inhibition; Red = Activation.

Finally, we performed gene set enrichment analysis to explore the differentially regulated KEGG pathway following EHT1864 single agent treatment further. In addition, KEGG analysis performed on our data showed apoptosis, mitochondrial

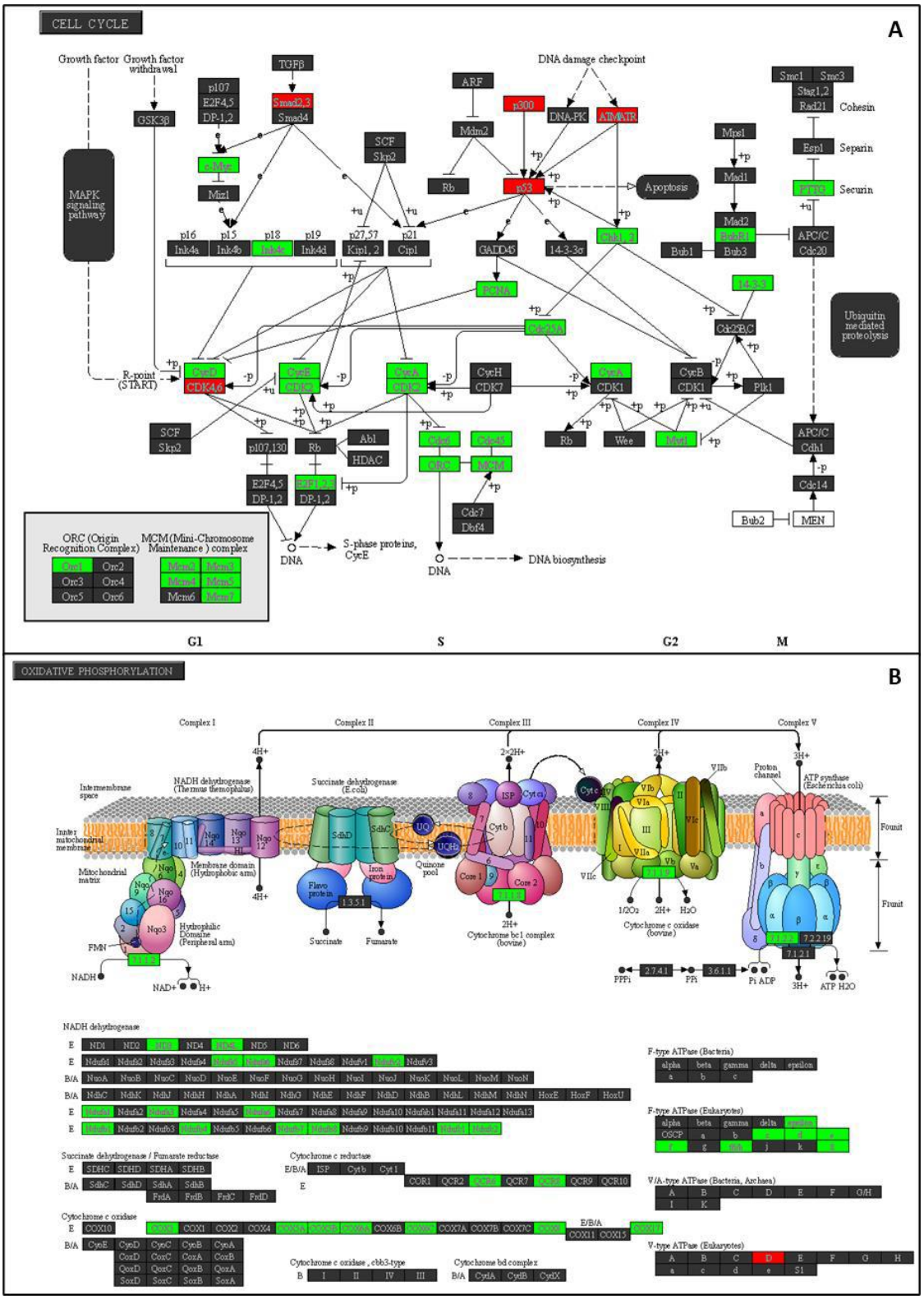


Figure 15 Top KEGG Pathways predicted by gene enrichment analysis: A. Cell Cycle. B. Oxidative phosphorylation

dysfunction, cell cycle arrest at the G2/M phase, and oxidative phosphorylation are among the top differentially regulated pathways (**Figure 15**).

Further, in accordance with the IPA analysis results, we could also observe changes in the MAPK pathway using our KEGG pathway analysis (**Figure 16**)

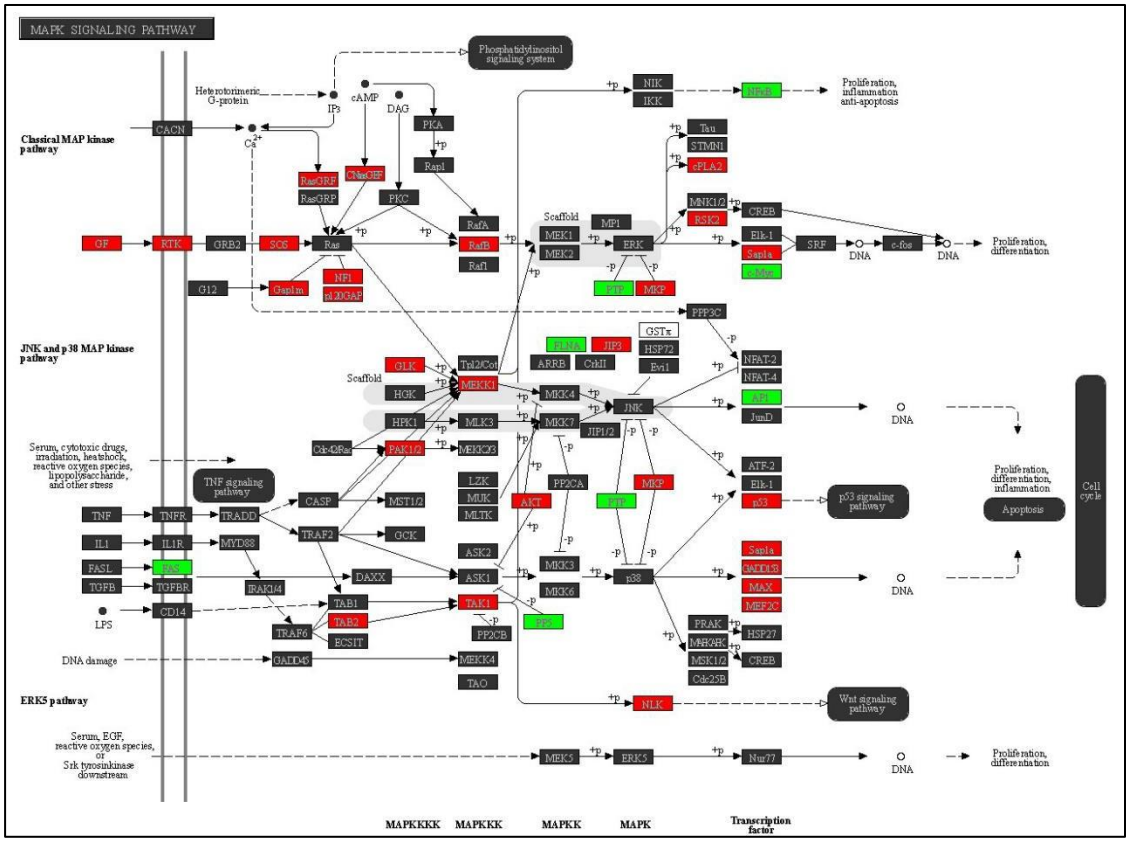


Figure 16 KEGG Pathway analysis: Effect of EHT1864 treatment on MAPK Pathway

CONCLUSION

Drug resistance is a major challenge in achieving complete and sustained therapeutic effects in myeloma chemotherapy.[42,44,107,108] Resistance to primary drugs like P.I.s and IMiDs may also lead to overdosing and unwanted exposure to ineffective anti-tumor agents, which may eventually increase the risk of negative side effects and the cost of drug development.[108,109] Using our secDrug algorithm, we have identified EHT1864, a Rac1 inhibitor, as a potential novel candidate for drug repurposing - a secondary choice in combination with P.I./IMiDs for the treatment of Relapsed/Refractory MM.

In this study, the EHT1864+Ixazomib combination was found to be potentially very effective when we presented single-cell transcriptomics as a unique screening approach for prioritizing secDrug combinations based on the sub-clonal expression of the drug targets. Next, we employed our HMCL panel as an *in vitro* model system exhibiting inter-individual variation in primary drug response/resistance to validate our prediction results. Our *in vitro* cytotoxicity results showed that EHT1864 exhibited significant single agent and synergistic cell killing activities in combination with Ixazomib (P.I.) and Lenalidomide (IMiD), especially in R/R MM cells. Further, our results also showed that EHT1864, in combination with IMiDs/P.I., was able to significantly lower the effective dose of both IMiDs/P.I. required to achieve the desired therapeutic response, thereby making the cell lines relatively more IMiDs/P.I. sensitive. We also found the synergy of EHT1864 with several other secDrugs (including an HSP inhibitor, CCT018159 and the ATM inhibitor CP466722) in resistant MM cell lines. Further, we found that EHT1864 was remarkably effective in increasing apoptotic activity. Next, we performed next-generation RNA sequencing analysis to identify several differentially expressed genes underlying the mechanisms of secDrug action. Pathway analysis following gene expression profiling of EHT1864-treated MM cell lines revealed the downregulation of several myeloma markers and pro-survival molecular pathways and genes, as well as the up-regulation of pro-

apoptotic markers, in addition to the identification of several molecular pathways associated with therapeutic efficacy in aggressive myelomas.

Currently, we are performing pre-vs-post-treatment single-cell RNA sequencing (scRNAseq) analysis to evaluate the intra-tumor efficacy of EHT1864 based on changes in the subclonal landscape of subclonal clusters within myeloma tumors. Next, we will perform single-cell proteomics (CyTOF or Cytometry time of flight) in patient-derived CD138+ bone marrow cells (*ex vivo*) from Mayo Clinic, MN, to establish the molecular pathways underlying secDrug efficacy and drug synergy. Our long-term goal is to perform CRISPR-based gene editing and functionally validate these mechanisms.

Our work lays a framework to establish a novel Rac1 inhibitor as a potent clinical trial-ready therapeutic option for the management of P.I. and IMiD-resistant Myeloma.

In addition, when we applied the secDrug algorithm to Genito-urinary cancers, we identified several agents as potential novel secondary drugs for the management of mCRPC[110]. Our plan is to validate the top PCa secDrugs as single agents and combination therapy candidates in aggressive lethal variants of PCa, particularly in cell types characterized by single-cell transcriptomics analysis as CSCs, TX-resistant or high expressors of drug target genes/pathways.

Thus, the secDrug algorithm promises to serve as a universal prototype for the discovery of novel drug combination regimens for treatment outcomes in any cancer type by enhancing sensitivity or overcoming resistance to standard-of-care drugs.

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