Study of crosstalk between progesterone receptor signaling and cellular stress pathways in breast cancer

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Abstract

Macroautophagy (autophagy) is intimately linked with cell death and allows cells to elude apoptosis. This has evoked clinical trials to combine autophagy inhibitors with other drugs with the aim of increasing the likelihood of cancer cells dying. However, the molecular basis for such effects is unknown. It has been demonstrated previously that synthetic progesterone, progestin R5020 induces cellular senescence in breast cancer cells with high levels of progesterone receptor B (PRB). The ligand-activated progesterone receptor has been reported to exert influence on cancer development by manipulating the autophagy pathway. Therefore, prolonged R5020 treatment upregulates autophagy in MCF-7 human breast cancer cells via a unique interplay between progesterone receptor B (PRB) and TFEB. These findings prompt an interesting strategy to exploit the senescence in order to check if anticancer drugs induce apoptosis synergistically with progestin R5020. Herein, I report the influence of combination treatment and co-treatment of R5020 with epigenetic modifiers, ER stress inducer and cytoskeleton disrupting drugs that act synergistically to produce synthetic lethal effect on the MCF-7 cells. Together, my findings help in hypothesising the molecular mechanisms that are involved in inducing cell death and unravel the possible link between the autophagy and apoptosis processes.

Keywords: Autophagy, apoptosis, MCF-7, PRB, progestin R5020, senescence, synthetic lethal effect, epigenetic modifications, synergy

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1. Introduction

Breast cancer is the most commonly diagnosed disease and the second leading cause of cancer-related death among women worldwide [1,2]. In about 90% of breast cancer cases, estrogen receptor α (ERα), progesterone receptor (PR), or the human epidermal growth factor receptor 2 (HER2/ERBB2) protooncogenic receptor are expressed. In many of these patients, treatment with anti-estrogens (e.g. aromatase inhibitors, tamoxifen, fulvestrant) and HER2-targeted agents has improved their survival significantly [3, 4]. However, despite these treatments, it is evident that some tumours acquire resistance to anti-estrogen and HER2-targeted therapies, causing recurrence of these tumours [5]. Estrogen and progesterone influence many hormonal functions in women, such as sexual development, pregnancy, childbirth, and menopause. If breast cancer cells possess estrogen receptors, the cancer is called ER-positive breast cancer and if the cells possess progesterone receptors, the cancer is called PR-positive breast cancer. If the cells do not have either of these 2 receptors, the cancer is called ER/PR-negative. About two-thirds of breast cancers are ER and/or PR positive. Until now, our perception of steroid hormone receptor function in cancer has mostly been limited to the study of a single receptor in the presence of its cognate or synthetic ligand. These approaches have helped reveal extraordinary insight into how these transcription factors, such as ER/PR, regulate gene expression and cell cycle progression [6,7].

Progesterone receptor (PR) is a member of the nuclear/steroid hormone receptor (SHR) family of ligand dependent transcription factors which is expressed primarily in female reproductive tissues. After binding its cognate steroid hormone, progesterone (P4), PR regulates the expression of gene networks to control development, differentiation, and proliferation of target tissues in endocrine-based cancers [8]. Progesterone activities are primarily mediated by its high-affinity receptors, which include the progesterone receptor (PR)-A and -B isoforms. The two isoforms are generally expressed at similar levels in the breast but the ratio can be altered in human breast tumours, where the PRA isoform predominates. PRB is the functionally important form in the mammary gland, while PRA is important for ovarian function[13. The two forms have distinct but overlapping transcriptional activities too [14].

Growing evidence suggests that progestins can exhibit both proliferative and antiproliferative activities on breast cancer cells *in vitro* independent of estrogen stimulation, suggesting that progesterone may act directly on target gene transcription in mammary cells [6]. Under hormone-depleted conditions in which oestrogen is removed from the growth media, progestin R5020 treatment can result in a modest increase in cell growth in MCF-7 breast cancer cell line models [12]. Progesterone mediates its biological activity following its interaction with progesterone receptor (PR) located within target cell nuclei [69]. The mechanism by which the PR modulates target gene transcription remains to be explored. However, it has been shown *in vitro* that the receptor directly interacts with the basal transcription machinery and stabilizes the formation of the transcription preinitiation complex [70,71].

Autophagy

Autophagy is an important catabolic process where the cytosolic cargo (consisting of cell debris, proteins and other substances to be recycled) is engulfed by the formation of a double membrane (autophagosomes) and then degraded through the fusion of autophagosomes with lysosomes. This process is essential for the maintenance of homeostasis in cellular stress responses and cell survival.

The first regulatory stage of autophagy is the de-repression of mTOR, a serine-threonine protein kinase that integrates intracellular and extracellular signals to modulate cell metabolism, growth, proliferation and survival. Certain pathways can regulate mTOR, including adenosine monophosphate-activated protein kinase (AMPK) and PI3K/AKT. Subsequently, mTOR can mediate the expression of several autophagy proteins, ultimately suppressing the autophagy process. Autophagy can be regulated through the AMPK pathway, and inhibition of the AMPK signaling pathway affects the process of autophagy in breast cancer [21]. Activated AMPK activates autophagy by targeting the ULK1 protein complex responsible for upregulating the protein complex involved in initiating the synthesis of phagophore. When mammalian target of rapamycin (mTOR) is inhibited, the activation of the ULK1, ATG101,

FIP200 and ATG13 protein complex is induced, which can mediate the initiation of autophagy. Phagophore generation is regulated by autophagy-related genes (ATGs). The Beclin1 complexes can be activated by Atg14L and UVRAG/Bif-1, and the latter is responsible for inducing the formation of autophagosomes. Two ubiquitin-like protein conjugation systems indulge in phagophore elongation into an autophagosome. Finally, the contents within the autophagosome are degraded when the autophagosome fuses with a lysosome. This cytotoxic autophagy may either independently lead to cell death or act as a precursor to apoptosis (figure 1) [72].

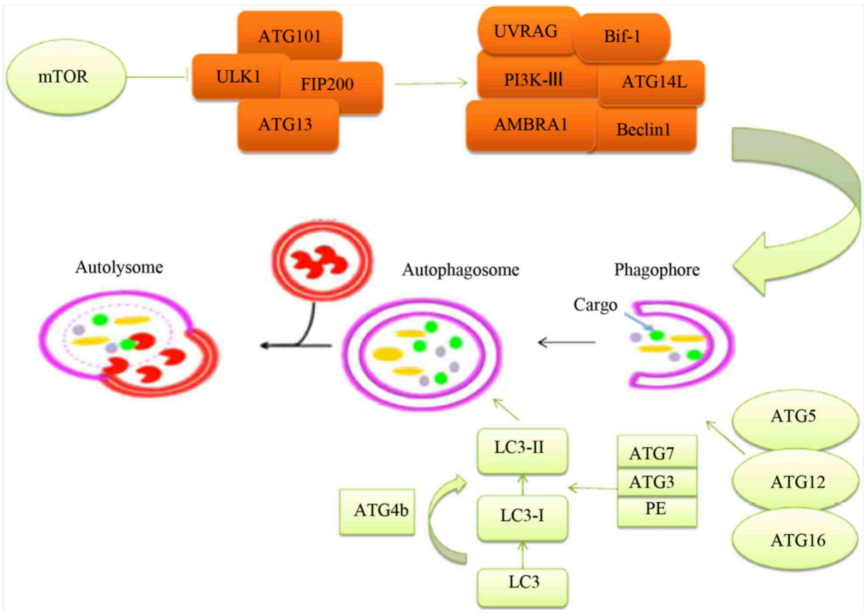


Figure 1: General process of autophagy in breast cancer cells. (figure adapted from Han *et al.*)

Apoptosis

Apoptosis is the result of an unsuccessful cytoprotective mechanism against intracellular and extracellular stressors. Apoptosis or programmed cell death is a key regulator of physiological growth control and regulation of tissue homeostasis characterized by chromatin condensation and DNA fragmentation. Killing of tumor cells by most anti-cancer strategies has been linked to activation of apoptosis signal transduction pathways in cancer cells such as the intrinsic and/or extrinsic pathway. In the intrinsic pathway of apoptosis, caspase activation leads to permeabilization of the outer mitochondrial membrane by proapoptotic proteins such as Bax/Bid. Outer mitochondrial membrane permeabilization is induced by multiple cytotoxic stimuli and proapoptotic signal-transducing molecules that converge at mitochondria. A group of proteins that are found in the space between the inner and outer mitochondrial membranes are released when the outer mitochondrial membrane is disrupted.

In response to external stimuli extrinsic apoptosis pathway apoptosis is triggered by specific death ligands binding at 'death' receptors on the cell surface. These receptors are members of the Tumour Necrosis Factor Receptor (TNFR) gene family, such as TNFR1 or FAS. This activates the caspase 8 activity and a series of signals leading to activation of other caspases finally leading to DNA cleavage and ultimately cell death [73] (figure 2).

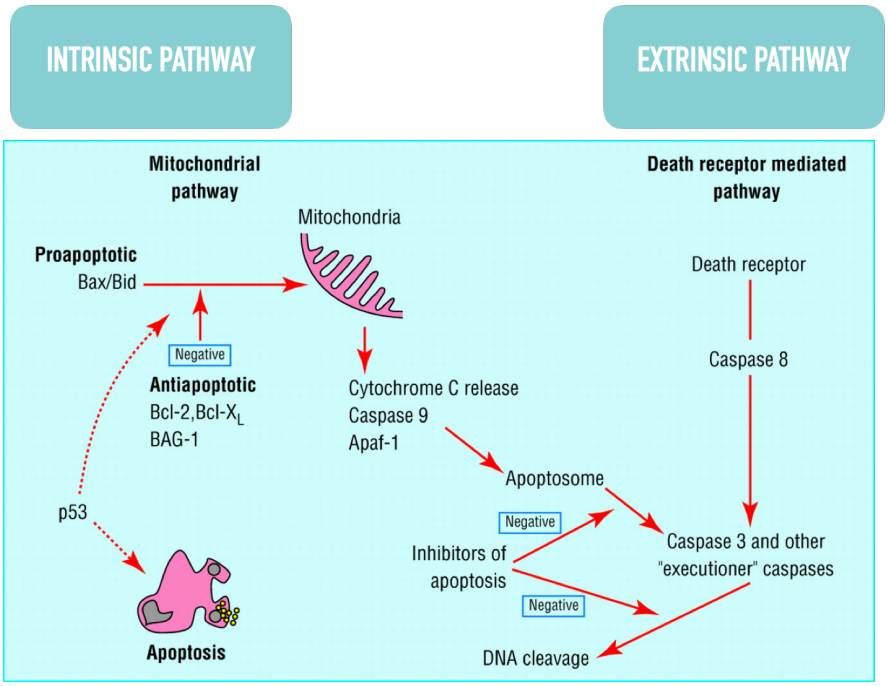


Figure 2: Apoptotic pathways involved in breast cancer (figure adapted from Marina, *et al*.)

Link between Autophagy and Apoptosis

Autophagy and apoptosis often occur in the same cell, mostly in succession in which autophagy precedes apoptosis [40]. This is because stress often stimulates an autophagic response, especially if the level of stress is not lethal. Apoptotic and non-apoptotic lethal programmes are activated when stress exceeds an intensity threshold or a critical duration. Usually autophagy constitutes a strategy to adapt to and cope with stress [42]. Nevertheless, if the cell commences apoptosis, autophagy can be inactivated, in part owing to the caspase-mediated cleavage of essential autophagy proteins. Beyond this general scenario, in particular circumstances autophagy or essential proteins involved in the autophagic process may promote cellular death, either by catabolizing critical portions of cells or facilitating the activation of apoptotic or necrotic scheme, respectively [41].

Correlation between autophagy and Ligand activated Progesterone Receptor B in MCF-7 cells

Progestin R5020 acts as a ligand for the PRB expressing cells. Previous studies in the lab showed that R5020 treated MCF-7 cells (for 96 hours) induces cellular senescence and proposed a working mechanism for R5020-induced autophagy in MCF-7 cells (Fig. 3). Although autophagy is critical for cell survival, there is also ample evidence for autophagy-associated cellular senescence [23] or cell death [24]. Hence, in cancer development, autophagy can be pro-tumorigenic or tumour suppressive. Previous studies in the lab have reported that ligand-activated progesterone receptor influences cancer development by manipulating the autophagy pathway.

In vitro studies have provided proof that progesterone and its synthetic mimic progestin initiate autophagy [25-33]. Notably, progesterone and progestin-induced autophagy evoke cytoprotective outcome in breast cancer by inducing senescence of cancer cells [35, 36]. This underscores the clinical relevance of progesterone and progestin in breast cancer therapy via modulation of the autophagy signalling pathway. It has been reported that prolonged treatment of progestin R5020 for 96 hours upregulates autophagy in MCF-7 human breast cancer cells via a novel interplay between progesterone receptor B (PRB) and TFEB [39]. PRB belongs to the nuclear receptor superfamily involved in modulating transcriptional response. Transcription factor EB is a transcriptional regulator of autophagy, as it is involved in promoting the expression of genes required for autophagosome formation, lysosome

biogenesis, and lysosome function. R5020 supposedly upregulates TFEB gene expression and protein levels in a PRB-dependent manner (refer figure 3). Additionally, R5020 enhances the co-recruitment of PRB and TFEB to each other to enable TFEB nuclear localization. TFEB induces the expression of autophagy and lysosomal genes to activate autophagy once it reaches the nucleus. Studies have shown that progesterone and progestin suppress the mammalian target of rapamycin (mTOR) to activate autophagy in various cell types [26, 27, 29-32]. Inhibition of mTOR is associated with autophagy upregulation. Interestingly, this study by Tan *et. al* showed that the autophagic responses mediated by R5020 in MCF-7 cells are unhampered by mTOR. This conclusion was reached as the hormone did not affect the phosphorylation and activation of the upstream regulator AKT as well as the effector mTOR. Instead, it was proposed that R5020-induced autophagy involves PRB-mediated nuclear translocation and activation of TFEB. This sheds light on the presence of multiple independent pathways in MCF-7 cells that autophagy upregulation does not influence the AKT-mTOR signalling pathway. Ligand-activated PRB also plays the role of a coactivator of TFEB to upregulate autophagy in MCF-7 cells [39].

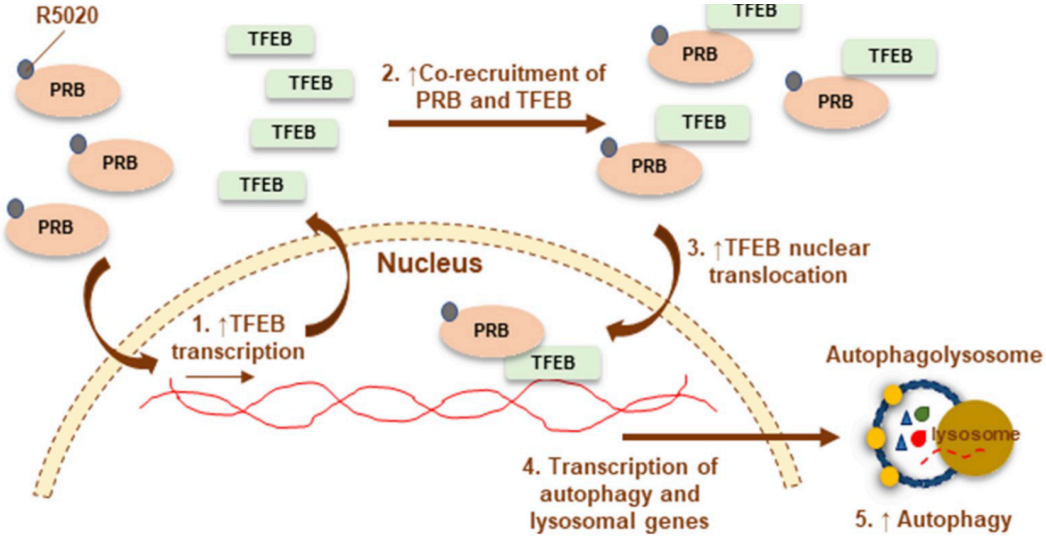


Figure 3: Working model showing that ligand-activated PRB upon prolonged R5020 treatment enhances TFEB expression and nuclear translocation to upregulate transcription of autophagy and lysosomal genes in MCF-7 cells [39].

Synthetic lethality principle

Synthetic lethality occurs when the simultaneous hampering of two genes results in cellular or organismal death. Synthetic lethality also occurs between genes and small molecules, and can be used to elucidate the mechanism of action of drugs. This concept is recently being explored because of the prospect of a new generation of anti-cancer drugs. It is a strategy that can be applied where two particular mutations result in cell death, but the cancerous cells only have one of those mutations. By artificially inducing the second mutation in the pair, the drug can induce death in the malignant cell. The drug induces mutations in the corresponding genes in healthy cells, too, but the healthy cells never had the first mutation. Only cancer cells end up with both mutations and die. In a sense, the therapy induces cancer to kill cancerous cells. The problem is in finding pairs. Thus, the aim of this approach was to screen anti-cancer drugs where cancer cells having a mutated gene are many times more vulnerable to inhibition (chemical/small molecule inhibitors) of its synthetic lethal interactor as compared to normal cells lacking gene mutation.

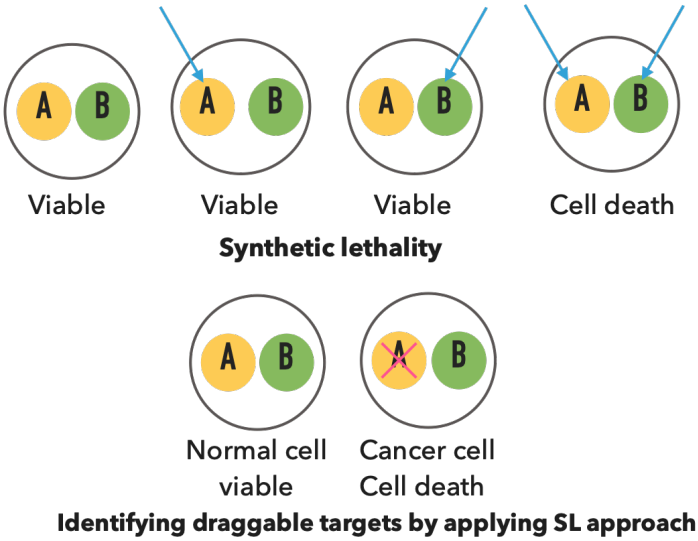


Figure 4: Schematic representation of synthetic lethality. Two genes are synthetic lethal only when their simultaneous inactivation results in cellular or organismal death. In this example, deletion of either gene A or gene B does not affect viability whereas inactivation of both at the same time is lethal. Similarly, if gene A is mutated and exogenous inhibition of synthetic lethal partner of gene A that is gene B by chemical inhibitor will lead to targeted cancer cell killing.

Combination therapy and co-treatment of drugs

The complexity of cancer highlights the need for a variety of treatment approaches, which is why a combination of one or more therapeutic interventions is often used to battle cancer. Combination therapy is a strategy of treatment in which drugs are administered in succession. This sort of therapy might achieve efficacy even with lower doses or less toxic drugs. It is capable of chemo-sensitizing cells, making an additional compound more potent. Although monotherapy is still a common approach, it is often deemed that even better results could potentially be obtained when these therapies will become rationally combined with others. Another treatment strategy called co-treatment is usually implemented where the drugs are treated simultaneously at the same time. In this treatment, addition of two or more drugs to the cells concurrently could be able to induce cellular death in a synergistic manner.

Combination therapy, a treatment modality that combines two or more therapeutic agents, is a cornerstone of cancer therapy. The amalgamation of anti-cancer drugs enhances efficacy compared to the mono-therapy approach because it targets key pathways in a characteristically synergistic or an additive manner, minimise drug resistance, or even fight against expected resistance [114]. Cancer drugs are most effective when given in combination.

Figure 5: Image representing principle of combination therapy for inducing mutations/changes in targeted manner in the cancer cells.

Hypothesis
In this study, I hypothesized that cells undergoing cellular senescence could be sensitized to apoptosis when treated with anti-cancer drugs leading to synthetic lethal effect in MCF-7 cells. The aim was to screen for drugs (HDAC inhibitors, cytoskeleton disruptors and ER stress inducer) that induce effective apoptosis in synergy with R5020 and further determine the molecular mechanisms and pathways adapted by them.

2. Materials and methods

2.1. Constructs

Human PRB was cloned into pcDNA3.1/Hygromycin (+) mammalian expression vector (Invitrogen) as described by Zheng *et al.* [74].

2.2. Cell culture

MCF-7 cell line was obtained from the American Type Culture Collection (ATCC). Cells were cultured in phenol red-containing Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque), supplemented with 7.5% Fetal Bovine Serum (HyClone) and 2mM L-glutamine (Life Technologies). The cells were grown in humidified atmosphere with 5% CO_2 at 37°C. Stable PRB overexpression in MCF-7 cells was established by S. Tan, *et al* where MCF-7 cells were transfected with pcDNA3.1-PRB using Lipofectamine 2000 (Invitrogen) with 1:3 DNA to Lipofectamine ratio. Selection of transfected cells was carried out in DMEM containing hygromycin B at 400 µg/ml. Positive clones were selected based on PRB protein expression by western blotting analysis. Validation was performed in consecutive 3 passages before experiments with stable clones started.

2.3. R5020 treatment

Synthetic progestin R5020 (Sigma) was dissolved in 100% ethanol (EtOH) to yield 1mM stock. 10 nM working concentration for R5020 was freshly prepared for each experiment by further diluting the stock in 100% EtOH. Prior to initiation of treatment, cells were plated in phenol red-free DMEM supplemented with 5% dextran-coated charcoal-treated FBS (DCC-FBS) (Life Technologies) for 48 h to remove the residual effect of hormones from the serum. Tantamount volume of 100% EtOH was used as the vehicle control treatment.

2.4. Drugs treatment paradigm

The drugs used for treatment were obtained from Sigma. Working concentrations from their respective stock solutions were reconstituted by serial dilution (table 1).

Table 1: List of drugs used and their working concentrations employed

Sl. no.	**Drugs used**	**Concentration (uM)**
1.	Panobinostat (LBH589)	0.2uM and 1uM
2.	Valproic acid (VPA)	1uM and 5uM
3.	Taxol	0.2uM
4.	Nocodazole	3uM
5.	Cytochalasin B	10uM
6.	Tunicamycin	5uM

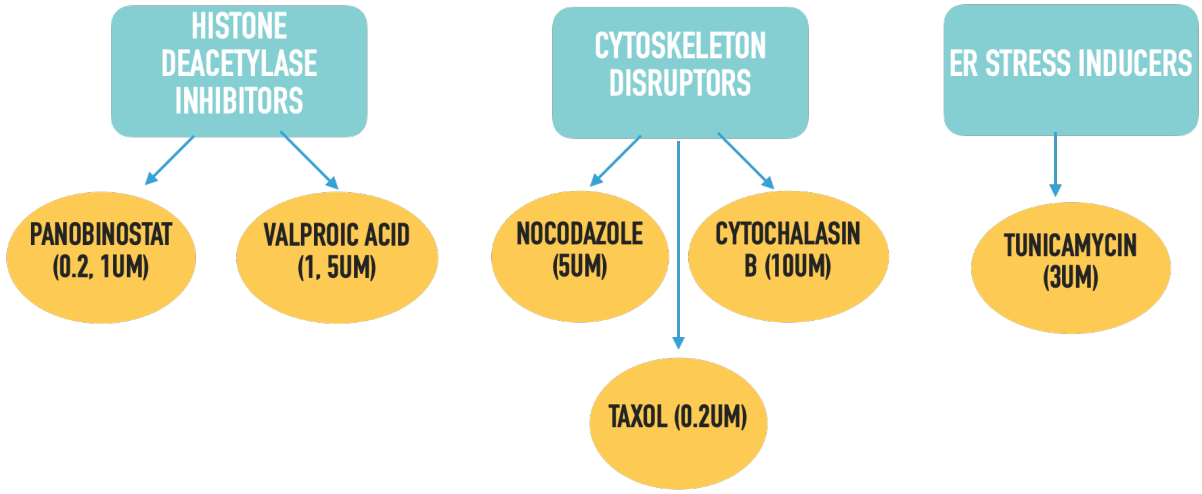


Figure 6: Approach for induction of apoptosis post progestin R5020 treatment. Three sets of drugs, HDACi, cytoskeleton disruptors and ER stress inducers were used to check for induction of cell death in cells undergoing cellular senescence.

2.5. Treatment scheme

Two treatment strategies were implemented *i. e.,* combination and co-treatment of drugs. Combination treatment involved the addition of drugs after prolonged R5020 treatment. Co-treatment involved the simultaneous treatment of drugs with R5020 at the same time. The incubation period was 24 hours and 48 hours respectively (refer figure 6).

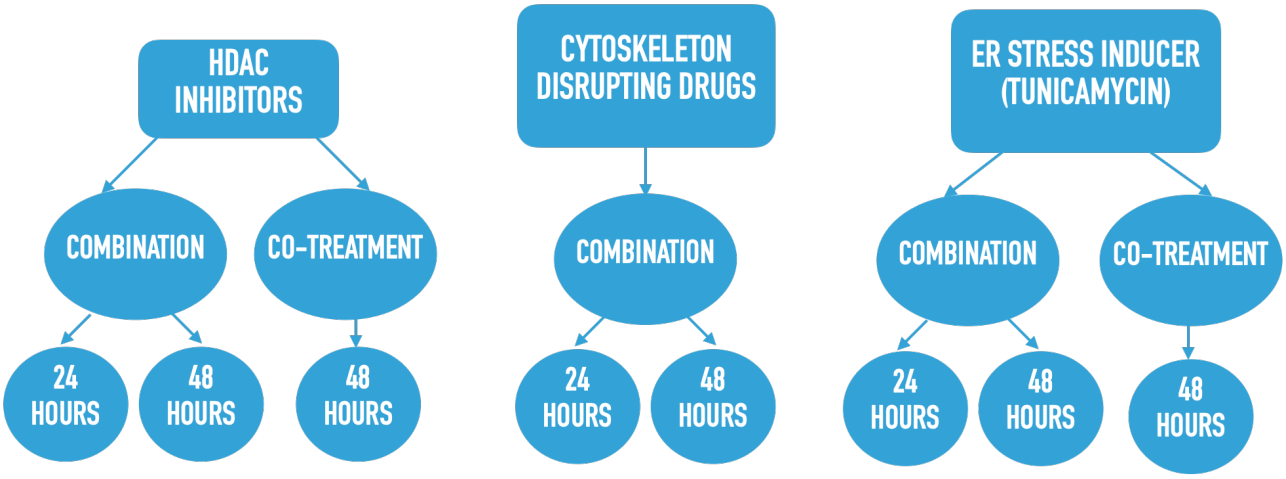


Figure 7: Treatment scheme implemented to induce apoptosis in the MCF-7 PRB 20 cells.

2.6. Cell morphology studies

The morphological features of the MCF-7 cells were studied after their respective treatments. The cells were checked under the microscope for distinct features and cellular structures. Images were acquired with Olympus IX71 inverted fluorescence microscope.

2.7. Cell viability assay

Cells were seeded at 50,000 cells per well for treatment with progestin R5020 and respective drugs, in 6-well plates. All experiments were performed at least three times in triplicate and the proportion of cells per treatment group was normalized to control wells. Cell viability was determined by Trypan Blue Exclusion assay where the cells were stained with trypan blue dye after their respective treatment

scheme. 1mL of the cell suspensions were centrifuged at 1000 rpm for 5 minutes. The collected pellet was dissolved in 200uL phenol red-free media from which 10uL of the cells were stained with 10uL trypan blue stain and mixed well. 10uL of this mixture was placed on the hemocytometer (Neubauer chamber) where live cells and dead cells were counted under the light microscope. Live cells appeared colourless while dead cells appeared blue. These cells were counted in four 1 x 1 mm squares of one chamber and the average number of cells per square was determined using the formula;

$$\frac{No.\,of\ live/dead\ cells\ in\ 4\ squares}{4} * 10^4 = \text{Total number of live/dead cells in 1mL}$$

Cell viability was calculated using the formula; $viable\ cells\ (\%) = \frac{(total\ number\ of\ live\ cells)*100}{(total\ number\ of\ cells)}$

2.7. Statistical analysis

Data are presented as the mean ± standard deviation. Two-way ANOVA test in R was used for the statistical analysis to determine the statistical significance between the effect drugs. Data were considered as statistically significant when $P < 0.05$.

3. Results

Progestin has been reported to induce autophagy [75-84] and the autophagic response has been mediated by activation of progesterone receptor isoform (PRB) [78-80]. Many chemicals kill cancer cells but their toxicity to normal cells limits their usefulness as anticancer drugs. Epigenetic and genetic alterations within cancer cells, as well as changes in their microenvironment, tends to increase their requirement for a particular molecular target (or targets) relative to normal cells, creating an opportunity for selectivity. Synthetic lethality is a principle in which two genes are said to be synthetically lethal if mutation of either gene alone is compatible with viability, but mutation of both genes induces death. Inhibiting the products of genes that are synthetically lethal to cancer-causing mutations should kill cells that consist of such mutations, while sparing normal cells. The challenge with this is to identify the relevant target (or targets) of compounds that score positively [85]. Thus, the aim of this approach was to screen anti-cancer drugs where cancer cells having a mutated gene are many times more vulnerable to inhibition (chemical/small molecule inhibitors) of its synthetic lethal interactor as compared to normal cells lacking gene mutation.

Unpublished data from the lab show that R5020 induces replicative senescence in MCF-7 cells expressing high levels of PRB. It was also previously demonstrated that ligand-activated PRB upon prolonged R5020 treatment enhances TFEB expression and nuclear translocation to upregulate transcription of autophagy and lysosomal genes in MCF-7 cells [84]. The objective of this study was to identify compound(s) that bring about the 'synthetic lethal' effect with R5020 so as to discover effective anti-tumoral therapy for PR-positive breast cancers. I determined the effects of R5020 in association with three sets of drugs, *i.e.,* HDAC inhibitors, cytoskeleton disrupting (CD) drugs and ER stress inducer in order to study the apoptosis induced by implementing two treatment strategies. First, combination treatment, refers to the use of two or more kinds of drugs that are used in sequence in various timing formats. Second, co-treatment refers to the treatment method where two or more drugs are treated at the same time. The approach for combining R5020 with anti-cancer drugs is to use drugs that work by different mechanisms, thereby decreasing the likelihood that resistant cancer cells will develop. When drugs with different effects are combined, each drug can be used at its optimal dose, hence reducing intolerable side effects. The effects of these treatments with regard to cell death/apoptosis are evaluated to determine the best combination of drugs that induce synthetic lethality.

Herein, I explored if prolonged (96 hour) R5020 treatment of MCF-7 cells followed by time-dependent treatment with HDAC inhibitors, CD agents and ER stress inducer promoted apoptosis and cell growth suppression. We also explored if co-treatment of these drugs with R5020 for 48 hours produced a significant decline in cell viability.

3.1. Treatment of MCF-7 cells with HDACi using combination treatment and co-treatment strategies

Two concentrations of LBH589 (0.2uM and 1uM) and VPA (1uM and 5uM) were tested on MCF-7 PRB cells respectively. The cells were initially treated with progestin R5020 for 96 hours further which they were subject to LBH and VPA treatments for 24 and 48 hours corresponding to combination therapy of anti-cancer drugs. Another treatment strategy was implemented where co-treatment of R5020 with LBH and R5020 with VPA simultaneously for 48 hours was carried out to determine the best treatment conditions for inducing synthetic lethality in the MCF-7 cells. Combination therapy and co-treatment strategies were used to screen for the HDACi drugs that induced maximum cell death in order to study the molecular mechanisms responsible for inducing synthetic lethality.

To distinguish the inhibitory effects of the drugs, the treated cells were subject to trypan blue staining and analyzed for live cells. The cells that did not take up the dye and were not stained were regarded as live cells and their numbers were counted in a hematocytometer. The cell viability was calculated using $viable\ cells\ (\%) = \frac{(total\ number\ of\ live\ cells)*100}{(total\ number\ of\ cells)}$

3.1.1. LBH589 and VPA induce cell death in both combination and co-treatment

24 hours treatment with HDACi drugs yields about 40% cell viability with LBH (0.2uM), 30% with LBH (1uM), 45% with VPA (1uM), 30% with VPA (5uM). While the vehicle control treated with R5020 yields about 50% cell viability. It can be noted that prolonged R5020 treatment itself produces 50% decline in cell viability (figure 8A). Addition of the HDACi drugs further promotes apoptosis/cell death. There is a 4, 10, 2.35 and 6 fold decrease in cell viability with 0.2uM, 1uM LBH, 1uM and 5uM VPA respectively from 24 to 48 hours. Therefore, there is a significant decrease in the cell viability in dose-dependent and time-dependent manner. Treatment of MCF-7 cells with HDACi drugs after prolonged R5020 treatment with LBH589 1uM being the most effective in inducing cell death.
In the case of co-treatment of progestin R5020 with the HDACi for 48 hours did not cause cell death as effectively as the combination treatment (figure 8C).

3.1.2. Co-treatment induced cell death is not as effective as combination treatment of HDACi with R5020

Although it is seen that 1uM LBH589 is more effective than VPA, VPA still has a considerable effect on inducing death of the cancer cells and it would be interesting to understand the mechanism behind it. As mentioned above, VPA 5uM has a better effect on reducing cell viability than a lower dose of 1uM at 48 hour treatment showing that the cell growth suppression is a dose & time-dependent phenomenon (figure 8). Significant decrease in cell viability (24%) was observed with co-treatment of LBH589 1uM while VPA 5uM showed 37% cell viability (figure 9). Although there was a decrease in cell viability during the co-treatment, it was not as effective as the combination treatment where prolonged R5020 induced senescence initially followed by cell death induced by HDACi.

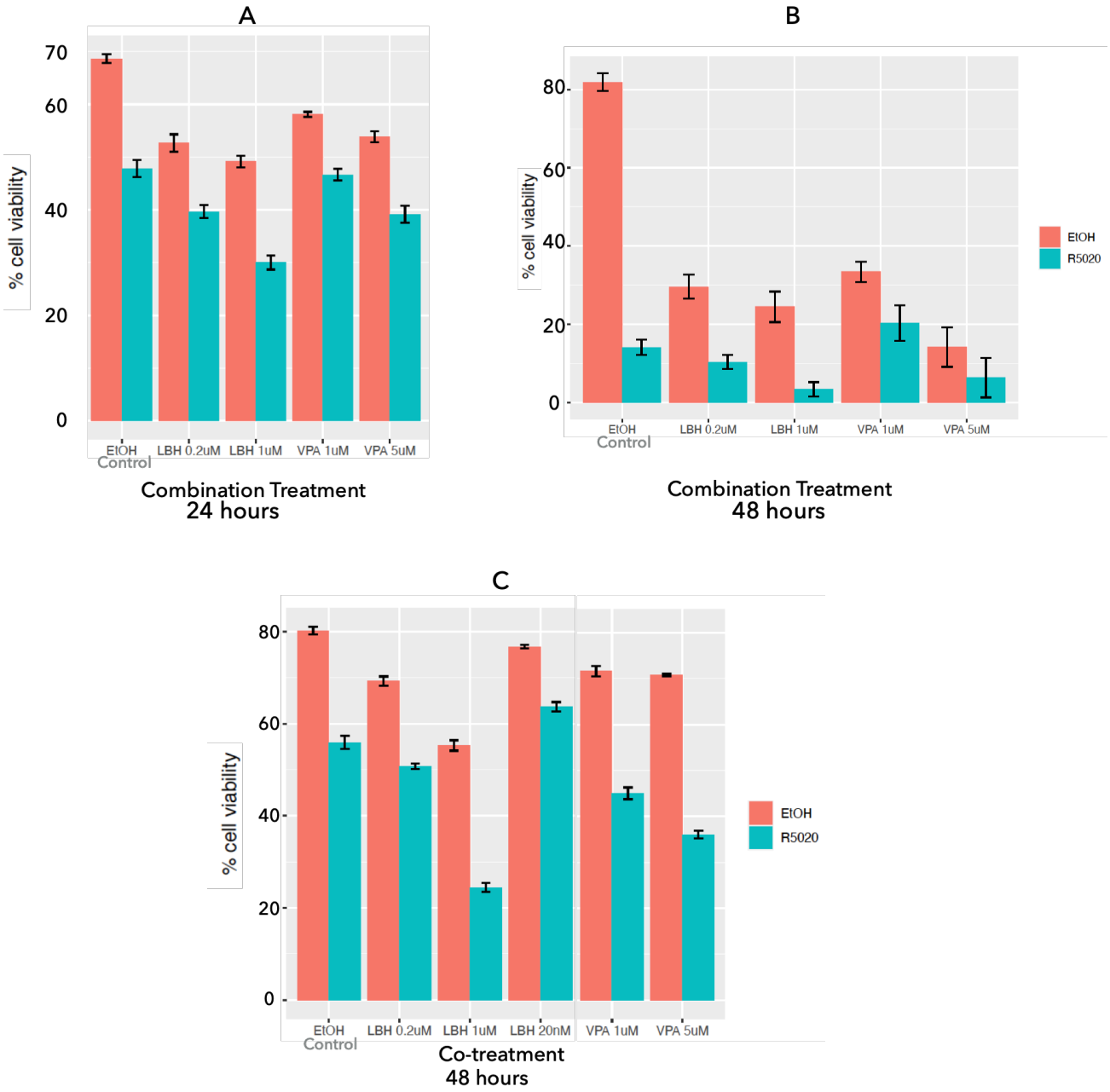


Figure 8: Statistical analysis showing dose and time-dependent effect of the LBH589 and Valproic acid drugs on the cell viability of MCF-7 cells. (A) 24 hours treatment of LBH589 and VPA showing a decline in cell viability with increasing drug concentrations. (B) 48 hours treatment of LBH589 and valproic acid showing a further decline in cell viability with increasing drug concentrations as compared to the 24 hour treatment. (C) Co-treatment of LBH589 and VPA with R5020 for 48 hours respectively. Significant decrease in cell viability (24%) was observed with co-treatment of LBH589 1uM while VPA 5uM showed 37% cell viability.

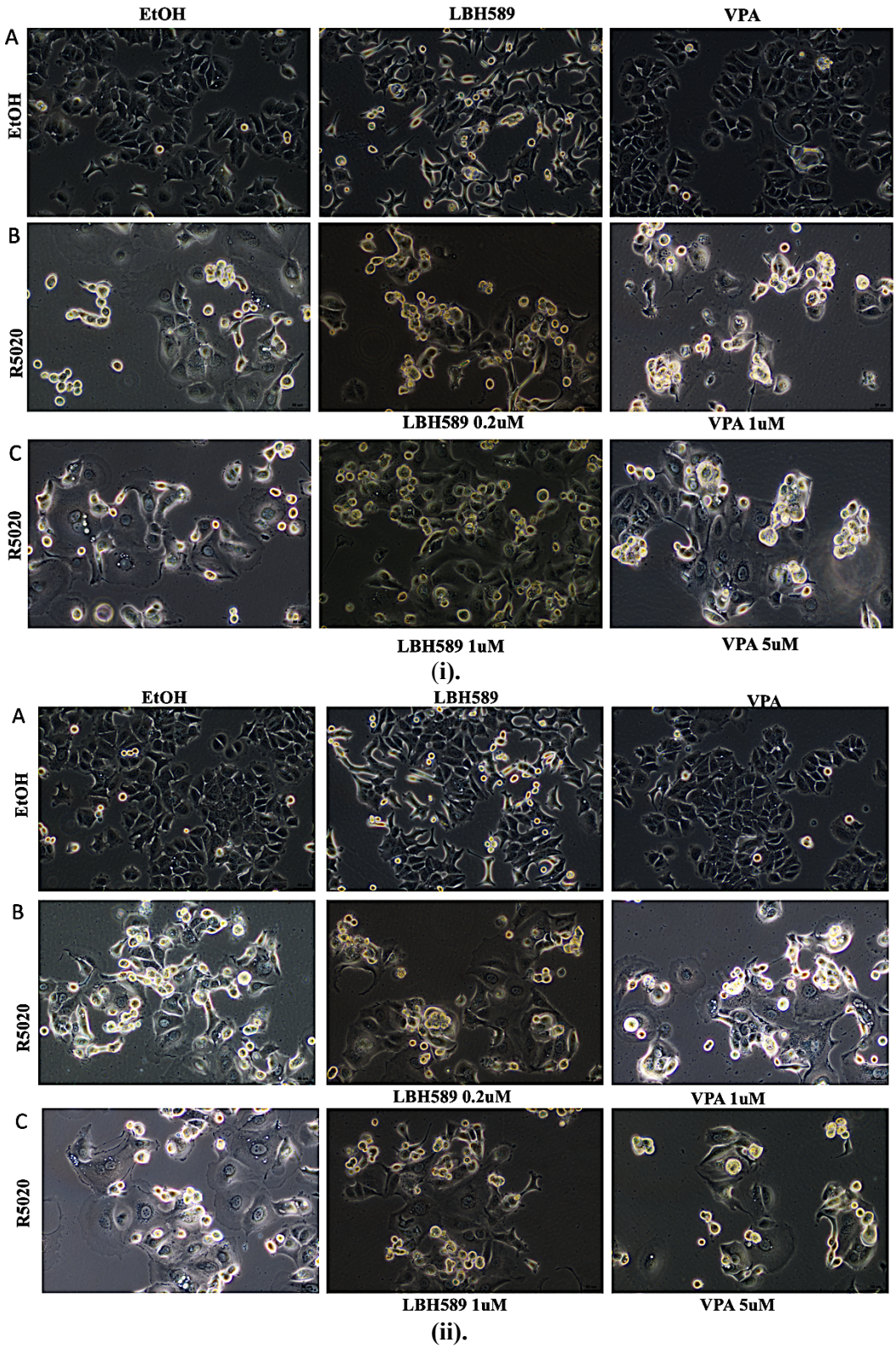


Figure 9: Fluorescence microscope image representing combination treatment of LBH589 (0.2uM & 1uM) and VPA (1uM & 5uM) with progestin R5020 in MCF-7 cells. (i). 24 hour combination treatment of HDACi with R5020. (ii). 48 hour combination treatment of HDACi with R5020. Vehicle control EtOH showed negligible cell death compared to 1uM LBH589 and 5uM VPA controls. R5020 (EtOH) control shows cells under extreme stress due to estrogen starvation and prolonged R5020 treatment leading to autophagy. With increase in dosage of the drugs there is increase in cell death where dispersed floating dead cells and live cells with disrupted morphology are observed. Highest dead cells can be seen in the cells treated with 1uM

LBH589 followed by a lower but significant decrease in viable cells treated with 5uM VPA. Controls treated with 1uM LBH and 5uM VPA (without R5020) have little effect on cell death. Due to extreme stress caused by autophagy and cytotoxicity, the cells underwent cell shrinkage, blebbing, disintegrated cell wall, disrupted nucleus and possible chromatin condensation. The floating dead cells are golden in colour and appear in suspensions.

In the case of co-treatment (figure 10) of R5020 with 1uM LBH589 for 96 hours, the simultaneous treatment yields only 9% cell viability while the treatment after prolonged R5020 yields 3% cell viability. This could mean that the both co-treatment and combination treatment are effective in inducing cell death with combination treatment being dominant.

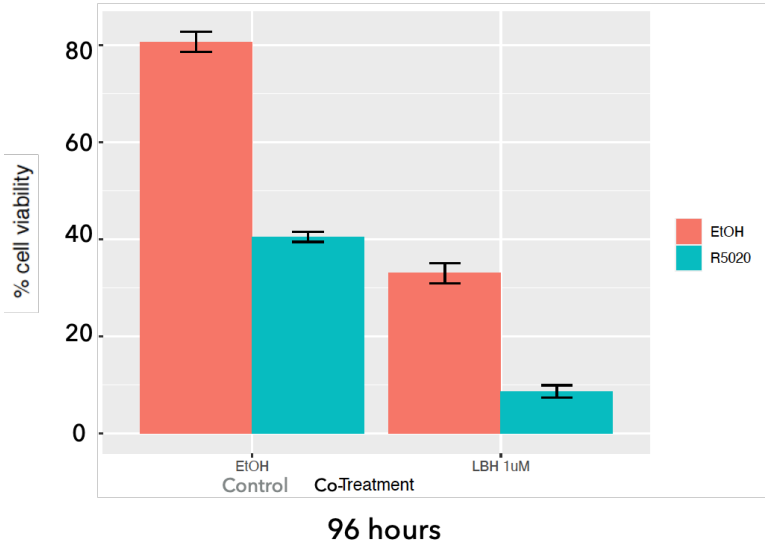


Figure 10: 96 hours co-treatment of 1uM LBH589 with R5020 showing a significant decline in cell viability (9%).

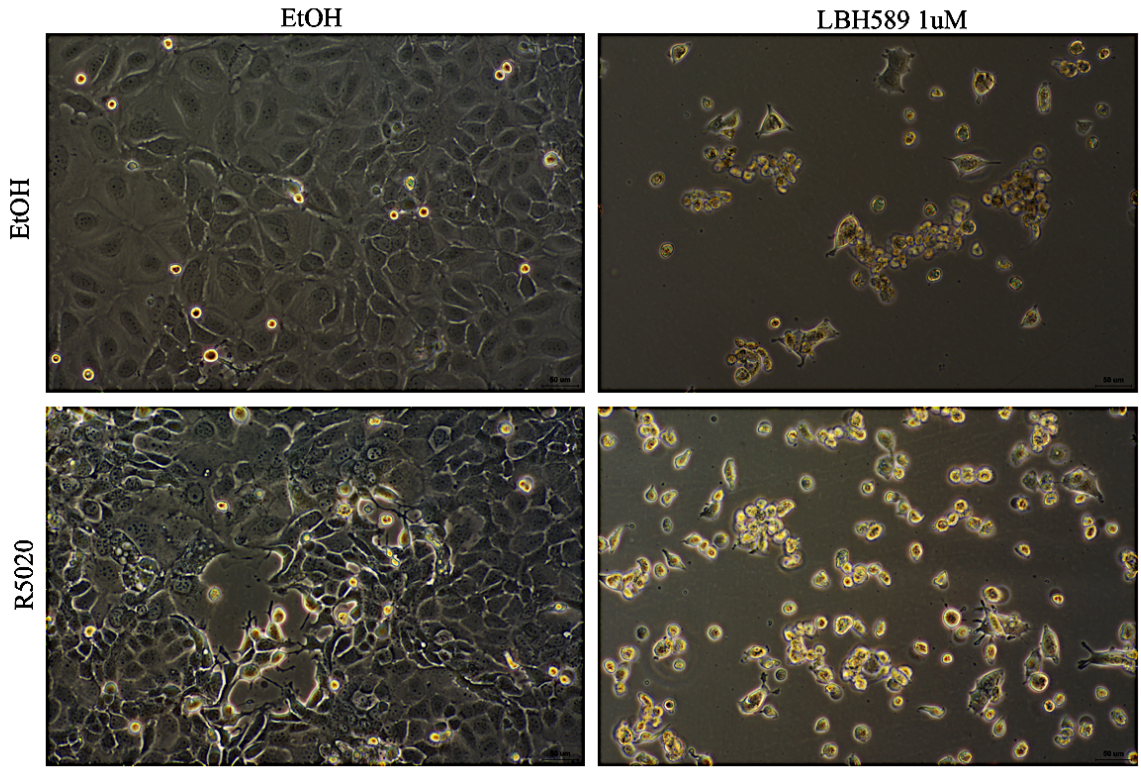


Figure 11: Co-treatment of 1uM LBH589 with R5020 for 96 hours showed a drastic increase in cell death compared to the controls. Statistical analysis showed about only 9% cell viability (figure 3). Golden

structures correspond to dead cells. Due to extreme stress caused by autophagy and cytotoxicity, the cells underwent cell shrinkage, blebbing, disintegrated cell wall, disrupted nucleus and possible chromatin condensation. The floating dead cells are golden in colour and appear in suspensions.

3.2. R5020-treated cells are sensitive to the cytoskeleton disruption effect of taxol, nocodazole and cytochalasin B

The MCF-7 cells were treated with CD drugs such as Taxol 0.2uM, Nocodazole 5uM and Cytochalasin B 10uM in combination therapy and co-treatment strategies where cells were subject to 24 and 48 hours treatment to screen the drugs that induced maximum cell death in order to study the molecular mechanisms responsible for inducing synthetic lethality.

Drug treatment after prolonged R5020 treatment (96 hours) yielded cell growth suppression in a time-dependent manner. There was significant decrease in the cell viability of cytochalasin B (CB) treated cells followed by nocodazole and taxol. CB had a major effect on the decline in cell viability after 48 hours of treatment as compared to nocodazole and taxol. It showed a cell viability of 18% post 48 hours when compared to 24 hours where it showed 35% cell viability indicating that it was indeed a time-dependent process (figure 12A and 12B). Nocodazole showed a considerable decline in cell viability of 34% at 24 hours to 20% at 48 hours. Treatment with taxol showed minimal decline in cell viability from 37% at 24 hours to only 25% after 48 hours treatment respectively indicating that it was least effective amongst other drugs. The effect of these drugs on EtOH control did not have any significant cell growth suppression as compared to treatment with R5020 indicating that the combination treatment is very effective in reducing the viability of cells. With respect to the drug controls there was no significant decrease in the cell viability.

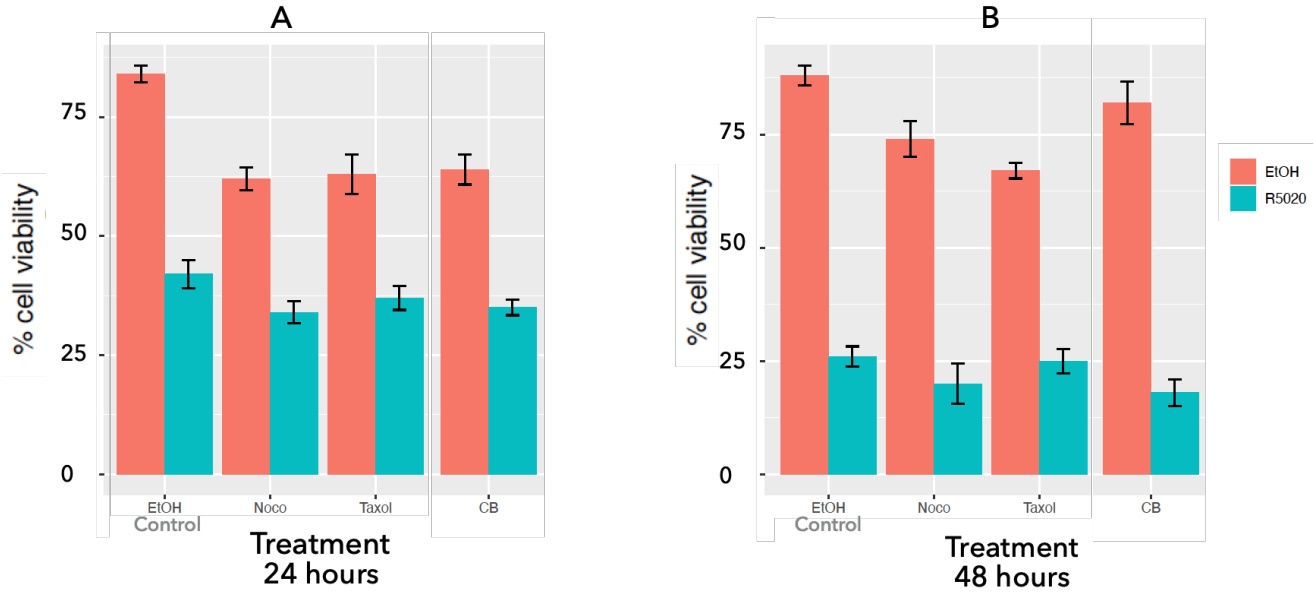


Figure 12: R5020-treated cells are more sensitive to the cytotoxic effect of cytoskeleton disruptors (A) 24 hours combination treatment showing very less decrease in the cell viability as compared to R5020 control. (B) 48 hours combination treatment showed that Cytochalasin B yielded a significant cell growth suppression with cell viability of only 18% followed by Nocodazole and Taxol. Taxol seems to have more or less no effect on cell viability just like R5020 control.

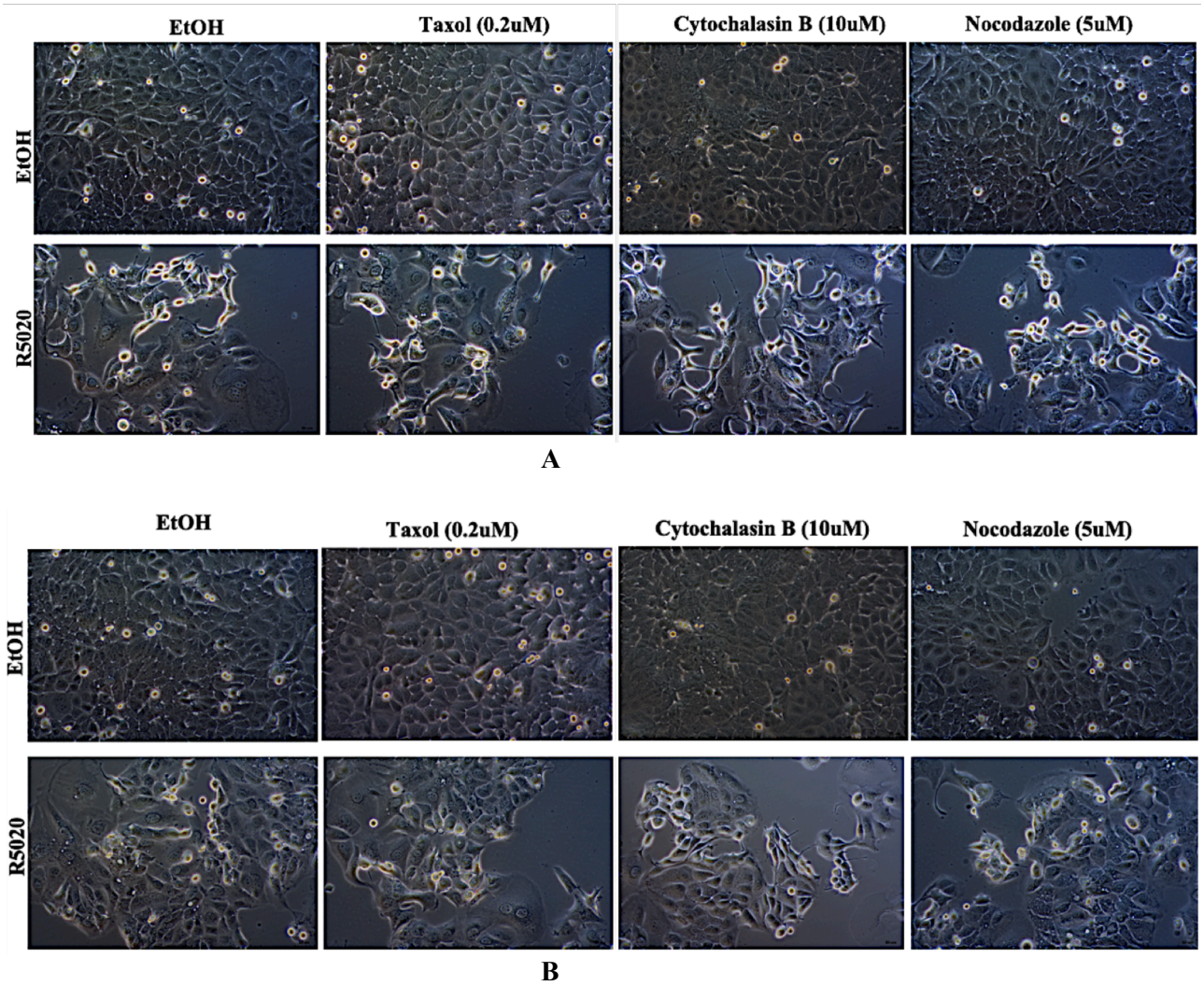


Figure 13: (A) Fluorescence microscope image representing 24 hour and (B) 48 hour combination treatment in MCF-7 cells. CB at 48 hours shows fewer overall cells compared to the other drugs indicating its efficiency in cell growth suppression and increased cell death. Indicates the time-dependent effect of inducing cell death. Vehicle control EtOH showed negligible cell death compared to drug controls. Due to extreme stress caused by autophagy and cytotoxicity, the cells underwent cell shrinkage, blebbing, disintegrated cell wall, disrupted nucleus and possible chromatin condensation. The floating dead cells are golden in colour and appear in suspensions.

3.3. R5020-treated cells are sensitive to the cytotoxic effect of ER stress inducer tunicamycin

The effect of tunicamycin on breast cancer cells was investigated in the present study. MCF-7 cells were treated with 3uM tunicamycin for 24 and 48 hours in combination treatment respectively. As presented in Fig. 14A and B, tunicamycin significantly inhibited the growth of MCF-7 cells in a time-dependent manner (24 and 48 hours), compared to the control cells ($P<0.01$). Co-treatment of Tunicamycin with R5020 for 48 hours yielded a cell viability of 25% just like LBH589 1uM (figure 14B). However, prolonged R5020 treatment seems to be essential for declining cell growth. With respect to the controls there was no significant decrease in the cell viability (figure 14A & B). This data suggests that Tunicamycin treatment may inhibit the growth and promote the apoptosis of breast cancer cells in a time-dependent manner.

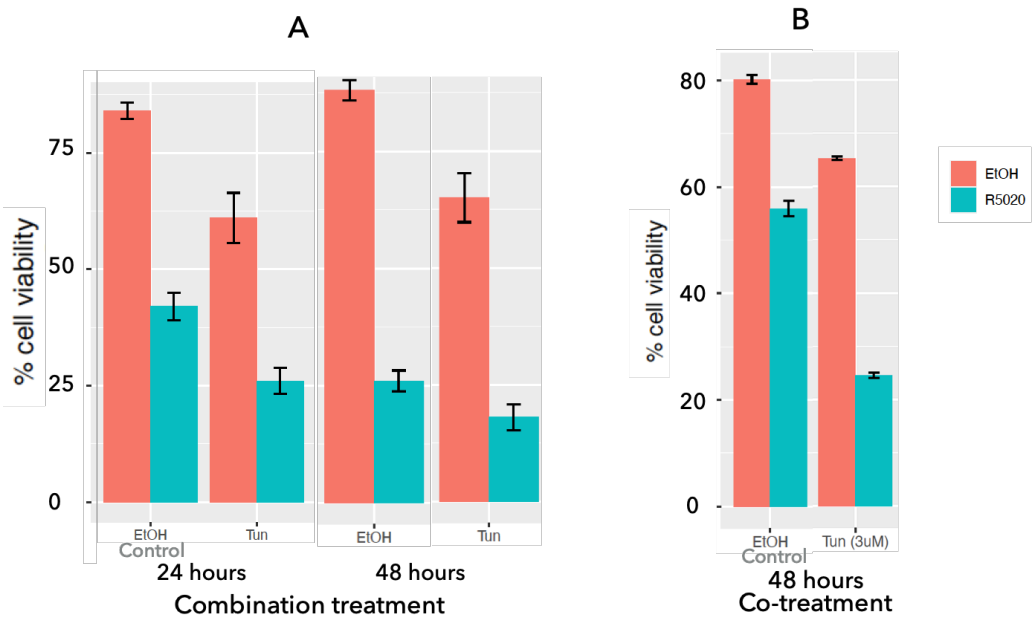
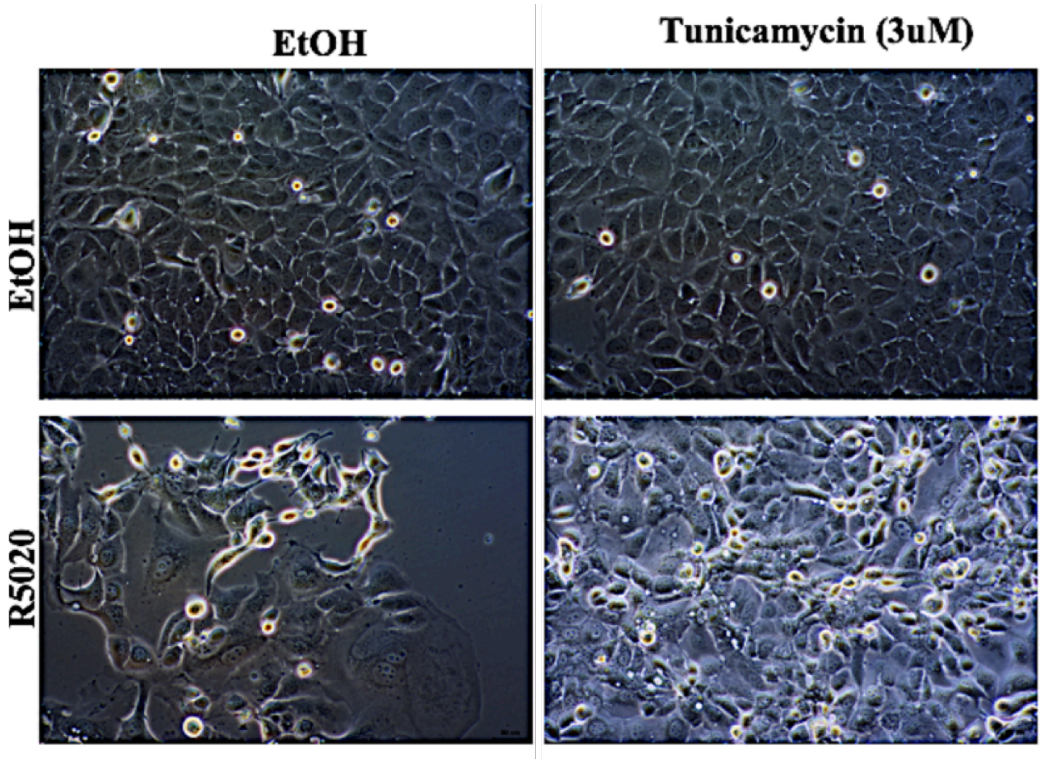


Figure 14: Statistical analysis showing the effect of combination and co-treatment of Tunicamycin with R5020. (A) Combination treatment of Tun with R5020 was carried out for 24 & 48 hours respectively. Results showed the time-dependent effect of Tun on the MCF-7 cells where 48 hours treatment yielded only 18% cell viability while 24 hours treatment yielded 25% viability. (B) Co-treatment of Tun with R5020 was conducted for 48 hours where a drastic decrease in cell viability (25%) compared to the controls was observed. However, combination treatment seems to be more effective than co-treatment.



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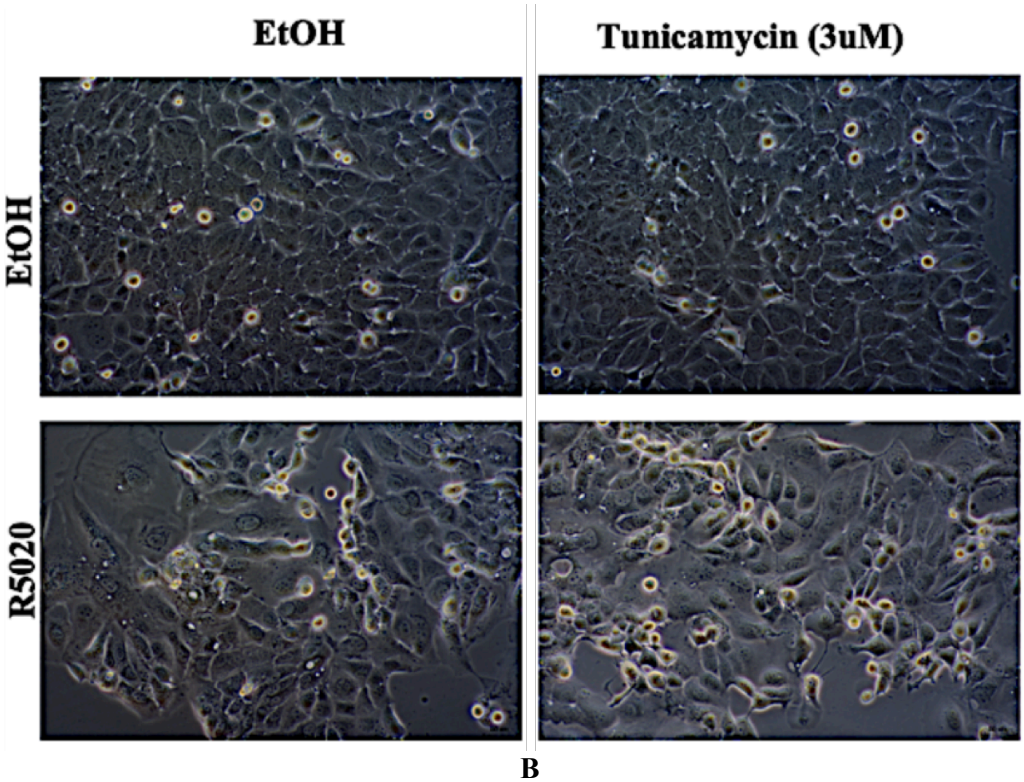

Figure 15: (A) Fluorescence microscope image representing 24 hour treatment of Tunicamycin with progestin R5020; combination treatment in MCF-7 cells. (B) Image representing 48 hour treatment of Tunicamycin with progestin R5020; combination treatment in MCF-7 cells. Vehicle control EtOH showed negligible cell death compared to control with drugs. Since the cells are under extreme stress due to estrogen starvation and prolonged R5020 treatment, there is a considerable amount of cell death in R5020 control. According to statistical analysis maximum cell death was observed in tunicamycin treated cells with a cell viability of only 26% at 24 hours and 18% at 48 hours of treatment indicating that cell death is a time-dependent process. Cell death is clearly visible in cells undergoing combination treatment. Maximum floating dead cells were observed in tunicamycin treated cells. Due to extreme stress caused by autophagy and cytotoxicity, the cells underwent cell shrinkage, blebbing, disintegrated cell wall, disrupted nucleus and possible chromatin condensation. The floating dead cells are golden in colour and appear in suspensions.

3.4. Morphology of MCF-7 cells (before and after treatment)

The untreated (control) MCF-7 cells displayed a rounded, epithelial cell–like morphology in 2D cultures (Fig. 2) with intact membrane maintaining their original morphology and close contact to each other even when the incubation was prolonged to 48 h. In contrast, the MCF-7 cells lost their original shape at 24 h of combination and co-treatment where cells were dispersed and detached. The cells were not in their polygonal or trigonal shape and obtained an elongated spindle-shape morphology. At 24 hours treatment, floating cells (dead cells) were identified and more suspension cells were observed at 48 hours. When the treatment was extended to 48 h, MCF-7 cells were clearly shrunk and had alienated morphology. Due to extreme stress caused by autophagy and cytotoxicity, the cells underwent cell shrinkage, blebbing, disintegrated cell wall, disrupted nucleus and possible chromatin condensation. The floating dead cells are golden in colour and appear in suspensions. (Refer figures 9, 11, 13, 15)

4. Discussion

Breast cancer is the second leading cause of cancer-related death among women worldwide [26]. Breast cancer development can be controlled by autophagy where it plays the roles of being pro-tumorigenic by supporting survival and proliferation of cancer cells while tumor suppressive by inducing cellular senescence and apoptosis [101, 102]. Hence, intrusions that promote or inhibit autophagy leading to cell death are potential cancer therapies. Progesterone and progestin play major roles in influencing

breast cancer development by exploiting the autophagy pathway [103-111]. Prolonged progestin R5020 treatment induces cellular senescence in MCF-7 cells via PRB-mediated nuclear translocation and activation of TFEB which is a master transcriptional regulator controlling the expression of autophagy and lysosomal genes [112]. In this study we hypothesized that cells induced with cellular senescence could be sensitized to apoptosis by treatment with anti-cancer drugs leading to lethal effect in MCF-7 cells. The aim of the combination therapy with two treatment strategies was to screen for drugs that induce effective apoptosis in synergy with R5020 and further determine the molecular mechanisms and pathways adapted by them.

LBH589 exerts cytotoxic activity in MCF-7 cells as it manifests impairment of cell viability, apoptosis induction and cell cycle arrest. The mechanisms underlying the cytotoxic effect of LBH589 on MCF-7 cell line includes apoptosis-induction and cell cycle arrest. In addition to apoptosis induction, LBH589 may cause G1 cell cycle arrest at low concentrations, and higher doses could result in G2-M arrest [93]. Immunofluorescence and nuclear staining experiments would be useful to confirm that LBH589 mediated mitotic arrest based on the presence of multiple nuclei. LBH589 is much more effective than VPA. Previous observations demonstarted that HDACi usually determine cytotoxicity at higher doses and induce G1 arrest at lower doses. The primary molecular mechanism of HDACi action is to alter the acetylation status of the core histone proteins, thereby facilitating chromatin remodeling with alteration in gene expression and cell differentiation. In agreement with this, we propose that LBH589 could acetylate histones of MCF-7 cancer cells, finally leading to the upregulation of p21 and the downregulation of cyclin D1 (figure 16A and B). Low doses of LBH589 (0.2uM) could lead to microtubule stabilization by increased tubulin acetylation and bundle formation. Tubulin acetylation being an corroborated marker of microtubule stability; the suppression of spindle-microtubule dynamics slow or block mitosis at the metaphase-anaphase transition, thus facilitating apoptotic cell death. I propose that LBH589 cytotoxic effects in MCF-7 cancer cells can be mediated through microtubule stabilization [93].

All HDAC inhibitors have been reported to activate either an extrinsic or intrinsic pathway or both of these cell death pathways in most cancer models [86]. LBH589 presumably decreases the expression of Bcl-2, Bcl-xL and XIAP, and enhance the expression of proapoptotic proteins, such as Bax and Bak, thus enhancing TRAIL-mediated cytotoxicity in MCF-7 cells by amplifying intrinsic as well as extrinsic apoptotic pathways [87].

Valproic acid causes hyperacetylation of the N-terminal tails of H3 and H4 and inhibits HDAC activity, probably by binding to the catalytic center and blocking the access of the substrate [88]. VPA is a potent inhibitor of class I HDACs [89, 90], but it also inhibits class II HDACs [91]. VPA influences differentiation and has antiproliferative effects in addition to induction of gene targeting of cyclin-dependent kinase inhibitor p21 (WAF1) [82]. p21 regulates cell-cycle progression, given the fact that it is involved in both G1-S and the G2-M transition. Anti-proliferative action, regulation of p21, cell-cycle arrest, apoptosis are the common effects of VPA on breast cancer cells. In this study, given that the MCF-7 cells have undergone autophagy prior to VPA treatment, the decline in cell numbers might be due to the reason that VPA caused sensitisation to apoptosis by causing cell-cycle arrest. With regard to the co-treatment of VPA with R5020 for 48 hours, it can be seen that there was much lesser cell growth suppression than in combination treatment. This suggests that for efficient cell growth suppression prolonged R5020 treatment is essential for apoptosis induction following the synthetic lethality principle.

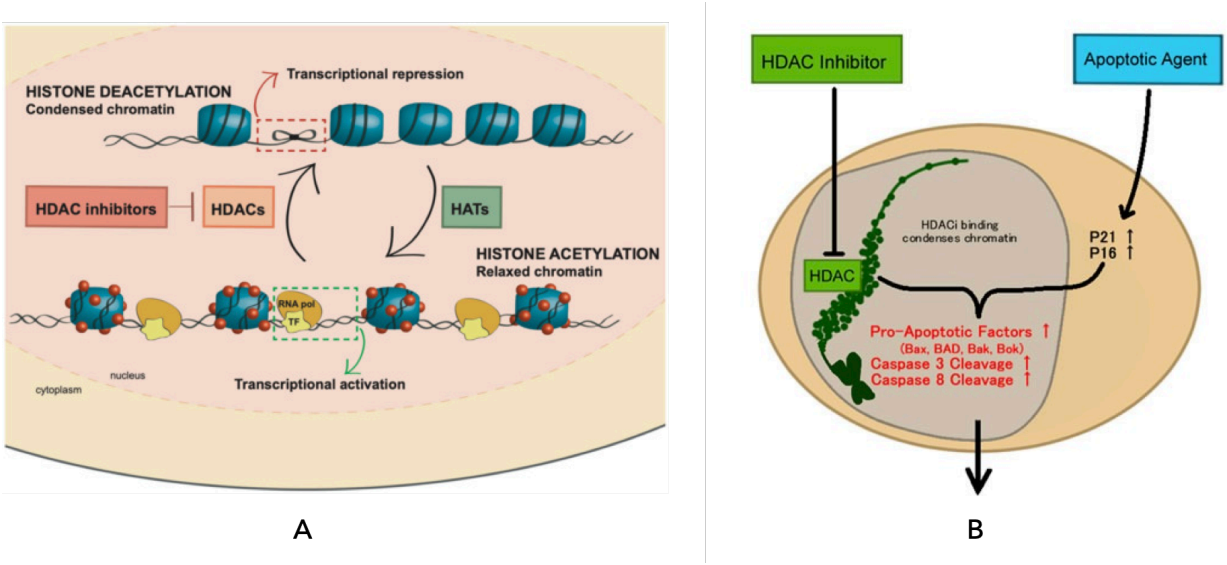

Figure 16: (A) HDAC inhibitor (HDACi) effect on chromatin remodeling. Histone deacetylases (HDACs) and histone acetyltransferases (HATs) are responsible for the balance of histone acetylation, and thereby regulate gene expression. Whereas HDACs deacetylate histones, promoting transcription repression, HATs are responsible for acetylating histones and inducing transcriptional activation. HDACi inhibits HDACs, and thus maintain an open chromatin conformation [figure adapted from Camino *et al*.]. (B) Apoptosis targeting agents in combination therapy. Many therapeutic agents aim to target factors that either upregulate proapoptotic factors or inhibit prosurvival mechanisms.

Cytochalasin B (CB) induces microfilament depolymerization that leads to apoptosis. CB inhibits actin polymerization and filament network build-up by binding to the growing barbed (+) end of microfilaments. This inhibition affects major steps of actin polymerization. It inhibits cell division by inhibiting cytokinesis thereby causing chromosome damage and chromosomal instability [98]. It was previously reported to decrease the number of motile cells. CB enhances tumor cell lysis and stasis by inhibiting the biosynthesis of phosphotidylcholine whose mechanism is associated with alterations of intracellular calcium ions [99]. Although cytokinesis is inhibited, mitosis is unaffected. As a cytokinesis inhibitor, CB disrupts the actin cytoskeleton and interferes with the development of the cleavage furrow. As a result, the cell is unable to divide, permeating a weakened cytoskeletal network. Further, the cell continues to form nuclei (multinucleated) and becomes enlarged [117]. This represents a substantial drug synergy between cytochalasin B and the R5020 (refer figure 17).

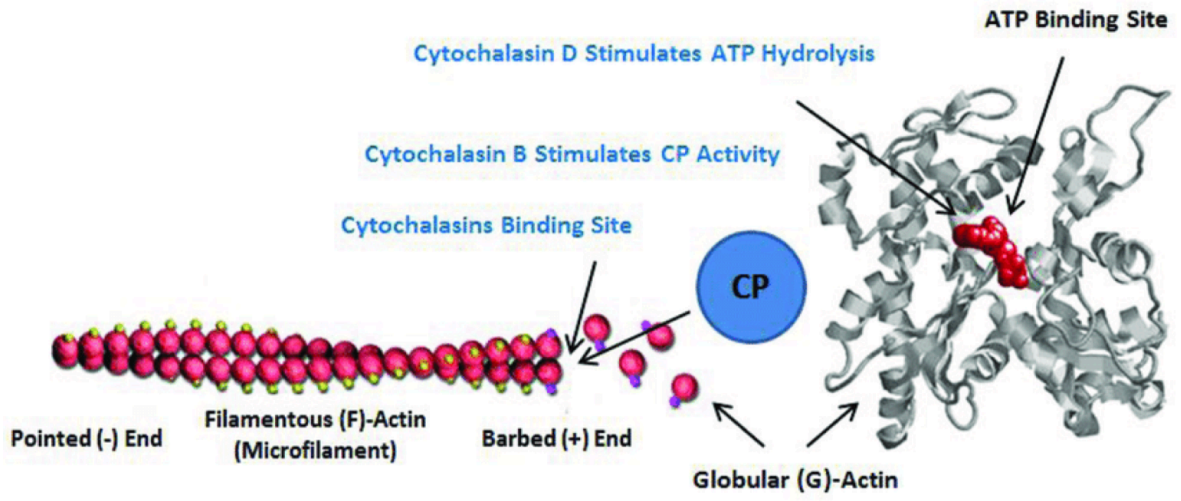

Figure 17: Cytochalasin B mediated microfilament depolymerization in MCF-7 cells [117].

Nocodazole induces microtubule depolymerization which affects cell division leading to apoptosis. Cells treated with nocodazole are known to arrest with a G2- or M-phase DNA content when analyzed by flow cytometry. Microscopy of nocodazole-treated cells from previous studies has showed that they do enter mitosis but cannot form metaphase spindles due to inability of microtubules to polymerise. There is absence of microtubule attachment to kinetochores which in turn activates the spindle assembly checkpoint, causing cell arrest in prometaphase. Prolonged arrest of cells in mitosis due to nocodazole treatment customarily results in cell death by apoptosis [96, 97]. In short, nocodazole treatment might cause morphology changes, spindle formation, kinase activation leading to upregulation of pro-apoptotic genes finally inducing apoptosis [118]. Prior induction of senescence by R5020 could sensitize the cells to mitotic arrest thereby leading to apoptosis (refer figure 18).

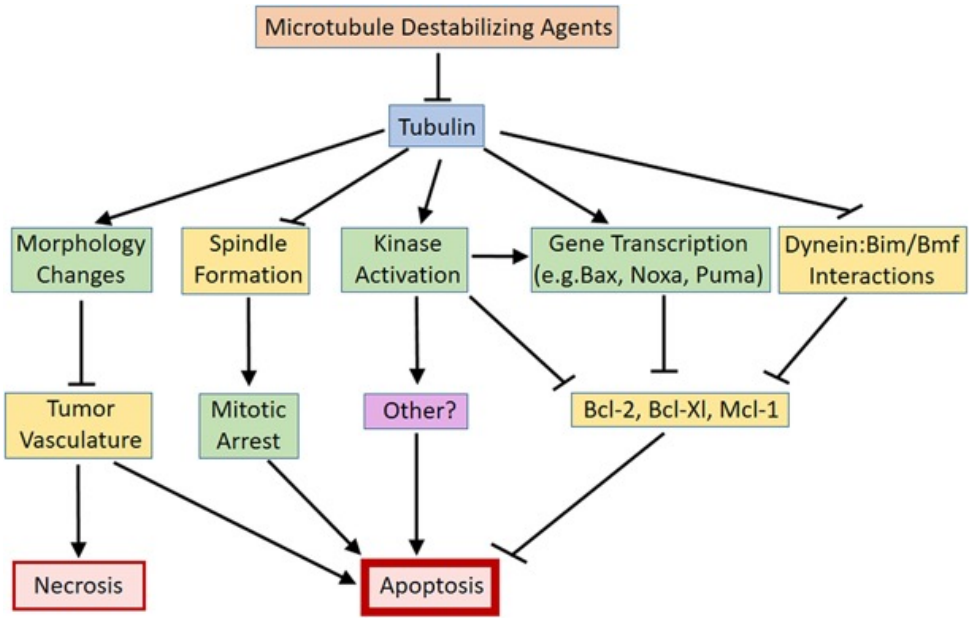

Figure 18: Consequences of microtubule disruption by nocodazole [118].

Taxol induces cytotoxicity by targeting tubulin in MCF-7 cells. Taxol (Paclitaxel) targets tubulin. Taxol-treated cells have defects in mitotic spindle assembly, chromosome segregation and cell division. It stabilizes the microtubule polymer and protects it from disassembly. Chromosomes are thus unable to achieve a metaphase spindle configuration which blocks the progression of mitosis and prolonged activation of the mitotic checkpoint triggers apoptosis [94, 95]. In the current study, the MCF-7 cells are subject to senescence by R5020 which might induce apoptosis due to blocking of mitosis and hence cell cycle progression.

Tunicamycin inhibits the growth of and promotes the apoptosis of breast cancer cells. According to Wang *et al*, it was revealed that tunicamycin significantly arrested the cell cycle of MCF-7 cells at the G1/G2 and S phase. Our results suggest that Tunicamycin treatment may inhibit the aggressiveness of and arrest the cell cycle of breast cancer cells. Tunicamycin is also said to inhibit N-linked glycosylation by preventing core oligosaccharide addition to nascent polypeptides leading to blocking of protein folding and transit through the ER and activates the unfolded protein response (UPR) to induce cell death. Tunicamycin inhibits glycoprotein biosynthesis in the ER which results in the accumulation of misfolded proteins to cause subsequent ER stress. It conversely triggers cell death and significantly enhances apoptosis induced by chemotherapy through exacerbating ER stress. Blocking ER stress-induced autophagy markedly could increase the apoptosis of MCF-7 cells under treatment with tunicamycin. Moreover, prior stress induced autophagy by R5020 mostly acts as an incentive in triggering the apoptosis of MCF-7 cells. Based on studies by Wang *et al*, tunicamycin treatment suppressed Akt and NF-κB expression in MCF-7 cells, relative to the control cells. It also inhibited migration and invasion in MCF-7 cells. Therefore, tunicamycin regulates the growth and aggressiveness of breast cancer cells through the Akt/NF-κB signaling pathway. [115]

Figure 10 [113] depicts a model summarizing the effect of tunicamycin-induced ER stress in CD44+/CD24- MCF-7 breast cancer cell. Tunicamycin causes accumulation of unfolded proteins in ER lumen inducing unfolded protein response in the cell. In this condition mRNA of XBP-1 (transcription factor that regulates the expression of genes important to the proper functioning of the immune system and in the cellular stress response) gets spliced by endoribonuclease domain of ER transmembrane protein IRE1 and XBP-1 protein is synthesized. Both XBP-1 and ATF6 are b-zipper transcription factors which induce expression of CHOP protein. CHOP protein is a pro-apoptotic factor which can provoke cell death via different pathways [113] (refer figure 19).

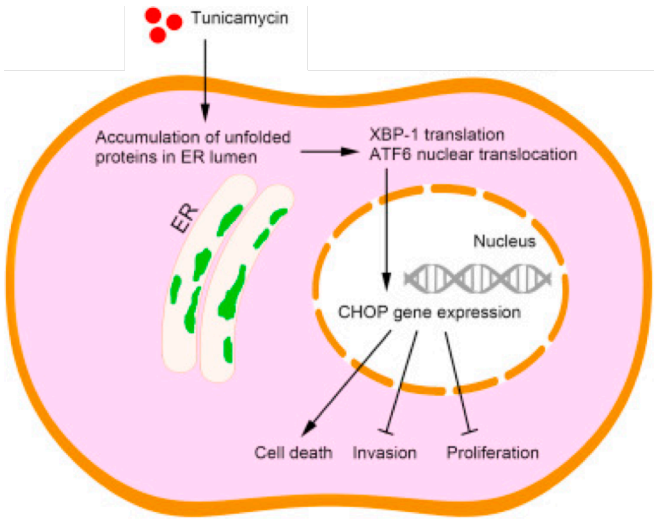

Figure 19: Model summarizing the effect of tunicamycin-induced ER stress in CD44+/CD24- MCF7 breast cancer stem cell.

The presence of estrogen and progesterone promotes cell proliferation while progestin R5020 inhibits cell proliferation in MCF-7 PRB cells. The progestin R5020 treatment induces autophagy in a PRB dependent manner co-ordinated by an interplay between ligand-activated PRB and TFEB. Autophagy can be pro-tumoral or anti-tumoral. Furthermore, addition of apoptosis inducing factors like HDACi, ER stress inducers, microfilament and microtubulin depolymerizing agents (CD agents) might retard the cell growth further by causing cell death and necrosis due to sensitization, using the various mechanisms described above. Since the cells are already under a lot of stress due to autophagy by R5020, it must be seemingly easier to induce apoptosis in an effort to reach balance for homeostasis. Although physiological levels of autophagy are essential for the maintenance of cellular homeostasis during various stress conditions, excessive or uncontrolled levels of autophagy are able to induce autophagy-dependent cell death. Given the hypothesis of the mechanisms involved in inducing apoptosis in association with progestin R5020, it is important to further explore the treatment strategies that would be effective in causing a synthetic lethal effect in breast cancer cells. It is essential to elucidate the activity of these drugs and how their action is regulated in different types of breast cancer in order to achieve a maximal therapeutic benefit.

5. Conclusion and future perspectives

Autophagy and apoptosis often occur in the same cell, mostly in succession in which autophagy precedes apoptosis. This is because stress often triggers an autophagic response, especially if the level of stress is not lethal. Apoptotic and non-apoptotic lethal programmes are activated when stress exceeds a critical duration or an intensity threshold. In many cases, autophagy constitutes a strategy to adapt to and cope with stress. If the cell commences apoptosis, autophagy can be inactivated. It has been demonstrated that R5020 induces autophagy in the MCF-7 cells. Due to the cellular stress caused by R5020, the cells turn out to be sensitive to apoptosis by the apoptosis inducing agents. Apoptosis was induced by epigenetic modifiers (LBH589 & VPA) in a dose and time-dependent manner. Similarly, the cytoskeleton disrupting agents and the ER stress inducer (Tunicamycin)

seemed to be cytotoxic to the MCF-7 cells in a time-dependent manner. 48 hours of combination treatment induced more cell death than 24 hour treatment. Combination treatment turned out to be more efficient in decreasing cell viability than co-treatment. This indicated that combination treatment enhances the sensitivity of cells to apoptosis due to existing stress caused by autophagy. It has to be noted that lower doses of the drugs also cause stress and induce cell death at a slower rate. The ER stress inducer Tunicamycin has a significant effect on inducing apoptosis and reducing cell migration and differentiation due to prior ER stress caused by R5020. Since this is just a preliminary evaluation of the cell viability, in the future, further validation of these drugs, and their comparisons with the expression profiles of breast cancer, will still be needed. Further analysis of the drug treatments can be carried out by employing techniques for quantitation of apoptotic cells and cell cycle analysis by flow cytometry. Cell viability test can be performed via MTT reduction assay. Western blot analysis of the apoptotic and autophagy markers to check the levels of expression would be beneficial in determining the existence of apoptosis or autophagy or both. Immunofluorescence studies for LC3 and puncta analysis would help to determine the presence of autophagy after the combination treatment. RT-PCR analysis to check for gene expression levels of apoptotic markers could help to predict the molecular mechanisms the markers are involved in. Further, combination treatment using these drugs with cytotoxics, aromatase inhibitors & prodrugs can pave the path for exploring different combinations of drugs that play an effective role in inducing apoptosis. Future therapy advances will likely include combination treatment using HDAC inhibitors, cytoskeleton disrupting agents, ER stress inducers and chemotherapy or other inhibitors. By combining therapeutics, we can simultaneously target multiple oncogenic signaling pathways and for effective cancer treatment and also overcome resistance. Their comprehensive study under different circumstances and environments is critical for the development of novel new treatment strategies for breast cancer. It is important to investigate the impacts of drugs beyond what they can achieve alone. Using them in combination can act as a multiplier and increase the sum of their benefits.

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