Curcumin in Turmeric as a plant-based therapy for breast cancer through inhibition of the mTOR signalling pathway

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Abstract— **With the incidence of cancer, and thus, breast cancer becoming more common, finding a treatment that would reduce the number of patients being lost in the follow-up period either due to complications associated with treatment or metastasis, is of utmost importance. Research suggests that relying on plant-based compounds may be the answer. Several papers point to substances in plants that have anti- cancer compounds which, when developed, can be used in medication for cancer. In this paper, we review curcumin (diferuloylmethane) found in turmeric (haldi), a common ingredient in medicinal and food products in India, as an inhibitory compound on the mTOR pathway which is followed in breast cancer cells. The advantage this treatment has is suggested to be extremely low, to none, cytotoxic effects on the cells. To this effect, this paper proposes a comparative study to be conducted between the synthetic drug rapamycin and natural curcumin, due to their similar targets in the pathways, in order to investigate which of the two could be a more effective therapeutic compound.**

***Index Terms*— breast cancer, curcumin, cytotoxicity mTOR pathway, rapamycin.**

I. INTRODUCTION & BACKGROUND INFORMATION

Cancer claims the lives of nearly 600,000 Americans on average, with Breast Cancer being one of the leading causes of death amongst women (Morgan et al.,2020). In the US, almost 30% of all cancers amongst women are attributed to breast cancer, with a survival rate of 21% for metastasised tumors that have spread to distant regions (taken: American Cancer Society). Curbing cancer before it reaches this stage (IV), requires interruption of the tumor pathway that inhibits the growth mechanism involved. This pathway is differentiated by the type and hormone receptor sensitivity of the tumor.

Several factors affect the initial incidence of invasive breast cancer (IBC), including genetic predisposition, mutations in the gene due to exposure to carcinogens, hormonal and reproductive cycle-related changes and lifestyle. A common mutation that is present in almost 0.25% of the population is the BRCA gene-related mutation. BRCA is translated to repair broken DNA in the breast cells, but people with mutation in the gene are unable to do so and thus more likely to develop breast cancer (Momenimovahed et al., 2019).
Invasive ductal carcinoma (IDL) and invasive lobular carcinoma (ILC) are the two types of IBC that are associated with a poor prognosis in patients, with IDL contributing to 80% of all IBCs. This means that this kind of diagnosis is associated with an unlikely or poor recovery for the patient. In many such cases, it is due to the metastasis of the tumor to the lymph node, bone, liver and lung. (taken: breastcancer.org). Both types demonstrate excessive cell proliferation that originates in a part of the breast; IDL begins in the milk ducts in the mammary glands, while ILC starts when there is a mutation in the lobules that produce milk in the breast. Both types spread outwards, damaging the nearby tissues and vessels to form a malignant tumor (taken: breastcancer.org).

Tumors can be hormone receptors - positive, negative, none or even both. Cells can have protein receptors embedded in their cell membranes that respond to hormones in the blood that allow them to grow. In the breast, normal breast cells have receptors that respond to progesterone and oestrogen, which aid in their development. However, when they become cancerous, the same receptors work in favour of the cancer, allowing the tumor to grow. Tumors that have a characteristic number of hormone receptors (HR) are known as positive or negative based on whether they are more for estrogen or/and progesterone (HR+/ ER+/ PR+). At least 66% of cases of IBC have one of these receptors, and the frequency increases with age. (taken: American Cancer Society) Another receptor commonly involved in IBC - almost 1 out of 5 cases - is the human epidermal growth factor receptor (HER2) which when present (HER2+) promotes the growth of the cancer cells. Another subtype of IBC is triple-negative breast cancer (TNBC) which has negative receptors for ER/PR/HER2. Treatment strategies, therefore, vary according to the subtype of cancer, based on the receptor susceptibility. (Ciriello et al., 2015)

Common therapies include hormone therapies, for example, which are effective against ER+/PR+/HER2+ breast cancers but not against those that are HR-. Chemotherapy and immunotherapy for TNBC are also effective unless the patient develops an acquired resistance. Acquired resistance can result in a relapse with the effect of preventing cell apoptosis, interrupting previously healthy pathways, disrupting the cell cycle and causing excessive production of the BRCP (breast cancer resistance protein) or the ABCG2 protein. (Nunnery et al.,2020)

Analysing the related pathways in detail will allow us to identify where and how they can be interrupted, and finding inhibitors might prevent the tumor from spreading past its initial stage (I). Some of these pathways will be summarised in this review, including mTOR and its signalling via cytokines and phosphoinositide 3-kinase (PI3K)/Akt, ER receptors and the activation of nuclear factor-kappaB (NFκB).

Historically, in Indian culture, *Ayurveda* (In Sanskrit: knowledge of life) is an ancient medical practice that relies on natural remedies to treat ailments (Jaiswal et al., 2017). In modern treatments, cancer drugs often have detrimental side effects on the patient. Most commonly, these include hair loss, fatigue, anaemia and nausea (taken: American Cancer Society). Drugs like Rapamycin (sirolimus), Afinitor (everolimus), Torisel (temsirolimus), and classical chemotherapeutics, are developed to inhibit these pathways by targeting specific proteins that interact with mTOR. Like other such drugs, these drugs have the side effect - rapamycin, for example - of causing leukopenia and thrombocytopenia in patients (Blagosklonny et al., 2019).

However, finding alternatives to these drugs in plants, as *Ayurveda* suggests, might decrease the likelihood of cytotoxic effects on cells and systems in the body. Amongst various attributes that make up *Ayurveda*, herbal remedies that rely on compounds extracted from plants have proven to be effective. Gingerol in ginger, resveratrol in grapes, genistein from soybean - all have cancer-repellant potential. In this paper, the focus is on the inhibitory mechanism of Curcumin (in turmeric) in the breast-cancer inducing pathway and propose an experiment to see its effectiveness in reducing metastasis.

II. Pathways of Breast Cancer

Cancer involves a complex signalling cascade, the origin and different controlling components of which are hard to trace. The mTOR pathway, particularly its over-activation, and mutation of PI3K/Akt as a regulator in its upstream pathway has been attributed with the progression of tumors in many cancers. Moreover, mTOR's interaction with other commonly mutated or affected cancer components like cytokines, nuclear-factor KappaB (NFκB) and hormone receptors are discussed.

A. mTOR Signalling Pathway in Breast Cancer

When you submit your final version, after your paper has been accepted, prepare it in two-column format, including figures and tables. The mechanistic (previously known as mammalian) target of rapamycin is a serine-threonine kinase, a part of the transferase family of enzymes, specifically the phosphoinositide 3-kinase (PIKK) family. mTOR has a complex signalling cascade that consists of many levels. Genetic alterations cause the hyperactivation of mTOR signalling and the aberrant production becomes a cause of tumor incidence and metastasis. Within this cascade, certain regulators 'cross-talk' with mTOR, either negatively (inhibitory fashion) or positively, the most important ones include phosphoinositide 3-kinase (PI3K)/Akt, nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK) and p53 (Conciatori et al.,2018) (Tian et al.,2019).

Under normal physiological conditions in eukaryotic cells, mTOR forms 2 complexes, namely mTORC1 and mTORC2. While mTORC1 regulates protein synthesis and consists of many levels. Genetic alterations cause the hyperactivation of mTOR signalling and the aberrant production becomes a cause of tumor incidence and metastasis. Within this cascade, certain regulators 'cross-talk' with mTOR, either negatively (inhibitory fashion) or positively, the most important ones include phosphoinositide 3-kinase (PI3K)/Akt, nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK) and p53 (Conciatori et al.,2018) (Tian et al.,2019).

Under normal physiological conditions in eukaryotic cells, mTOR forms 2 complexes, namely mTORC1 and mTORC2. While mTORC1 regulates protein synthesis and autophagy, mTORC 2 plays a key role in the translation of kinases of the AGC family and in cell survival in response to growth factors and nutrient availability (Hare et al., 2017). Consequently, mTOR has been associated with metabolic processes, protein synthesis and the growth and survival of cells normally, and, in cancerous conditions, because of the mutations, its pathways' components sustain.

mTORC1 communicates with five other components that work together to carry out its function. Most notably, this complex consists of the mTOR protein itself, proline-rich AKT substrate 40 kDa (PRAS40) & Deptor, which is the DEP-domain- containing mTOR-interacting protein. When activated, mTORC1 phosphorylates PRAS40 and Deptor - which on their own have the effect of reducing its signalling - reducing their effect on mTORC1 and having a positive effect on further signalling (Moschetta et al., 2014).

In protein synthesis, lipid and nucleotide production, mTORC1 has a positive controlling effect through downstream effectors, while limiting macrophage activity. The transmission for the signal of cap-dependent mRNA translation is done this way. Phosphorylation of the eukaryotic initiation factor 4E - binding protein 1 (4E-BP1) and p70 ribosomal S6 kinase (S6K1) at Thr389 aids in the protein synthesis mechanism. The phosphorylation prevents 4E-BP1 from binding to eIF4E at Thr37/46, Thr70 and Ser65 allowing the cap-dependent translation to occur (reviewed by Richter and Sonenberg, 2005). S6K1 activity is stimulated as well increasing mRNA biogenesis, cap-dependent translation of mRNA, rRNA through protein phosphate 2A and ribosomal protein through proteins like S6K1 aly (SKAR), eEF2K and ribosomal protein S6 (reviewed by Ma and Blenis, 2009) (Laplante et al., 2009).

Another important factor involved in the regulation of mTOR activity is the tuberous sclerosis complex, TSC, consisting of hamartin (TSC1) and tuberin (TSC2). Both act as GAP (GTPase) activators for Ras homolog enriched in brain (Rheb). TSC1 and TSC2 convert Rheb into an inactive GDP-bound complex negatively regulating mTORC1. The GTP-Rheb form binds with mTORC1 for its function (Long et al.,2003; Inoki et al.,2003; Sancak et al., 2007). Mutations that inactivate this mechanism, therefore, can lead to tuberous growths of large tumor cells (Crino et al., 2009).

The pathway that is most commonly focused on in relation to mTOR is the PI3K (phosphoinositide 3-kinase) pathway. PI3K is an upstream regulator of mTORC1, through TSC. IGF-1 and insulin activate PI3K, thereby generating PIP3 from PIP2 which is present and attached to membranes. Effectors in the downstream paths, PDK1 and Akt, are stimulated through their PH domains. Akt, in particular, is phosphorylated by PDK1 on Thr308 and Ser473, and once activated through this mechanism, affects TSC. At multiple

areas, Akt phosphorylates TSC2 further at S939 and T1462, detailing it as its association with TSC1 is weakened and positively affecting the mTORC1 signalling. This is because TSC2 cannot behave as the GAP activator. Another way Akt negatively affects mTORC1 is by binding PRAS40 with 14-3-3 proteins inducing an inhibitory effect. Furthermore, Ras-dependent ERK1 and 2 (extracellular signal-regulated protein kinase) also phosphorylate TSC2. Further downstream RSK also acts on TSC2 on S1798. These different factors have a combined effect on TSC2 and the mTOR pathway (Alayav et al., 2016). An overview showing the interaction of the various factors of this process is given in Fig 1.1 (Fan et al., 2017) (Laplante et al., 2009).

In breast cancer, mutations affecting mTOR pathways, the most common alteration in PI3K signalling, loss of PTEN expression or increase in ErbB receptors, may result in tumor proliferation. Lying upstream of mTOR, these changes in the signalling system increase the activity of mTOR and as a result, the cell growth of the tumor accelerates. Base changes and deletion in the PIK3CA gene, that codes for PI3K, and AKT are typical of breast cancer cases. The mutations cause changes to helical domains and kinases (TSC/AKT) which are activators of mTOR, increasing its signalling in the process (Tian et al., 2019).

Research suggests that the mTORC1 signalling cascade has a more profound oncogenic effect than mTORC2. Even though the mTORC2 pathway has been associated with Akt, however, the rictor expression required is lower in breast cancer tumors than in normal conditions, which might mean that mTORC2 doesn't play as prominent a role as its counterpart.

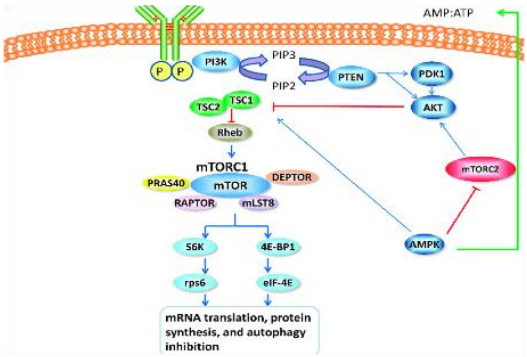

Fig 1.1 mTOR Pathway & its Factors

B. Role of mTOR and Hormone Receptors in IBC

As discussed in earlier sections, ER+ & PR+ breast cancers have better outcomes than Her-2/neu positive tumors. Her-2/neu (epidermal growth factor tyrosine kinase) receptor is known to control the differentiation and growth of epithelial cells. Its overexpression is common to one-fifth of breast cancers and has shown a poor prognosis (Gil et al., 2014). Immunohistochemistry can prove the presence of HR+ tumors in cancers. Commonly, these cancers are then subjected to endocrine therapies but risk developing resistance.

IBC cells depend on estrogen and progesterone for cell survival and proliferation. ERα, or nuclear transcription factor, forms a ligand with estrogen to allow the expression of the genes that control growth. mTORC1 has a link to estrogenic signalling in two ways: (1) Estrogen is an activator of mTORC1 and (2) mTORC2 has a role to play in ERα activity. MAPK and PI3K pathways have been shown to be upstream effectors of mTORC1 and regulate ERα transcriptional activity caused by estrogen (Alayev et al., 2016).

Phosphorylation of ERα on S167 by mTORC1 through S6K1 is an example of an indirect link between mTOR and estrogen. The Raptor of mTOR binds to ERα, which when stimulated by estrogen translocates it to the nucleus. mTOR also phosphorylates ERα on S104 and S106 implicating mTORC in the causative mechanism of ER+ IBC.

C. mTOR & Nuclear Factor kappaB

Nuclear factor-kappaB (NFκB) is a proinflammatory transcription factor and is activated in breast cancer. Its activation causes tumors to become invasive and more untreatable because of hormone insensitivity and reduced apoptotic effect. Angiogenesis and metastasis have also been linked with the transcription factor. Its inhibition can lead to a better prognosis amongst patients due to the increased apoptotic potential of tumor cells in response to drugs and endocrine therapy (Aggarwal et al., 2004).

Five complexes belong to the NF-κB family, activated downstream by cytokines like TNF and IL-1, T-cell activation amongst other effectors. Controlling NFκB involves the phosphorylation of IκB proteins that are degraded to form NFκB dimers. The IKK complex of which the IκB proteins are part includes IKKα and IKKβ subunits (Dan et al., 2008). Akt, the upstream effector in the mTOR pathway, has been reported to regulate NFκB activity. Some papers suggest that Akt works via IKK promoting the transactivation potential and phosphorylation of NF-κB allowing metastasis and angiogenesis in this manner. mTORC1 is activated by Akt via phosphorylating TSC2 that activates GTPase Rheb and has an adverse regulatory effect on mTOR (Inoki et al., 2003). ATP levels and AMPK cellular activity also seem to have an effect on mTOR because of a controlling mechanism Akt has on them. The correlation between factors in the mTOR pathway and those involved in NFκB have an established effect on one another in tumorigenesis, apoptosis and growth (Zou et al., 2020).

III. CURCUMIN AS AN INHIBITOR OF IBC

Curcumin (diferuloylmethane) is a dietary antioxidant compound derived from turmeric, which comes from the rhizome of the plant Curcuma longa. Specifically, curcumin belongs to the group of curcuminoids apart from demethoxycurcumin and bisdemethoxycurcumin in turmeric, which has been proved as having beneficial effects on the body. Curcumin's potential as an anticancer compound is because of its proven ability to have an inhibitory effect on different types of tumors. Amongst its wide range of targets are transcription factors like NF-kappaB, TNF; receptors like EGFR and HER2; kinases like tyrosine kinases and serine/threonine kinases. The following section gives an insight into how curcumin down-regulates and inhibits the mechanisms discussed above for IBCs.

A. *Curcumin & mTOR*

A study conducted not only showed curcumin's effect on human rhabdomyosarcoma cells but, more notably, its effect on mTOR because of the central role it played in the process of growth and survival of the rhabdomyosarcoma cells. Curcumin inhibited the phosphorylation of S6K1 and 4E-BP1 mediated by mTORC1 in breast (MCF-7), cervical (HeLa), colon (HT29), and prostate (DU145) cancer cells (Beevers et al., 2015).

Subsequently, curcumin was identified as an independent inhibitor of mTOR. Upstream kinases that affect mTORC1 signalling, namely insulin-like growth factor 1 receptor (IGF-IR) and phosphoinositide-dependent kinase 1 (PDK1), did not play a role in its inhibitory mechanism. In fact, protein phosphatase 2A (PP2A) and TSC 1 & 2 did not seem to be involved in the mechanism according to the study, which tested curcumin's effects on mTOR in the presence of PP2A and AMPK inhibitors. Instead, curcumin was shown to dissociate Raptor from mTORC1, establishing curcumin as an effective agent for the mTOR pathway (Beevers et al., 2015).

B. *Curcumin & Nuclear Factor kappaB*

Curcumin has induced apoptosis and prevented the metastases of breast cancer in cell lines. The inhibitor of KappaB or IkappaB phosphorylation is prevented by curcumin which targets it to reduce the nuclear translocation i.e. exit of RNA from the nuclear of NFκB. In the long run, this means that the metastatic, proliferating effect of NFκB is prevented which is another reason why curcumin is endorsed for cancer chemoprevention (Zou et al., 2020).

C. *Curcumin & Estrogen Receptors*

Lastly, the effect curcumin will have on HR+ breast cancers, particularly those that have developed resistance is important. This can be a de-novo or acquired resistance resulting in the relapse of a patient into cancer. One study investigated the effect of three phytocompounds that would mimic estrogen - or 'phytoestrogen' - to be hypothetically used in therapies by binding to receptors while imitating estrogen. Comparing enterolactone, quercetin and curcumin in breast cancer cell lines led to the conclusion that curcumin has an effect on gene transcription showing an estrogenic effect that could be useful when using endocrine treatment for HR+ breast cancers. Although the effect of 17-beta-estradiol, which was the control in the experiment, on the cell lines was more significant than that of the 'phytoestrogens', the results demonstrate the ability of curcumin as a therapeutic in the treatment. Since the 'phytoestrogens' stimulated the transcription of the gene, albeit not to the same extent as endogenous estrogen does, they show estrogen-mimicking abilities and could be used in endocrine therapy once resistance develops (Bachmeier et al., 2010).

IV. Proposed Research: Comparative Study between Rapamycin and Curcumin on Invasive Breast Cancer

i. *Curcumin & Introduction to Rapamycin*

Curcumin has been shown to display anticancer properties across various tumor types and cell lines. Studies in various models have demonstrated its inhibitory activity of multiple mechanisms of carcinogenesis. In order to assess the efficacy of curcumin as a substitute for rapamycin and its analogues, it would be necessary to investigate its effectiveness as an inhibitor in comparison to Rapamycin in in-vivo studies.

ii. *Rapamycin*

Rapamycin, also called Sirolimus, belongs to the macrolide class of drugs that are used for immunosuppressive purposes and is commonly used in kidney transplants to prevent the rejection of the transplanted kidney. Busca et al., 1996; Grewe et al., 1999 showed its ability to inhibit various cancer cell lines in human tissue culture medium and xenograft models, namely B16 melanoma, P388 leukaemia and Panc-1 pancreatic carcinomas. This has allowed Rapamycin to be used in a wide variety of cancer therapies /9Findley et al., 2007).

Rapamycin's inhibitory mechanism involving the signalling of mTORC1 is an allosteric mechanism. Once rapamycin interacts with receptor FK-506 binding protein 12 (FKBP-12) it causes it to bind to the FKBP12 rapamycin binding domain in mTOR. This has the result of dissociating the Raptor in the mTORC1 complex leading to its inhibition. mTORC1's interaction with 4E-BP1 and S6K1 (mentioned above) allows for the translation of RNA, therefore, its inhibition (by rapamycin) leads to the hypo-phosphorylation of 4E-BP1. As a consequence, eIF4E binds to 4E- BP1 and the eIF4F initiation mechanism is prevented from initiating cap-dependent translation of mRNA. In this way, Rapamycin prevents protein synthesis and thus, cell proliferation.

When administered at high doses, rapamycin inhibits cell division (through the mechanism above) which has the effect of decreasing the red blood cells, platelets or leukocytes in circulation causing thrombocytopenia, anaemia and leukopenia as a result. Apart from this, stomatitis and myositis may also be side effects of rapamycin when used chronically. In rare cases, rapamycin can cause noninfectious interstitial pneumonitis in patients. However, typically its effects are asthenia (fatigue), fever and increasing susceptibility to bacterial infections (due to its neutrophil function) (Blagosklonny et al., 2019).

iii. *Proposed Methodology*

The experiment procedure was conducted in a biohazard safe laboratory. The proposed experiment will test the effects of rapamycin and curcumin, separately on the models by measuring the cytotoxicity effect on the cells as well as tumor inhibition through multiple parameters. The experiment will involve a subtype of the hormone-dependent subtype of breast cancer.

When determining the model for the experiment, several factors needed to be addressed. Tree shrews, mice, zebrafish could all be potential models because of a similar breast cancer mechanism and genetic similarity they share with humans. Mice, however, seemed to be the best alternative

because of their anatomical, physiological and genetic similarity to humans, their ability to reproduce, in response to cancer along with the variety of strains of mice available. That being said, the mouse model is not without drawbacks. Mice can tolerate higher concentrations of drugs and thus be administered higher doses of drugs during experimentation, which may lead to human clinical trials failing since humans cannot reach the same level of blood concentration. (Kim & Baek., 2010)

Using spontaneous breast cancer models in mice, initially at least, seemed most appropriate since the animals aren't artificially treated and are exposed to carcinogens, they resemble a similar etiology to humans. However, the frequency and incidence of such tumors are different according to the strain. This coupled with the relatively long experimental periods, non-synchronisation, low incidence rate and a higher latency made me rule it out. (Zeng et al., 2020). Similarly, induced breast cancer models which involve the treatment of animals with chemical, or biological carcinogens artificially by injecting them, intaking them orally or through whole-body treatment were unreliable because of pathological differences and should rather be used to study prevention & etiology. (Mollard et al., 2011). Instead, I propose to use transplanted models. Despite it being technically challenging, low costs, short cycles, high tumour incidence and less variation make it a logical choice. (Russo & Russo et al., 1996)

Transplanted models transplant induced or spontaneous tumorous cells into a model organism. My experiment will require a xenograft model for breast cancer cells in mice. (DeRose et al., 2011). Xenograft models could include the transplant of mice tumour cells in immunodeficient mice (to see the full effect of the drug without competition from the immune system), or human tumour cells in immunodeficient mice. My experiment makes use of the latter technique.

Cell-derived tumours (CDX) are transplanted into immunodeficient mice that lack T-cells (nude mice) or T & B-cells (NOD-SCID mice). CDXs' have the disadvantage of having slightly different pathologies, gene expressions and microenvironments which is why I would propose to use PDX (Patient-derived xenograft) models that utilise human tumor specimens and aren't cultured in-vitro. PDX models are closer to human patients in pathological characteristics, genetic aberrations and drug response. Micro-environments in immunodeficient mice cannot mimic the micro-environment in human tumours making immunotherapy research more challenging, which is why humanised PDX(Hu-PDX) model can be used for this research. This method involves injecting the mice intravenously with PDMCs or CD34+ HSCs before tumour transplantation. (Meraz et al., 2019) (Zeng et al., 2020)

For this experiment, the BT20 human breast cancer cell line (subtype: HER2) was subcutaneously injected in 25 nude mice (strain) after the mice were treated with PDMCs, in order to develop IDC in the mice. The number of cells used in inoculation was 6.25 x 106. Transplantation sites can be orthotopic or ectopic with subcutaneous being a type of the latter. (Mollard et al., 2011). The latency period is approximately 3 weeks for this kind of model (Zeng et al., 2020).

After a period of three weeks has passed, Rapamycin and Curcumin can be added in doses to see the effect on cell growth. The mice can be divided into 3 distinct groups: the control, those who will be treated with rapamycin, and those who will be given curcumin. The two drug therapies will be administered at different concentrations, therefore, the curcumin and rapamycin groups should be further divided into 2 subgroups - high-dose and low-dose. The concentrations for any 2 comparable groups will be of equal concentration to analyse the effect of the drugs in determining which is more potent in destroying or limiting the tumorous growth. I propose the total equivalent low-dose (inhibits S6K1 pathway) concentration that should be administered to the curcumin and rapamycin groups should be 1.5 mg/kg/day (± 0.5) (Guba et al.) and high-dose (inhibits both S6K1 and 4EBP1 pathway) total equivalent concentration should be 3.6 g/day for a time-period of 4 months or more (Gupta et al., 2012).

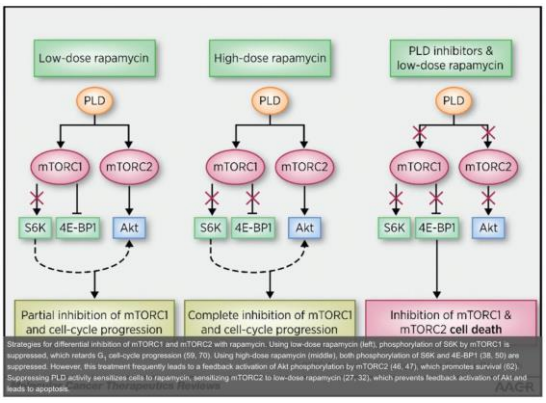

Fig 1.2: Effect of low and high dose Rapamycin on mTOR pathways (Mukhopadhyay et al.,)

The influence of rapamycin and curcumin on the tumour through mTOR, AKT and protein expression can be detected by Western Blotting. The drugs would induce apoptosis and the subsequent cell viability could be detected using the MTS Assay, TUNEL Assay and LDH Assay. Bioluminescence biomarkers and CEA tumour markers can also be used to track the cancer growth.

Other than this, monitoring tumour size through X-rays and MRIs can also be done to see the growth and change at a macro level and monitor the change in the tumour diameter and height. The effect of curcumin and rapamycin treated groups can be compared to that of the control, where the tumour was allowed to progress, and to one another to determine which of the two is more effective.

iv. *Hypothesis, Results & Discussion*

Over the course of the treatment, the effect of the different inhibitors will be assessed by their performance determined by the assays listed that are essentially methods used to determine cell cytotoxicity. Cytotoxicity is the destruction of living cells due to chemicals or mediator cells unable to carry out their function. These conditions are very common in cancer, and various methods can be used to track the cancer this way. Cytotoxic compounds can induce healthy cells to

undergo apoptosis or necrosis. Formazan dyes, protease biomarkers and ATP concentration can all be signs to determine cytotoxicity. The dyes are chromogenic products that are reduced from tetrazolium salts by dehydrogenases (released during necrosis/apoptosis) like LDH (lactate dehydrogenase). Tetrazolium salt examples can be MTS or MTT.

Dying cells have cellular membranes that are in the process of breaking down, allowing cytoplasmic material to be released and extracellular substances (like fluorescent dyes) to enter. The LDH assay works on this premise. These processes are vital otherwise and have specific hallmarks that are identified and used when monitoring diseases. In pathological conditions, these processes (apoptosis, protein degradation) either speed up or slow down and differ from the normal condition greatly. Tracking cancer depends on this alteration to happen, specifically apoptosis, necrosis and autophagy.

Similarly, during apoptosis, chromatin condensation, DNA degradation and cell shrinkage occurs. The TUNEL Assay (Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling) identifies and 'labels' the DNA fragments using fluorophores that attach to the 3' - hydroxyl open ends that are formed to indicate the occurrence of apoptosis.

Regardless of the parameters being used, a consistent procedure must be established. Variation in cells per well during an assay or equilibration period might affect the results of the assay and their maintenance as well as handling must be homogenised for consistency and accuracy in the results.

Predicted results can be calculated after a period of at least 4 months wherein the mice will be kept in controlled conditions. Table 1 combines a few hypothesised observations. It indicates the size of the tumour (initially and after treatment), measured using x-rays, CT scans or MRIs and the results of the MTS Assay for all groups (curcumin, rapamycin and the control).

Table 1: Predicted results for mice groups.

Group	Mean Initial Tumour Diameter (cm)	Mean Tumour Diameter after Treatment (cm)	MTS Assay Results (cell viability %)
Control group	$0.5 < x < 3$	$2 < x < 6$	100
Low-Dose Curcumin Group	$0.5 < x < 3$	$0.75 < x < 4$	90
High-Dose Curcumin Group	$0.5 < x < 3$	$0.3 < x < 3$	80
Low-Dose Rapamycin Group	$0.5 < x < 3$	$0.25 < x < 3$	80
High-Dose Rapamycin Group	$0.5 < x < 3$	$1 < x < 5$	60

For the MTS Assay, the number of cells placed in the 'well' (96 well-plate) is important because it differs from cell line to cell line. The time at which the drug, which is being investigated, is added also makes a difference; it must be added during the growth stage and not when cells have reached confluence. Once the cells are added to a solution (containing growth solution and treated with a carrier – 0.05% ethanol) followed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4- sulfophenyl)-2H-tetrazolium (MTS) assay using the Promega kit. Time must be given for the cells to soak up in the reagent, after which an absorbance plate set at a particular wavelength (490 nm) must be read. The time at which the plate can be read is also very specific for reliable results. The efficacy of the drug being tested is then calculated, by comparing the ratios of absorbance between the control and wells that had cells with the drug. For the MTS Assay, the lower the value, the more potent the drug is (Natarajan et al.).

In my experiment I hypothesise that the value of MTS Assay (as a relative percentage) in terms of cell viability will be lowest in the group treated with high-dose of rapamycin, followed by high-dose curcumin. The difference in cytotoxicity between low-dose and high-dose curcumin won't be statistically significant since curcumin, in general, doesn't have much of an adverse effect on cells. The low-dose rapamycin will be less fatal in its effects on the cells. In terms of cell toxicity, curcumin will not be as damaging as rapamycin on the tissues of the mice. As a result, the relative absorbance of the rapamycin treated groups will be lower indicating a greater cytotoxicity.

For the results of the X-Ray/CT scan, as seen in the table above, I predict that the tumour diameter measured through these scans will show the smallest tumour diameter, and perhaps even shrinkage of the tumour, in the low-dose rapamycin group. This may be attributed to the fact that rapamycin has been an important drug since its discovery in the 1990s, which has allowed it to be developed into an effective therapeutic. However, curcumin has a lot of potential. Not only is it less cytotoxic, as suggested by the MTS assay, but it is also quite effective when administered and will slow down the tumour growth, maybe even reduce it at a higher dose. The lower-dose will not be as effective in reducing tumour growth, but it will slow it down, nonetheless, and will have the least toxic effect. The high-dose rapamycin group will have a strong reaction to the tumour. I hypothesise that while the high-dose may be effective at removing the tumour, because it is also the most toxic at such a micro-environment, there will be counterintuitive effect on the cancerous growth i.e. its cytotoxicity may lead to the unwanted effect of an acceleration or an ineffective inhibition of the cell proliferation.

Low-dose rapamycin therapy, therefore, seems like the best drugs in our arsenal against breast cancer. However, development of curcumin as a therapeutic will let its targeting mechanism and low cytotoxic effect on cells to be used as an anticancer product (Gupta et al., 2013).

V. Conclusion & Future Studies

This review detailed the pathway outlined in multiple papers that breast cancers follow. Curcumin was identified as a potential drug due to its inhibitory effect on the pathway. The experiment proposed, included a comparative study between rapamycin and curcumin as drugs that inhibit the mTOR pathway. Results suggested that while rapamycin was more effective, as it had a greater inhibitory effect on the tumour at a lower concentration, it was also more cytotoxic in comparison to curcumin which even at a higher concentration had a lower adverse effect on cells.

This suggests there might be a trade-off between effectivity and cytotoxic effect in drugs. However, the success of curcumin as a plant-based therapeutic cannot be diminished. Moreover, rapamycin is a developed drug having been put to use for decades, whereas curcumin isn't. This might play a role in shaping the results as well since rapamycin is more 'familiar'.

Future studies could entail conducting a similar comparative study on organisms that share a closer genetic history to humans, like primates, to better understand the impact the drugs will have before moving to clinical trials for the same. Since curcumin's prospects as an inhibitor have been demonstrated in multiple cancers, future research could focus on developing its effectiveness and target potential. Studies could also be done to see whether a combined therapy between curcumin and rapamycin may be most effective and less cytotoxic when treating breast cancer specifically. Lastly, seeing curcumin's effect as a preventive substance for cancer can also be demonstrated through population studies that involve administering it regularly in a group, and seeing if it affects the incidence of cancer.

Curcumin isn't the only plant-based therapeutic that has immense scope as an anti-cancer drug. Many such compounds exist, waiting to be discovered.

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