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**Research Paper**

# Exploring Osimertinib-Abemaciclib Interactions: BioAssay-Based Analysis and Solid Dosage-form development

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## ABSTRACT

Lung cancer remains a leading cause of cancer-related mortality, and the emergence of resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) such as osimertinib continues to limit long-term therapeutic success in EGFR-mutated non-small cell lung cancer (NSCLC). Co-targeting EGFR signaling and cell-cycle regulation via cyclin-dependent kinase 4/6 (CDK4/6) inhibition represents a rational approach to enhance tumor control and delay resistance.The present work aimed to evaluate the cytotoxic and antiproliferative potential of a combination of osimertinib and abemaciclib using onion root tip and brine shrimp lethality bioassays, and to formulate and evaluate an immediate-release–immediate-release (IR–IR) bilayer tablet for their simultaneous delivery in lung cancer therapy. Cytotoxicity was assessed by brine shrimp lethality assay using methotrexate as a standard, while antiproliferative activity was determined through Allium cepa root growth inhibition and mitotic index (MI) reduction at 100 µg/mL. A bilayer tablet containing an osimertinib layer and an abemaciclib layer was prepared by direct compression using suitable diluents, binders, superdisintegrants, glidants and lubricants, and evaluated for weight variation, hardness, friability, thickness, disintegration time and in vitro dissolution. Individually, osimertinib and abemaciclib displayed moderate lethality in the brine shrimp assay, whereas the combination produced 80% mortality, higher than either single drug and slightly lower than methotrexate, indicating a possible synergistic or additive cytotoxic effect. In the Allium cepa assay, the combination markedly reduced root length (2.4 mm) and MI (2.2%) compared with individual treatments, while remaining marginally less inhibitory than methotrexate, confirming enhanced antiproliferative activity of the dual regimen. The optimized bilayer tablet met pharmacopoeial limits, showing hardness of approximately 4.53 kg/cm², friability 0.293%, thickness 5.466 mm, disintegration time 14.26 minutes, and cumulative drug release of 85.78% for osimertinib and 67.05% for abemaciclib, with release best described by the Korsmeyer–

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Peppas model. Overall, the study demonstrates that the combination of osimertinib and abemaciclib exhibits improved preliminary cytotoxic and antiproliferative effects over monotherapy and can be successfully incorporated into an IR–IR bilayer tablet, supporting further evaluation in advanced in vitro and in vivo lung cancer models

## INTRODUCTION

### 1.1 Introduction of disease

### 1.1.1 Lung Cancer

It is a group of disease characterized by the uncontrolled growth and division of abnormal cells, which have the potential to invade or spread to other parts of the body. This growth can form masses known as tumors although not all cancers from solid tumors.A number of benign and malignant tumors occur in the lungs but the primary lung cancer, commonly termed bronchogenic carcinoma, is the most common (95%).

Bronchogenic carcinoma

The term bronchogenic carcinoma is commonly used for cancer of the lungs which includes carcinomas arising from the respiratory epithelium lining the bronchi, bronchioles and alveoli.

### 1.1.2 WHO Classification of Lung Tumours.[1]

I. EPITHELIAL TUMOURS

A. Benign
1. Papilloma
2. Adenoma
B. Dysplasia and carcinoma in situ
C. Malignant

Bronchogenic carcinoma

1. Squamous cell (epidermoid) carcinoma
2. Small cell carcinoma
i) Oat cell carcinoma
ii) Intermediate cell carcinoma
iii) Combined oat cell carcinoma

3. Adenocarcinoma
i) Acinar adenocarcinoma
ii) Papillary adenocarcinoma
iii) Bronchiolo-alveolar carcinoma
iv) Solid carcinoma with mucus formation
4. Large cell carcinoma
5. Adeno-squamous carcinoma

Other carcinomas
1. Pulmonary neuroendocrine tumour (carcinoid tumour)
2. Bronchial gland carcinomas
i) Adenoid cystic carcinoma
ii) Mucoepidermoid carcinoma

II. SOFT TISSUE TUMOURS
(Fibroma, fibrosarcoma; leiomyoma, leiomyosarcoma; lipoma, chondroma, haemangioma, lymphangioma, granular cell myoblastoma)

III. PLEURAL TUMOURS
A. Benign mesothelioma
B. Malignant mesothelioma
IV. MISCELLANEOUS TUMOURS
1. Carcinosarcoma
2. Pulmonary blastoma
3. Malignant melanoma
4. Malignant lymphoma
V. SECONDARY TUMOURS

**1.1.3 INCIDENCE AND CLASSIFICATION**. Lung cancer is the most common primary malignant tumour in men and accounts for nearly 30% of all cancer deaths in both sexes in developing countries. Currently, the incidence of lung cancer in females in the United States has already exceeded breast cancer as a cause of death in women. Cancer of the lung is a disease of

middle and late life with peak incidence in 55-65years of age, after which there is gradual fall in its incidence of late, there has been slight decline in lung cancer deaths in males due to smoking cessation efforts which started in the West 4 decades back and has started yielding results. However, worldwide the scene on its incidence and prognosis are quite grim; data from International Agency for Research on Cancer estimate that worldwide by the year2030 there would be about 10 million deaths per year from lung cancer.[1]

**1.1.4 ETIOLOGY**. The high incidence of lung cancer is associated with a number of etiologic factors, most important of which is cigarette smoking.[1]

**1. Smoking**. The most important factor for high incidence of all forms of bronchogenic carcinoma is tobacco smoking. About 80% of the lung cancer occurs in active smokers. A number of evidences support the positive relationship of lung cancer with tobacco smoking.

**i) Total dose:** There is a direct statistical correlation between death rate from lung cancer and the total amount of cigarettes smoked e.g. an average regular smoker has 10 times greater risk of developing lung cancer than a non-smoker. The risk of smokers of more than 2 packs (40 cigarettes) per day for 20 years is 60-70 times greater than a non-smoker. Cessation of smoking by a regular smoker result in gradual decline in the chances of developing lung cancer.After10 years of abstinence from smoking, the risk declines but never returns to the non-smoker level. Pipe and cigar smokers, though have higher risk than non-smokers but are at lesser risk than cigarette smokers.

**ii) Histologic alterations**: The association of tobacco smoking is strongest for squamous cell carcinoma and small cell carcinoma of the lung. More than 90% of smokers have sequential epithelial changes in the respiratory tract in the form of squamous metaplasia, dysplasia and carcinoma in situ.

**iii) Mechanism**: How tobacco smoking causes lung cancer is not quite clear. However, following facts have been observed:Analysis of the tar from cigarette smoke has revealed a number of known carcinogens (e.g. polycyclic aromatic hydrocarbons, nitrosamines) and tumour promoters (e.g. phenol derivatives). In experimental animal studies, it has been possible to induce cancer by skin painting experiments with smoke-tar.[1]

2. Other factors.

Although smoking is the dominant etiologic factor in lung cancer, 15% cases of lung cancer occur in non-smokers, more so in women probably related to hormonal factors. A few other factors implicated in lung cancer are as follows:

i) Atmospheric pollution. There is increased risk of developing bronchogenic carcinoma in non-smokers living in industrialised and smoky cities than in the less polluted rural areas. It is possible that specific industrial pollutants may be at fault as evidenced by high rates for lung cancer in people living in the neighbourhood of petrochemical industries.
ii) Occupational causes. There are a number of well-established occupational causes of lung cancer. These include workers exposed to asbestos, radiation of all types, bis-ethers, nickel, beryllium, arsenic, metallic iron and iron-oxide. Some industrial carcinogens and cigarette smoking have co-carcinogenic effect, particularly in uranium mines and asbestos workers.

iii) Dietary factors. Susceptibility to respiratory cancers is increased in vitamin A deficiency. Smokers with low vitamin A intake have a greater risk of lung cancer than those with vitamin A-rich diet. The incidence of lung cancer is inversely related to socioeconomic level reflecting their dietary pattern.
iv) Chronic scarring. Peripheral adenocarcinomas occur more frequently in areas of chronic scarring caused by chronic inflammatory changes, old tuberculosis, asbestosis, chronic interstitial fibrosis, old infarcts and in scleroderma.

**1.1.5 MOLECULAR PATHOGENESIS.** Molecular studies have revealed that there are several genetic alterations in cancer stem cells which produce clones of malignant cells to form tumour mass. Following genetic changes have been found:

1. Activation of growth-promoting oncogenes: Mutation in K-RAS oncogene has been seen as the dominant change in lung cancer. Besides, there is mutation in tyrosine kinase domain of EGFR oncogene in cases of adenocarcinoma lung in non-smokers. Other mutations include BRAF, PIK3CA and MYC family, and over expression of bcl-2 and other anti apoptotic proteins.[2]

2. Inactivation of tumour-suppressor genes. Inactivation of tumour suppressor genes has been found as another molecular mechanism in lung cancer. Many tumour suppressor genes have been found on chromosome 3p in lung cancer cases. These include inactivation of p53 and Rb gene. besides, some tumour-acquired promoter genes have been identified in lung cancer e.g. p16, RASSFIA etc, which cause loss of normal function of growth-regulatory tumour suppressor genes.[2]

3. Autocrine growth factors. Studies have shown that lung cancer is a multistep process—initiator carcinogen causing mutation, followed by action of tumour promoters. Nicotine acts as both initiator as well as promoter carcinogen. derivatives of nicotine in smoke unmask and expresses nicotine acetylcholine receptors which activate the signalling pathway in tumour, blocking the apoptosis. Promoters also include several hormones which are elaborated by lung cancer by autocrine pathway as part of paraneoplastic syndrome.[2]

4. Inherited predisposition. Although not common, but there are a few examples of inheritance of lung cancer as under:
i) Patients of Li-Fraumeni syndrome who inherit p53mutation may develop lung cancer.
ii) Clinical cases of retinoblastoma having mutation in Rb gene are predisposed to develop lung cancer if they live up to adulthood.
iii) First-degree relatives of lung cancer patients have a 2-3fold higher risk of developing lung cancer in their lifetime.
iv) Mutations of cytochrome P450 system have been identified in lung cancer patients; P450 metabolises chemical carcinogen in tobacco smoke.

5. Molecular targets for therapy and survival prediction.
Knowledge on the insight into molecular biology and pathogenesis of lung cancer has applications in discovering newer molecules for targeted therapy, predicting response to treatment and survival:
i) EGFR mutations and NSCC therapy: It has been reported that 70% cases of NSCC have over expression of EGFR protein or amplification of the EGFR gene. EGFR belonging to ERBB (HER) family of proto oncogenes through mutation in its tyrosine kinase (TK) domain plays a role in both extracellular and intracellular signalling resulting in tumour cell proliferation, metastasis and anti apoptotic action. Targeted molecular therapy

against these mutations in EGFR include EGFR-TK inhibitor oral therapy.[3]

ii) VEGF and monoclonal therapy: Although not mutated, VEGF is excessively produced in lung cancer and contributes to tumour angiogenesis. Monoclonal antibody therapy against EGFR in conjunction with chemotherapy has been used for curtailing tumour angiogenesis in lung cancer.

iii) Molecular signature gene for prediction: Recent proteomic studies at research level have shown that each patient has unique protein pattern in the serum (i.e. molecular signatures) which may be used for early diagnosis, predict drug resistance, response to treatment and survival, but these are yet to be applied in clinical settings.[3]

### 1.1.6 EPIDERMIOLOGY

In 2020, according to the research, approximately 2.21 million cases were detected, and 1.8 million mortalities were caused by lungs cancer (Sharma 2022). The report presented by the World Health Organization (WHO) in 2020, shows that lungs cancer is the deadliest among all kinds of cancers, that is said according to the death rate that is calculated as 1.80 million (World Health Organization 2022). Figure shows the details of extinction because of cancer in 2020 according to WHO Lungs cancer is one of those diseases in which early-stage diagnosis and disease management play a crucial role in proper treatment.[7]

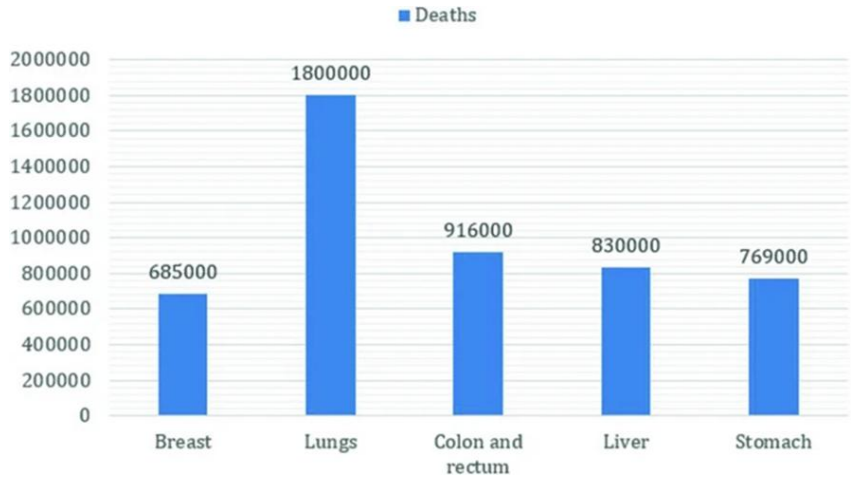



**Figure 1: The details of extinction because of cancer in 2020 according to WHO**

A schematic representation which summarizes all genetic mutations, the frequency of their detection, what metabolic pathway they are involved in, and what subtype of lung cancer they are typical for, is shown in Table 1.

**Table 1. Summary of lung cancer genetic mutations with the frequency of their detection, the metabolic pathway in which they are involved, and for which lung cancer subtype they are typical.[8]**

| Gene | Frequency [%] | Metabolic Pathways | Type of Lung Cancer |
|---|---|---|---|
| EGFR | 30–40 | PI3K/AKT, RAS/RAF/MEK/ERK | Adenocarcinoma Squamous cell |

| Gene | Frequency [%] | Metabolic Pathways | Type of Lung Cancer |
|---|---|---|---|
| | | | carcinomas Large cell carcinoma |
| KRAS | 20–30 | MAPK, PI3K-AKT | Adenocarcinoma Large cell carcinoma |
| ALK | 3–7 | STAT3, mTOR, PI3K, Ras and MEK. | Adenocarcinoma Squamous cell carcinomas Large cell carcinoma |
| BRAF | 1–3 | MAPK/ERK | Adenocarcinoma Large cell carcinoma |
| ROS1 | 1–2 | JAK/STAT, PI3K/AKT, and MAPK/ERK | Adenocarcinoma Squamous cell carcinomas Large cell carcinoma |
| PD-L1 | 20–30 | JAK/STAT, PI3K/Akt/mTOR, MAPK/ERK, NF-κB e TGF-β | Adenocarcinoma Squamous cell carcinomas |

| Gene | Frequency [%] | Metabolic Pathways | Type of Lung Cancer |
|---|---|---|---|
| MET | 3–4<br>1–6<br>0.2–0.3 | PI3K/AKT and MAPK/ERK | Adenocarcinoma Large cell carcinoma |
| RET | 1–2 | RAS/MAPK/ERK, PI3K/AKT | Adenocarcinoma Large cell carcinoma |
| NTRK | 0.1–0.2 | PIK3/PLCγ/MAPK | Adenocarcinoma Large cell carcinoma |
| PIK3CA | 2–4 | PI3K/AKT/mTOR | Adenocarcinoma Squamous cell carcinomas Large cell carcinoma |
| HER2 | 1–3 | MAPK, PI3K/AKT, protein kinase C and STAT, | Adenocarcinoma Squamous cell carcinomas Large cell carcinoma |
| STK11 | 20–30 | Dysfunction of the AMPK pathway | Adenocarcinoma Squamous cell carcinomas |

| Gene | Frequency [%] | Metabolic Pathways | Type of Lung Cancer |
|---|---|---|---|
| | | | Large cell carcinoma |

## 1.2 Introduction of drug

### 1.2.1 Osimertinib

Osimertinib is an oral, third-generation, irreversible epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) that selectively inhibits both EGFR-TKI–sensitizing and EGFR T790M resistance mutations. We compared osimertinib with standard EGFR-TKIs in patients with previously untreated, EGFR mutation–positive advanced non–small-cell lung cancer (NSCLC).[9,19,20]The discovery of epidermal growth factor receptor (EGFR) mutations and subsequent demonstration of the efficacy of genotype-directed therapies with EGFR tyrosine kinase inhibitors (TKIs) marked the advent of the era of precision medicine for non-small-cell lung cancer (NSCLC). First- and second-generation EGFR TKIs, including erlotinib, gefitinib and afatinib, have consistently shown superior efficacy and better toxicity compared with first-line platinum-based chemotherapy and currently represent the standard of care for EGFR-mutated advanced NSCLC patients. However, tumors invariably develop acquired resistance to EGFR TKIs, thereby limiting the long-term efficacy of these agents. The T790M mutation in exon 20 of the EGFR gene has been identified as the most common mechanism of acquired resistance. Osimertinib is a third-generation TKI designed to target both EGFR TKI-sensitizing mutations and T790M, while sparing wild-type EGFR. Based on its pronounced clinical activity and good safety profile demonstrated in early Phase I and II trials, osimertinib received first approval in 2015 by the US FDA and in early 2016 by European Medicines Agency for the treatment of EGFR T790M mutation-positive NSCLC patients in progression after EGFR TKI therapy. Recent results from the Phase III AURA3 trial demonstrated the superiority of osimertinib over standard platinum-based doublet chemotherapy for treatment of patients with advanced EGFR T790M mutation-positive NSCLC with disease progression following first-line EGFR TKI therapy, thus definitively establishing this third-generation TKI as the standard of care in this setting. Herein, we review preclinical findings and clinical data from Phase I–III trials of osimertinib, including its efficacy in patients with central nervous system metastases. We further discuss currently available methods used to analyze T790M mutation status and the main mechanisms of resistance to osimertinib. Finally, we provide an outlook on ongoing trials with osimertinib and novel therapeutic combinations that might continue to improve the clinical outcome of EGFR-mutated NSCLC patients.[10,17,18]

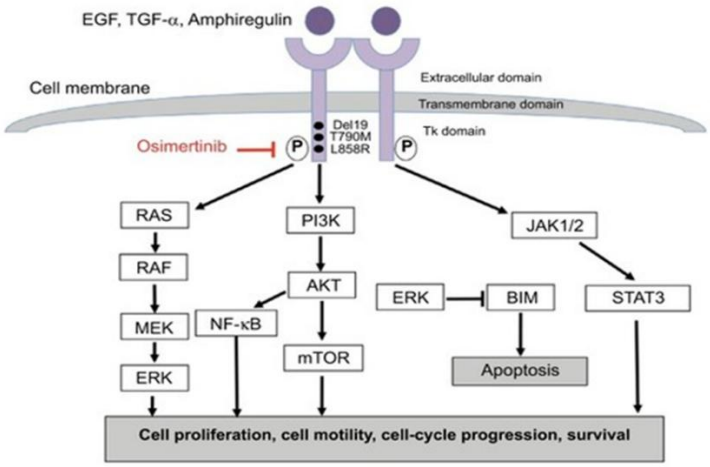



**Figure 2: EGFR pathway and mechanism of action of osimertinib**

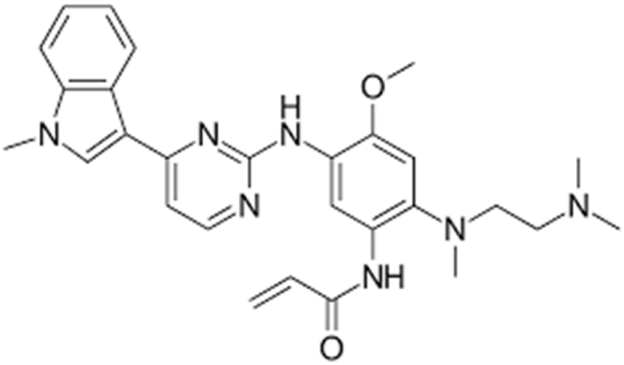



**Figure 3: Structure of Osimertinib**

Drug Profile Table no.2

| Parameter | Details |
|---|---|
| Generic name | Osimertinib; available as osimertinib mesylate. |
| Brand name | Tagrisso. |
| Drug class | Third-generation, irreversible EGFR tyrosine kinase inhibitor. |
| Dosage form | Film-coated oral tablets. |
| Available strengths | 40 mg and 80 mg tablets. |
| Route of administration | Oral. |
| Mechanism of action | Selectively and irreversibly inhibits mutant EGFR, including exon 19 deletion, L858R, and T790M mutations, with lower activity against wild-type EGFR. |
| Main indications | EGFR mutation-positive NSCLC, including metastatic disease, adjuvant treatment after resection, and certain unresectable or advanced settings depending on labeling. |
| Standard adult dose | 80 mg once daily, with or without food. |
| Absorption | Median time to maximum concentration is about 6 hours; steady state is reached in about 15 days. |

### 1.2.2 Mechanisms of Resistance

Osimertinib is a mono-anilino-pyrimidine compound that corresponds to the molecular formula C28H33N7O2 and possesses a molecular weight of 596 g/mol. Osimertinib irreversibly binds to mutant EGFR via its acrylamide group that forms a covalent bond with the Cys797 residue located in the ATP-binding site of mutant EGFR.The mechanisms of resistance to osimertinib are generally divided into two categories: (a) those occurring as a genetic alteration at the EGFR gene (on-target mechanisms) and (b) those involving different genetic alterations and activation of other pathways (off-target mechanisms). Evidence from the clinical trials and real-world registries has revealed differential mechanisms when osimertinib is given as first-line or second-line treatment, underlying the discrepancies in the selection pressure and clonal evolution of tumor cells. The relative frequencies of resistance mechanisms that have been reported from clinical trials and representative real-world cohorts are summarized in Table 3.[11,14,15,16]

**Table 3. Relative frequencies of resistance mechanisms that have been reported from clinical trials and representative real-world cohorts.**

| Author (Year) | Number of Patients | Line of Therapy | EGFR-Dependent Mechanisms (On-Target) | EGFR-Independent Mechanisms (Off-Target) |
|---|---|---|---|---|
| Papadimitrakopoulou et al. (2018)[12] | 73 | 2nd line | T790M loss (49%)<br>C797 mutations (15%; 10 patients with C797S, 1 patient with C797G) | MET amplification (19%)<br>HER2 amplification (5%)<br>PIK3CA amplification (4%)<br>BRAFmut (V600E) (4%)<br>KRAS mutation (1%)<br>PIK3CA mut (E545K) (1%)<br>FGFR/RET/NTRK fusions (4%) |
| Oxnard et al. (2018) | 41 | 2nd line | T790Mloss (63%)<br>C797S (22%) | SCLC transformation (15%)<br>MET amplification (10%)<br>BRAF mutation (5%)<br>PIK3CA mutation (5%)<br>KRAS mutation (2%)<br>CCDC6-RET fusion (2%)<br>FGFR fusion (2%)<br>BRAF fusion (2%) |
| Ramalingam et al. (2018)[13] | 91 | 1st line | C797S (7%) | MET amplification (15%)<br>HER2 ampl, PIK3CAmut, RAS mut (2–7%) |
| Enrico et al. (2019) | 31 | Any | C797S (29%)<br>L817Q (6%)<br>EGFR amplification (3%) | Oncogenic fusions (RET, MET, BRAF, ALK, FGFR3, and NTRK1) 16%<br>BRAF mutation (V600E) 6% (co-existing with C797S)<br>MET amplification (3%)<br>HER2 amplification (3%) |

| Author (Year) | Number of Patients | Line of Therapy | EGFR-Dependent Mechanisms (On-Target) | EGFR-Independent Mechanisms (Off-Target) |
|---|---|---|---|---|
| | | | | KRAS mutation (3%)<br>PIK3CA mutation (3%) |
| Mehlman et al. (2019) | 73 | Any | T790M loss (68%)<br>C797S (12%) | MET amplification (11%)<br>Histologic transformation (9% of patients who underwent a tissue biopsy)<br>HER2 amplification (3%)<br>BRAF mutation (V600E) (1%) |
| Lee et al. (2021) | 34 | 2nd line | T790Mloss (65%)<br>C797S (12%) | SCLC transformation (9%)<br>Squamous cell carcinoma transformation (5%)<br>MET amplification (15%) |
| Akli et al. (2022)[21] | 27 | 1st line | C797S (11%) | MET amplification (15%)<br>HER2 amplification (4%)<br>SCLC transformation<br>RET fusion (4%) |
| Nie et al. (2022) | 21 | 1st line | C797S (24%)<br>L718Q (5%)<br>EGFR amplification (1%) | MET amplification (29%)<br>HER2 amplification (10%)<br>PTEN loss (5%)<br>PIK3CA mutation (5%) |

### 1.3 Abemaciclib

Abemaciclib (LY2835219) is a small-molecule inhibitor of CDK4 and CDK6 that is structurally distinct from other dual inhibitors (such as palbociclib and ribociclib) and notably exhibits greater selectivity for CDK4 compared with CDK6. Consistent with its activity against CDK4 and CDK6, abemaciclib inhibits RB phosphorylation and leads to G1 arrest in RB-proficient cell lines. In a colorectal cancer xenograft model used to develop an integrated pharmacokinetic/pharmacodynamic model, abemaciclib can be dosed orally on a continuous schedule to achieve sustained target inhibition and demonstrates not only durable cell-cycle inhibition but also single-agent antitumor activity. Tumor growth inhibition is observed in multiple other human cancer xenograft models, including those derived from non–small cell lung cancer (NSCLC), melanoma, glioblastoma, and mantle cell lymphoma. Abemaciclib distributes across the blood–brain barrier and prolongs survival in an intracranial glioblastoma xenograft model, suggesting potential efficacy against primary and metastatic tumors involving the central nervous system.[22]LY2835219 (abemaciclib) was identified via compound and biochemical screening by scientists at Eli Lilly and Company Research Laboratories and selected for its biological activity and highly selective inhibition of the complexes CDK4/ cyclin D1 (IC50 =2 nmol/L) and CDK6/cyclin D1 (IC50 =10 nmol/L), with no activity against other CDK/cyclin

complexes or cell-cycle-related kinases within the nanomolar ranges, except for inhibition of CDK9 at IC50 at least five times higher (Figure 2). The compound was shown to act as a competitive inhibitor of the ATP-binding domain of the CDK4 and CDK6 and to be 14 times more potent against CDK4 than against CDK6. In comparison to palbociclib and ribociclib, abemaciclib shows higher selectivity for the complex CDK4/cyclin D1, with IC50 values five times lower than those of the two other compounds[23]

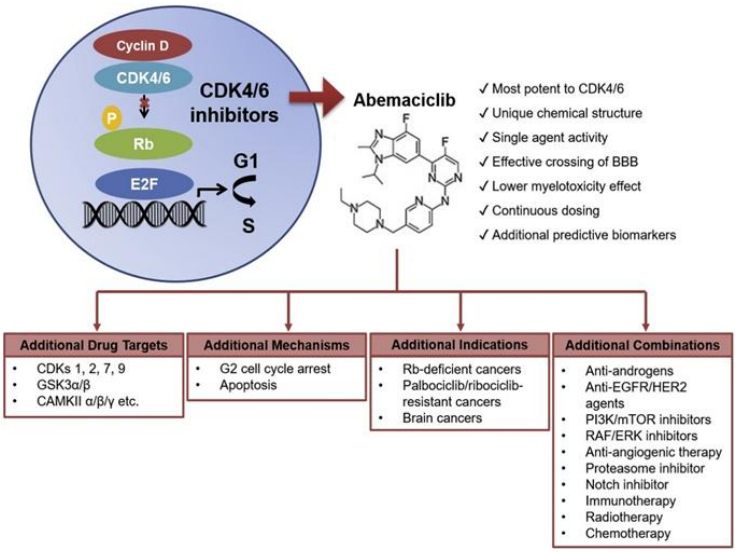



**Figure 4 additional mechanism of abemaciclib**

Over the last decade, advances in molecular translational research have heralded major breakthroughs in the understanding, diagnosis, and management of lung cancer, particularly for the more common (~80%) non–small cell lung cancer (NSCLC). NSCLC is subclassified by histology and driver mutations such as mutated KRAS and activating mutations in the epidermal growth factor receptor (EGFR) tyrosine kinase (TK) domain. The two most common EGFR-TK domain mutations are exon 19 deletions (60%) and L858R missense substitutions resulting in constitutive activation of the receptor without ligand binding. Constitutive activation of receptors or protein kinases stimulates a complex cascade of cross-signaling pathways leading to uncontrolled growth, proliferation, and survival. Successful targeted therapies in NSCLC involve the identification and inhibition of these upregulated pathways by small molecule tyrosine kinase inhibitors (TKI) or receptor monoclonal antibodies. Although EGFR-TKIs have been useful in the treatment of EGFR-mutant NSCLC, most responses have not proved to be durable with many patients progressing after 7 to 12 months. The most frequent mechanism (~50%) is concurrent acquisition of novel mutation in exon 20 of EGFR, encoding for T790M making tumors refractory to the existing EGFR-TKI therapy. Apart from EGFR-TKI, radiotherapy either alone or in combination with chemotherapy, remains the primary modality of treatment for patients with stage III NSCLC. For stage I and II NSCLC, radiotherapy is an alternative curative option to surgery for patients who are medically inoperable or refuse surgery. Overall radiotherapy is an important palliative treatment modality to treat symptoms from the primary or bone or brain metastases and improve patients' quality of life.

Despite these medical interventions, 5-year survival rates of NSCLC patients are less than 5%. Hence, there is an urgent need to target other signaling pathways or design combination therapy that is more effective than first-line single agents while balancing toxicity and costs. Other than the EGFR or MEK/ERK pathway, cyclin D kinase 4/6 (CDK4/6) activity is typically deregulated and overactive in various cancers including NSCLC.[28]CDK4 and CDK6 are cyclin-dependent kinases that control the transition between the G1 and S-phase of the cell cycle. A major target of CDK4 and CDK6 during cell-cycle progression is the retinoblastoma protein (RB). When RB is phosphorylated, its growth-suppressive properties are inactivated. Selective CDK4/6 inhibitors "turn off" these kinases and dephosphorylate RB, resulting in a block of cell-cycle progression in mid-G1 preventing proliferation of cancer cells. Recent studies have identified poor responses to EGFR-TKIs in lung adenocarcinoma patients with the altered CDK4/6-RB pathway. In preclinical mouse models, KRAS-driven lung cancer is highly dependent on CDK4, and genetic or pharmacologic ablation of CDK4 activity has effects on both the establishment and maintenance of these tumors. Palbociclib (PD0332991), a selective CDK4/6 inhibitor, has been shown to sensitize lung cancer cells to EGFR-TKI, gefitinib. In addition, the MEK inhibitor (trametinib) in combination with palbociclib has significant anti-KRAS-mutant NSCLC activity and radiosensitizing effects in preclinical models. Silencing CDK4 in breast cancer cells leads to enhanced radiosensitivity, indicating targeting the CDK4/6-cyclin D axis is a rational approach for enhancing tumor IR response. Currently, there are three highly selective CDK4/6 inhibitors (palbociclib, ribociclib, and abemaciclib) approved by FDA for hormone receptor positive and Her2 negative breast cancer patients. Currently, several clinical trials are underway to evaluate these agents as monotherapy or combination therapy for various solid tumors. In the present study, we investigated the effects of abemaciclib alone and in combination with IR on NSCLC in vitro and in vivo.[24]

### 1.3.1 CDK4 and 6 and Cell Cycle Regulation

Cellular division requires a well-orchestrated transition through the phases of the cell cycle. A family of serine/threonine kinases known as cyclin-dependent kinases (CDKs) plays an important role in regulating cell cycle progression. The catalytic activity of CDKs is controlled by their association with cyclins, which allows CDKs to act upon substrates to trigger coordinated molecular events that drive cellular proliferation. Transient expression of cyclins allows for distinct roles of cyclin-CDK heterodimers during different phases of the cell cycle. CDK4 and CDK6 operate during the G1 to S phase transition, and their enzymatic activities are governed by the D-type cyclins (Cyclin D1, D2, and D3), which are expressed in response to extracellular signals including stimulatory mitogens, estrogen, cytokines, differentiation inducers, cell-cell contacts, and other spatial cues. Once active, the cyclin D-CDK4/6 holoenzyme phosphorylates a wide range of substrates, including the retinoblastoma (Rb) tumor suppressor protein. Rb binds to and represses the E2F group of transcription factors, which control the expression of an array of genes involved in DNA replication, G1 to S phase progression, and mitosis. As a result of phosphorylation of Rb by activated CDK4 and 6-D-cyclin complexes, E2F is released, facilitating the transcription of S phase genes. While Rb may be the most clinically relevant substrate of CDK4 and 6, Anders et al performed a comprehensive screening of CDK4 and CDK6 in vitro substrates in an effort to investigate the full spectrum of

molecules phosphorylated by CDK4 and 6. The analysis revealed a considerable number of proteins which may have potential clinical relevance across many tumor types.

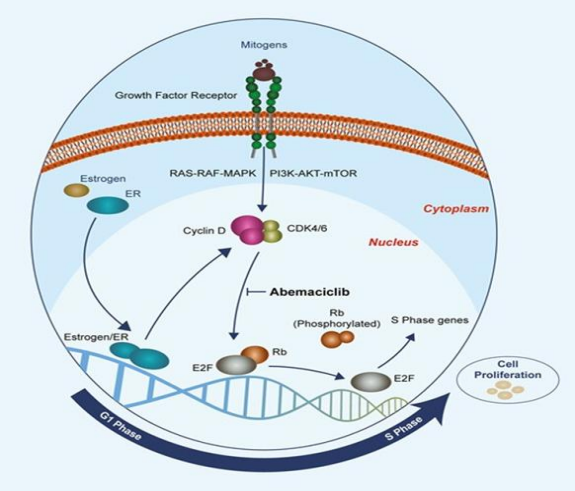



**Figure 5.**

Depiction of the CDK4 and 6 mechanism of action. Upstream signalling promotes the activation of the D-cyclin-CDK4 and 6 complex. This complex phosphorylates Rb, releasing E2F, resulting in the transcription of genes required for transition into S Phase. Abemaciclib inhibits the phosphorylation of Rb, inducing cell cycle arrest. Abbreviations: CDK4/6, cyclin-dependent kinase 4/6; ER, estrogen receptor; E2F, E2 factor; Rb, retinoblastoma; MAPK, mitogen-activated protein kinase; PI3K[25]

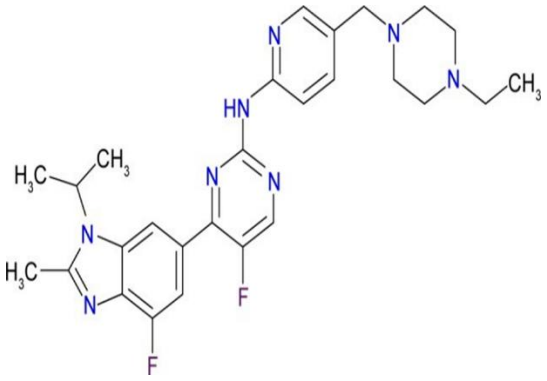


**Figure 6: Stucture of Abemaciclib**

**Drug Profile Table no. 4**

| Parameter | Details |
|---|---|
| Generic name | Abemaciclib. |
| Brand name | Verzenio. |
| Drug class | Antineoplastic agent; selective CDK4 and CDK6 inhibitor. |
| Dosage form | Film-coated oral tablets. |
| Available strengths | 50 mg, 100 mg, 150 mg, and 200 mg tablets. |
| Route of administration | Oral. |
| Mechanism of action | Inhibits CDK4 and CDK6, preventing phosphorylation of retinoblastoma protein and blocking progression from G1 to S phase in the cell cycle. |

| | |
|---|---|
| Main indications | Used in HR-positive, HER2-negative early or advanced breast cancer in combination with endocrine therapy or as monotherapy in selected settings according to product labeling. |
| Standard adult dose | Common doses include 150 mg twice daily with endocrine therapy or 200 mg twice daily as monotherapy, depending on indication. |
| Absorption | Median time to maximum concentration is about 8 hours after oral dosing. |
| Protein binding | Approximately 96% bound to plasma proteins. |
| Metabolism | Primarily metabolized in the liver by CYP3A. |
| Elimination half- life | About 18 hours. |
| Excretion | Eliminated mainly in feces, with a smaller amount in urine. |
| Common adverse effects | Diarrhea, neutropenia, nausea, abdominal pain, fatigue, infections, anemia, leukopenia, decreased appetite, vomiting, and alopecia. |

1.4 Combination effect

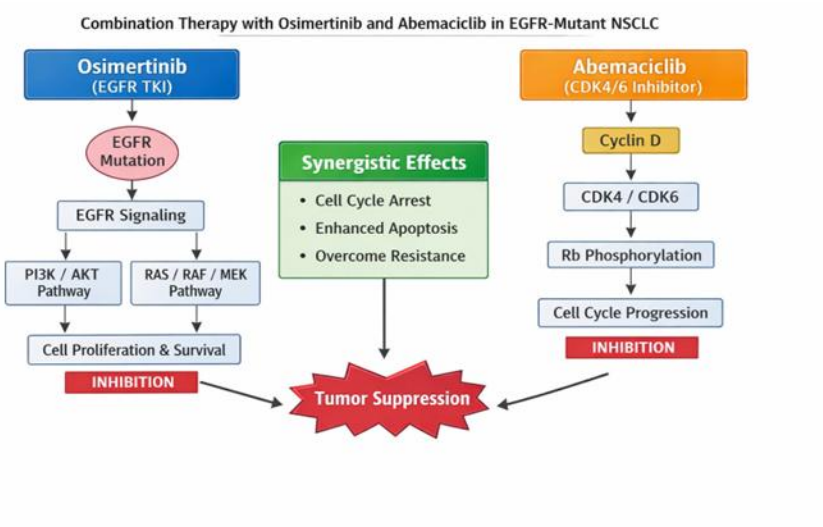



**Figure 7: Combination effect**

The flowchart illustrates the comprehensive mechanism of action and synergistic therapeutic effect of the combination of osimertinib and abemaciclib in the treatment of non-small cell lung cancer (NSCLC) harboring epidermal growth factor receptor (EGFR) mutations. This combination strategy is based on dual inhibition of critical oncogenic signaling pathways and cell cycle regulatory mechanisms, which together contribute to enhanced tumor suppression and delayed drug resistance.The mechanism begins with the presence of activating mutations in the EGFR gene, which leads to continuous activation of the EGFR signaling pathway. Under normal physiological conditions, EGFR regulates cell growth, proliferation, and survival. However, in NSCLC, mutations in EGFR result in constitutive activation of its tyrosine kinase domain, leading to persistent downstream signaling even in the absence of ligand binding. This abnormal activation stimulates multiple intracellular pathways, most notably the phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) pathway and the rat sarcoma/rapidly accelerated fibrosarcoma/mitogen-activated protein kinase (RAS/RAF/MEK) pathway. These pathways promote cellular proliferation, inhibit apoptosis, and enhance tumor survival.[83]Osimertinib, a third-generation EGFR tyrosine kinase inhibitor, acts at this stage by selectively and irreversibly

binding to the mutant EGFR receptor, including the T790M resistance mutation. By inhibiting EGFR phosphorylation, osimertinib effectively blocks the activation of downstream signaling pathways. As a result, there is a significant reduction in tumor cell proliferation and an increase in programmed cell death (apoptosis). However, despite initial responsiveness, cancer cells often develop resistance to osimertinib through activation of alternative signaling pathways or downstream effectors.One of the most important resistance mechanisms involves the activation of the cell cycle regulatory pathway mediated by cyclin-dependent kinases 4 and 6 (CDK4/6). EGFR signaling is closely linked to the regulation of cyclin D expression, which forms a complex with CDK4/6 to drive the progression of the cell cycle from the G1 phase to the S phase. In resistant tumor cells, cyclin D expression may remain elevated or become independent of EGFR signaling, allowing continued cell proliferation even in the presence of EGFR inhibition.[84]Abemaciclib, a selective CDK4/6 inhibitor, targets this resistance pathway by directly inhibiting the activity of CDK4 and CDK6 enzymes. Under normal conditions, CDK4/6 phosphorylates the retinoblastoma (Rb) protein, leading to its inactivation and release of E2F transcription factors, which promote DNA synthesis and cell cycle progression. Abemaciclib prevents Rb phosphorylation, thereby maintaining it in an active, hypophosphorylated state. This results in sequestration of E2F transcription factors and arrest of the cell cycle in the G1 phase, effectively halting tumor cell proliferation.The combination of osimertinib and abemaciclib provides a synergistic therapeutic effect by targeting both upstream and downstream components of the cancer growth pathway. Osimertinib reduces the expression of cyclin D by inhibiting EGFR signaling, while abemaciclib directly blocks CDK4/6 activity, ensuring complete suppression of the cell cycle progression. This dual blockade leads to a more robust inhibition of tumor growth compared to monotherapy.In addition to inhibiting proliferation, the combination enhances apoptotic signaling pathways, leading to increased cancer cell death. It also reduces the likelihood of resistance development by simultaneously targeting multiple critical pathways required for tumor survival. The final outcome of this combined mechanism, as depicted in the flowchart, is effective tumor suppression characterized by decreased proliferation, increased apoptosis, and improved therapeutic response.Overall, this integrated approach represents a rational and promising strategy in the management of EGFR-mutated NSCLC. By overcoming the limitations of single-agent therapy and addressing key resistance mechanisms, the combination of osimertinib and abemaciclib holds significant potential for improving clinical outcomes in patients with advanced lung cancer.[85]

### 1.5 Bilayer tablet

Bilayer tablets have some key advantages compared to conventional monolayer tablets. For instance, such tablets are commonly used to avoid chemical incompatibilities of formulation components by physical separation. In addition, bilayer tablets have enabled the development of controlled delivery of active pharmaceutical ingredients with pre-determined release profiles by combining layers with various release patterns, or by combining slow-release with immediate-release layers. Bi-layer tablet is suitable for sequential release of two drugs in combination, separate two incompatible substances and also for sustained release tablet in which one layer is immediate release as initial dose and second layer is maintenance dose.

### 1.5.1 Need of bilayer tablets

1. Controlling the delivery rate of single or two different active pharmaceutical ingredients.
2. To modify the surface area available for API by swellable/erodible barriers for modified release.
3. To separate incompatible Active pharmaceutical ingredient (APIs) from each other.
4. To control the release of API from one layer by utilizing the functional property of the other layer.
5. For the administration of fixed dose combinations of different APIs.

### 1.5.2 Advantages

1. Cost is subordinate compared to all other oral dosage form.
2. Maximum chemical and microbial stability over all oral dosage form.
3. Offensive odor and bitter taste can be masked by coating technique.
4. Flexible Concept.
5. They offer greatest dose accuracy and least content variability.
6. Easy to swallowing with least tendency for hang up.
7. Suitable for large scale production

### 1.5.3 Disadvantages

1. Some drugs resist compression into dense compacts due to low density character.
2. Bitter tasting drugs or drugs that are sensitive to oxygen may require coating.
3. Difficult to swallow in case of children and unconscious patients.
4. Drugs with poor wetting, slow dissolution properties may be difficult to formulate.

### 1.5.4 Ideal characteristics of bilayer tablets

1. It should be free from defects like chips, cracks, discoloration and contamination.
2. It should have sufficient strength during its production, packaging, shipping and dispensing.
3. It should have the chemical and physical stability overtime.
4. It releases the agents in a predictable and reproducible manner.
5. It must have a chemical stability and shelf-life.

### 1.5.5 Challenges in bilayer tablet manufacturing

Conceptually, bilayer tablets can be seen as two single-layer tablets compressed into one. In Practice, there are some manufacturing challenges.
Delamination: Tablet falls apart when the two halves of the tablet do not bond completely.
Cross-Contamination: When the granulation of the first layer intermingles with the granulation of the second layer results cross contamination occurs. Proper dust collection goes a long way toward preventing cross contamination.
Production yields: To prevent cross contamination, dust collection is required which leads to losses. Thus, bilayer tablets have lower yields than single layer tablets.
Cost: Bilayer tableting is more expensive than single layer tableting for several reasons. First, the tablet press costs more. Second, the press generally runs more slowly in bilayer mode. Third, development of two compatible granulations is must, which means more time spent on formulation development, analysis and validation.

### 1.5.6 Single sided press

Various types of bi-layer presses have been designed over the years. The simplest design is a single sided press with both chambers of the double feeder separated from each other. Each chamber is gravity- or forced-fed with a different powder, thus producing the two individual layers of the tablet.

When the die passes under the feeder, it is at first loaded with the first-layer powder followed by the second-layer powder. Then the entire tablet is compressed in one or two steps (two = pre- and main compression). The two layers in the die mix slightly at their interface and in most cases bond sufficiently so that no layer-separation occurs when the tablet is produced. This is the simplest way of producing a bilayer tablet.

**Limitations of single-sided press**

Various types of bi-layer presses have been designed over the years. The simplest design is a single-sided press with both chambers of the double feeder separated from each other. Each chamber is gravity- or forced-fed with a different powder, thus producing the two individual layers of the tablet. When the die passes under the feeder, it is at first loaded with the first layer powder followed by the second-layer powder. Then the entire tablet is compressed in one or two steps (two = pre- and main compression). The two layers in the die mix slightly at their interface and in most cases bond sufficiently. So that no layer-separation occurs when the tablet is produced. This is the simplest way of producing a bilayer tablet. It undergoes certain limitation as follow.

No weight monitoring/control of the individual Layers.

No distinct visual separation between the two Layers.

Very short first layer-dwell time due to the small compression roller, possibly resulting in poor de-aeration,capping and hardness problems. This may be corrected by reducing the turret-rotation speed (to extend the dwell time) but with the consequence of lower tablet.output. Very difficult first-layer tablet sampling and sample transport to a test unit for in-line quality control and weight recalibration to eliminate these limitations, a double-sided tablet press is preferred over a single-sided press. A double-sided press offers an individual fill station, pre -compression and main compression for each layer. In fact, the bi-layer tablet will go through 4 compression stages before being ejected from the press

1.5.7 Double-sided tablet presses:

Double-sided tablet presses have been specifically designed and developed for the production of quality bi-layer tablets and provide:

'displacement' weight monitoring/control for accurate and independent weight control of the individual layers

low compression force exerted on the first layer to avoid capping and separation of the two individual layers

increased dwell time at pre-compression of both first and second layer to provide sufficient hardness at maximum turret speed

maximum prevention of cross-contamination between the two layers

a clear visual separation between the two layers

maximised yield[26]

**1.5.8 Preparation of Bilayer Tablet**

Bilayer tablets are prepared with one layer of drug for immediate release with the second layer designed to release drug later, either as a second dose or in an extended release form8. The bilayer tablets with two incompatible drugs can also be prepared by compressing separate layers of each drug so as to minimize area of contact between two layers. An additional intermediate layer of inert material may also be included.Compaction To produce adequate tablet formulation, certain requirements such as sufficient mechanical strength and desired drug release profile must be met. At times, this may be difficult task for formulator to achieve these conditions especially in bilayer tablet formulation where double compression technique is involved, because of poor flow and compatibility characteristic of the

drug which will result in capping and/or lamination. The compaction of a material involves both the compressibility and consolidation. Compression: it is defined as reduction in bulk volume by eliminating voids and bringing particles into closer contacts.Consolidation: it is the property of the material in which there is increased mechanical strength due to interparticulate interaction (bonding). The compression force on layer 1 was found to be major factor influencing tablet delamination.[27]

## 2.1 AIM

To evaluate the cytotoxic and antiproliferative potential of the combination of Osimertinib and Abemaciclib using onion root tip and brine shrimp lethality assays, and to formulate and evaluate a bilayer immediate-release tablet for simultaneous drug delivery in lung cancer therapy.

## 2.2 OBJECTIVE

1. Biological Evaluation Objectives

-To study cytotoxic activity using Onion Root Tip Assay
Evaluate root growth inhibition
Assess mitotic index reduction
-To perform Brine Shrimp Lethality Assay
Determine % mortality
Calculate $LC_{50}$ values
-Compare toxicity of individual drugs vs combination
To analyze synergistic effect of the combination
Compare individual vs combined response
Identify enhanced cytotoxic potential

**2. Formulation Objectives**

To develop a bilayer tablet (IR–IR) containing:

Osimertinib
Abemaciclib
To select suitable excipients for rapid drug release
Superdisintegrants
Binders, diluents, lubricants
To prepare tablets using direct compression.

## 2.3 RATIONALE

Lung cancer remains one of the leading causes of cancer-related mortality worldwide, and despite advancements in targeted therapy, drug resistance and incomplete tumor suppression remain major challenges. Osimertinib is a third-generation EGFR inhibitor that specifically targets mutated EGFR in non-small cell lung cancer, providing effective tumor suppression. However, resistance mechanisms often develop over time.On the other hand, Abemaciclib inhibits cyclin-dependent kinases 4 and 6, thereby blocking cell cycle progression from G1 to S phase. This complementary mechanism suggests that combining EGFR inhibition with CDK4/6 inhibition may produce synergistic anticancer effects, improving therapeutic outcomes and delaying resistance.Before proceeding to advanced in-vitro or in-vivo models, preliminary screening using simple, cost-effective bioassays is essential. The onion root tip assay is a well-established model for evaluating cytotoxicity and genotoxicity by observing mitotic inhibition and chromosomal abnormalities. Similarly, the brine shrimp lethality assay serves as a rapid and economical method to assess general toxicity and biological activity of compounds.Following confirmation of cytotoxic potential, formulation of a bilayer immediate-release (IR–IR) tablet offers several advantages. It allows simultaneous delivery of both drugs, improves patient compliance, reduces pill burden, and prevents drug–drug incompatibility by separating the two drugs into different layers. Moreover, immediate release ensures rapid onset of action, which is

crucial in cancer therapy.Thus, this study integrates biological evaluation and pharmaceutical formulation, aiming to develop an effective combination therapy approach for lung cancer using a novel bilayer tablet system.

## 3 REVIEW OF LITERATURE

Siegel RL et al. (2024)[29] reported a comprehensive analysis of global cancer statistics with a specific focus on lung cancer incidence and mortality trends. The objective of the study was to evaluate changes in disease patterns in relation to declining smoking rates and increasing environmental exposure. The authors utilized large-scale epidemiological databases and observed that although smoking-related lung cancer cases are gradually decreasing in developed countries, there is a notable rise in lung cancer incidence among non-smokers, particularly in younger populations and females. The study demonstrated that exposure to fine particulate matter (PM2.5), occupational carcinogens, and genetic susceptibility significantly contribute to this shift in disease pattern. Furthermore, disparities in healthcare access, delayed diagnosis, and lack of screening programs were identified as key contributors to high mortality rates. The authors also emphasized the importance of public health interventions, including pollution control, smoking cessation programs, and early detection strategies. The study concluded that lung cancer epidemiology is evolving and requires a multidisciplinary and preventive approach for effective control.Ettinger DS et al. (2024)[30] reported updated clinical practice guidelines for the diagnosis and management of lung cancer, particularly non-small cell lung cancer (NSCLC). The objective of the study was to standardize treatment approaches based on recent advancements in molecular biology and clinical trials. The authors reviewed extensive clinical evidence and recommended comprehensive biomarker testing, including EGFR mutations, ALK rearrangements, KRAS mutations, and PD-L1 expression, prior to initiating therapy. The study demonstrated that biomarker-driven therapy significantly improves treatment outcomes by enabling personalized medicine. Furthermore, the use of combination therapies involving immunotherapy and chemotherapy was found to enhance overall survival and progression-free survival. The authors also discussed treatment sequencing, management of adverse effects, and strategies to overcome resistance. The study concluded that precision medicine is now the foundation of modern lung cancer treatment and should be implemented in routine clinical practice.Garon EB et al. (2023)[31] reported long-term follow-up data on the use of immune checkpoint inhibitors in advanced lung cancer. The objective was to assess the durability of response and long-term survival benefits associated with pembrolizumab therapy. The study included patients with high PD-L1 expression and evaluated clinical endpoints such as overall survival, response rate, and safety profile. The results demonstrated that a significant proportion of patients achieved durable responses lasting several years, which is rarely observed with conventional chemotherapy. Furthermore, patients experienced improved quality of life and fewer severe adverse effects. The authors highlighted that immunotherapy enhances the immune system's ability to recognize and eliminate tumor cells. However, variability in response due to tumor heterogeneity and immune microenvironment differences was also noted. The study concluded that immunotherapy represents a paradigm shift in lung cancer treatment and offers long-term clinical benefits in selected patients.Dagogo-Jack I et al. (2023)[32] reported an in-depth analysis of acquired resistance mechanisms in targeted lung cancer therapy. The

objective was to identify molecular alterations responsible for treatment failure in patients receiving targeted agents such as EGFR and ALK inhibitors. The study demonstrated that secondary mutations, activation of alternative signaling pathways, and phenotypic transformation contribute to drug resistance. Furthermore, tumor heterogeneity and clonal evolution were identified as major challenges in maintaining long-term treatment efficacy. The authors emphasized the importance of continuous molecular monitoring using advanced diagnostic tools such as liquid biopsy. The study concluded that overcoming resistance requires the development of next-generation inhibitors and combination treatment strategies.Zhang Y et al. (2022)[33] reported the application of nanotechnology in lung cancer drug delivery systems. The objective of the study was to enhance the therapeutic efficacy and specificity of anticancer drugs while minimizing systemic toxicity. The authors demonstrated that nanoparticle-based drug delivery systems improve drug targeting to tumor tissues through enhanced permeability and retention (EPR) effect. Furthermore, these systems allow controlled drug release, improved pharmacokinetics, and reduced adverse effects. The study also highlighted the potential of multifunctional nanoparticles for combined therapy and imaging. The authors concluded that nanomedicine represents a promising approach for improving lung cancer treatment outcomes in the future.Hanahan D et al. (2022)[34] provided an updated framework of the "hallmarks of cancer," offering a comprehensive understanding of tumor biology. The objective was to describe the fundamental biological processes involved in cancer development and progression. The study highlighted key hallmarks such as sustained proliferative signaling, evasion of apoptosis, induction of angiogenesis, activation of invasion and metastasis, and immune evasion. Furthermore, emerging concepts such as tumor-promoting inflammation, metabolic reprogramming, and cellular plasticity were discussed. The authors concluded that understanding these mechanisms is essential for identifying novel therapeutic targets and developing effective treatment strategies for lung cancer.Bade BC et al. (2022)[35] reported the effectiveness of low-dose computed tomography (LDCT) screening in early detection of lung cancer. The objective was to evaluate its impact on mortality reduction among high-risk populations, particularly smokers. The study demonstrated that LDCT screening significantly reduces lung cancer mortality by detecting tumors at an early stage when they are more amenable to treatment. Furthermore, implementation of screening programs was found to be cost-effective and beneficial in reducing disease burden. The authors concluded that early detection through screening is a key strategy in improving survival outcomes.Hirsch FR et al. (2021)[36] reported advancements in molecular diagnostics and targeted therapy for lung cancer. The objective was to assess the role of next-generation sequencing (NGS) in identifying actionable mutations. The study demonstrated that NGS allows simultaneous detection of multiple genetic alterations, enabling personalized treatment approaches. Furthermore, targeted therapies such as EGFR and ALK inhibitors were shown to significantly improve progression-free survival compared to conventional chemotherapy. The authors concluded that molecular profiling is essential for precision oncology.Reck M et al. (2016)[37] reported a clinical trial evaluating the efficacy of immunotherapy compared to chemotherapy in lung cancer patients. The objective was to assess the role of PD-1 inhibitors in improving survival outcomes. The study demonstrated that immunotherapy significantly increased overall survival and provided durable responses. Furthermore, it was associated with

fewer adverse effects compared to chemotherapy. The authors concluded that immunotherapy is a major breakthrough in lung cancer treatment.Herbst RS et al. (2018)[39] reported a comprehensive review of non-small cell lung cancer biology and treatment strategies. The objective was to evaluate recent therapeutic advancements. The study demonstrated that targeted therapy and immunotherapy have significantly improved survival rates. Furthermore, combination therapies were found to enhance treatment efficacy. The authors concluded that integrated treatment approaches are necessary for optimal patient care.Lynch TJ et al. (2004)[40] reported a landmark study on EGFR mutations and their role in lung cancer treatment. The objective was to evaluate the effectiveness of EGFR inhibitors. The study demonstrated that patients with EGFR mutations respond better to targeted therapy compared to chemotherapy. Furthermore, improved survival rates and reduced toxicity were observed. The authors concluded that EGFR-targeted therapy represents a significant advancement in lung cancer management.Ramalingam SS et al. (2020)[41] reported the final overall survival analysis of the landmark FLAURA trial to determine the long-term clinical benefit of osimertinib in patients with previously untreated EGFR mutation-positive advanced non-small cell lung cancer (NSCLC). The primary objective of the study was to assess whether the significant progression-free survival advantage observed earlier could translate into improved overall survival. The authors conducted a randomized, double-blind, phase III clinical trial comparing osimertinib with standard first-generation EGFR tyrosine kinase inhibitors such as gefitinib and erlotinib. The study demonstrated that osimertinib significantly prolonged overall survival, with a median survival advantage of several months over standard therapy. Furthermore, osimertinib showed superior central nervous system (CNS) efficacy due to its enhanced ability to penetrate the blood-brain barrier, thereby reducing the incidence and progression of brain metastases. The treatment was also associated with a more favorable safety profile, including lower rates of severe dermatological and gastrointestinal toxicities. The authors emphasized that osimertinib delays the development of resistance mutations, particularly T790M, and improves patient quality of life. The study concluded that osimertinib should be considered the preferred first-line therapy for EGFR-mutated NSCLC due to its sustained efficacy, improved survival outcomes, and manageable toxicity profile.Soria JC et al. (2018)[42] reported the primary results of the FLAURA trial, focusing on progression-free survival and treatment efficacy of osimertinib compared to first-generation EGFR inhibitors. The objective of the study was to evaluate whether osimertinib could provide superior disease control in treatment-naïve patients with EGFR-mutated NSCLC. The authors enrolled a large cohort of patients and randomly assigned them to receive either osimertinib or standard EGFR-TKI therapy. The study demonstrated a significantly longer progression-free survival in the osimertinib group, indicating delayed disease progression. Furthermore, osimertinib exhibited a higher objective response rate and longer duration of response. The authors also reported improved CNS activity, highlighting its effectiveness in preventing and treating brain metastases, which are common complications in lung cancer. In addition, the safety analysis revealed fewer grade 3 or higher adverse events compared to standard therapy. The authors concluded that osimertinib represents a major advancement in targeted therapy and provides a new standard of care for first-line treatment.Mok TS et al. (2017)[43] reported the AURA3 phase III clinical trial evaluating the efficacy of osimertinib in patients with T790M-positive NSCLC who had

experienced disease progression following first-line EGFR-TKI therapy. The objective of the study was to compare osimertinib with platinum-based chemotherapy in this resistant population. The study demonstrated that osimertinib significantly improved progression-free survival and overall response rate compared to chemotherapy. Furthermore, patients receiving osimertinib experienced fewer severe adverse effects and improved symptom control. The drug also showed superior intracranial activity, effectively managing brain metastases. The authors discussed the mechanism of action of osimertinib, which involves irreversible inhibition of mutant EGFR, including the T790M resistance mutation, while sparing wild-type receptors. This selectivity reduces off-target toxicity and enhances therapeutic efficacy. The study concluded that osimertinib is the treatment of choice for patients with acquired resistance mediated by T790M mutation.Reungwetwattana T et al. (2018)[44] reported a detailed analysis of the central nervous system (CNS) activity of osimertinib in patients with EGFR-mutated NSCLC. The objective was to evaluate the drug's ability to penetrate the blood-brain barrier and control intracranial disease. The study demonstrated that osimertinib achieved high CNS response rates and significantly reduced the risk of CNS progression compared to earlier EGFR inhibitors. Furthermore, patients with existing brain metastases showed marked tumor regression, indicating strong intracranial efficacy. The authors highlighted that osimertinib's molecular structure allows it to effectively cross the blood-brain barrier, which is a major limitation of earlier therapies. The study concluded that osimertinib provides substantial clinical benefit in patients with brain metastases and should be preferred in such cases.Wu YL et al. (2020)[45] reported subgroup analyses of osimertinib efficacy across different demographic and ethnic populations. The objective was to determine whether clinical outcomes varied among patient subgroups, particularly in Asian populations where EGFR mutations are more prevalent. The study demonstrated consistent improvements in progression-free survival and response rates across all subgroups. Furthermore, the safety profile remained favorable, with manageable side effects such as diarrhea and rash. The authors emphasized that osimertinib's efficacy is independent of ethnicity, age, or gender, supporting its universal applicability. The study concluded that osimertinib is a globally effective therapy for EGFR-mutated NSCLC.Janne PA et al. (2015)[46] reported phase I clinical trial results focusing on dose escalation, safety, and preliminary efficacy of osimertinib. The objective was to determine the optimal therapeutic dose and evaluate its antitumor activity. The study demonstrated high response rates, particularly in patients with T790M mutation. Furthermore, adverse effects were generally mild and manageable, supporting long-term use. The authors concluded that osimertinib has a favorable therapeutic index and significant clinical potential.Cross DA et al. (2014)[47] reported the discovery and preclinical development of osimertinib as a third-generation EGFR inhibitor. The objective was to design a molecule capable of selectively targeting mutant EGFR while overcoming resistance mechanisms. The study demonstrated that osimertinib irreversibly binds to mutant EGFR, including T790M, with high specificity. Furthermore, preclinical models showed potent antitumor activity and reduced toxicity compared to earlier inhibitors. The authors concluded that osimertinib represents a significant advancement in targeted drug design.Johnston S et al. (2020)[48] reported the final analysis of the MONARCH 3 trial, which evaluated the efficacy and safety of abemaciclib in combination with non-steroidal aromatase inhibitors as initial endocrine-based therapy in patients with hormone receptor-positive (HR+),

HER2-negative advanced breast cancer. The primary objective of the study was to determine whether the addition of abemaciclib could significantly improve progression-free survival compared to endocrine therapy alone. The authors conducted a randomized, double-blind, placebo-controlled phase III clinical trial involving postmenopausal women. The study demonstrated that abemaciclib significantly prolonged progression-free survival and improved overall response rates. Furthermore, sustained tumor regression and delayed disease progression were observed across multiple patient subgroups. The mechanism underlying these outcomes involves selective inhibition of cyclin-dependent kinases 4 and 6 (CDK4/6), which regulate cell cycle progression from the G1 to S phase, thereby preventing uncontrolled tumor cell proliferation. The authors also noted that abemaciclib has continuous dosing capability due to its distinct toxicity profile compared to other CDK4/6 inhibitors. The safety analysis revealed manageable adverse effects, with diarrhea being the most common, which could be controlled with dose modification and supportive care. The study concluded that abemaciclib in combination with endocrine therapy represents a highly effective first-line treatment strategy for HR+/HER2− advanced breast cancer.Goetz MP et al. (2017)[49] reported the results of the MONARCH 2 trial, which assessed abemaciclib in combination with fulvestrant in patients with HR+/HER2− advanced breast cancer who had progressed on prior endocrine therapy. The objective of the study was to evaluate whether abemaciclib could overcome endocrine resistance. The study demonstrated a significant improvement in progression-free survival in the abemaciclib plus fulvestrant group compared to fulvestrant alone. Furthermore, the combination therapy resulted in higher objective response rates and better disease control. The authors explained that abemaciclib inhibits CDK4/6 activity, leading to cell cycle arrest and reduced tumor cell proliferation. Additionally, the study highlighted that abemaciclib exhibits greater selectivity for CDK4 compared to CDK6, which may contribute to its unique efficacy and safety profile. The authors concluded that abemaciclib is an effective option for patients with endocrine-resistant breast cancer.Dickler MN et al. (2016)[50] reported early-phase clinical trial data on abemaciclib, focusing on safety, pharmacokinetics, and preliminary efficacy. The objective was to determine the optimal dosing regimen and evaluate antitumor activity. The study demonstrated promising clinical responses in patients with advanced breast cancer. Furthermore, pharmacokinetic analysis showed favorable absorption and sustained plasma levels. The authors concluded that abemaciclib has significant therapeutic potential and warrants further investigationPatnaik A et al. (2016)[51] reported a phase I dose-escalation study evaluating abemaciclib in patients with various solid tumors. The objective was to assess safety, tolerability, and preliminary efficacy across different cancer types. The study demonstrated that abemaciclib exhibits broad antitumor activity and a manageable safety profile. Furthermore, early signs of efficacy were observed in breast cancer patients. The authors concluded that abemaciclib is a promising candidate for targeted cancer therapy.Gelbert LM et al. (2014)[52] reported the preclinical development of abemaciclib. The objective was to design a selective CDK4/6 inhibitor with potent anticancer activity. The study demonstrated that abemaciclib effectively inhibits CDK4/6 activity, leading to cell cycle arrest and suppression of tumor growth. Furthermore, preclinical models showed significant tumor regression and favorable pharmacokinetic properties. The authors concluded that abemaciclib represents a novel and effective targeted therapy for cancer treatment

Naz S, et al.(2018)[53] demonstrated that Abemaciclib exerts significant antitumor activity in non-small cell lung cancer (NSCLC) by targeting the cyclin-dependent kinase 4 and 6 (CDK4/6) pathway, which plays a critical role in regulating cell cycle progression. The study revealed that abemaciclib effectively inhibits phosphorylation of the retinoblastoma (Rb) protein, thereby inducing G1 phase arrest and suppressing tumor cell proliferation. Furthermore, the authors showed that abemaciclib enhances radiosensitivity in NSCLC models, indicating its potential role not only as a monotherapy but also in combination with other treatment modalities. Mechanistically, abemaciclib was found to impair DNA damage repair following radiation exposure and inhibit key signaling pathways such as mTOR, leading to metabolic stress and reduced tumor survival. These findings highlight the importance of CDK4/6 inhibition in controlling tumor growth and improving therapeutic response in lung cancer.Turner NC, et al. (2018)[54] described the broader role of CDK4/6 inhibitors in cancer therapy, emphasizing that dysregulation of the cyclin D–CDK4/6–Rb pathway is a hallmark of uncontrolled cellular proliferation in many cancers, including lung cancer. The authors highlighted that abemaciclib, due to its continuous dosing and higher selectivity for CDK4, provides sustained inhibition of tumor growth. They also emphasized that combining CDK4/6 inhibitors with targeted therapies enhances treatment efficacy by simultaneously blocking multiple oncogenic pathways, thereby reducing the likelihood of resistance development and improving overall therapeutic outcomes.Patnaik A, et al. (2016)[55] reported in early-phase clinical studies that abemaciclib demonstrates promising antitumor activity in patients with advanced solid tumors, including NSCLC. The study highlighted that abemaciclib has a manageable safety profile and can be administered continuously, which allows prolonged inhibition of CDK4/6 activity. The findings support the clinical potential of abemaciclib as both a monotherapy and as part of combination regimens in lung cancer treatment, particularly in cases where resistance to standard therapies has developed.La Monica S, et al. (2021)[56] investigated the therapeutic potential of combining Osimertinib with Abemaciclib in epidermal growth factor receptor (EGFR)-mutated non-small cell lung cancer (NSCLC) models exhibiting different mechanisms of resistance to EGFR-targeted therapy. The authors demonstrated that osimertinib, a third-generation EGFR tyrosine kinase inhibitor, effectively suppresses tumor growth by selectively targeting EGFR mutations; however, prolonged treatment leads to the development of acquired resistance, primarily due to activation of alternative signaling pathways, including dysregulation of the cyclin D–CDK4/6–retinoblastoma (Rb) axis. Their study provided clear evidence that hyperactivation of the CDK4/6 pathway, reflected by increased phosphorylation of the Rb protein, plays a critical role in enabling tumor cells to bypass EGFR inhibition and continue proliferating despite osimertinib therapy. In this context, abemaciclib, a potent and selective CDK4/6 inhibitor, was evaluated for its ability to target this resistance pathway. The findings revealed that abemaciclib significantly inhibited cell proliferation, reduced clonogenic survival, and induced cellular senescence in both osimertinib-sensitive and resistant NSCLC cell lines, indicating its strong antiproliferative activity through effective blockade of cell cycle progression at the G1 phase.Husain H, et al. (2024)[57] conducted a clinical investigation (ClinicalTrials.gov Identifier: NCT04545710) to evaluate the efficacy and safety of combining Osimertinib with Abemaciclib in patients with epidermal growth factor receptor (EGFR)-mutant non-small cell lung cancer (NSCLC) who had developed resistance to prior osimertinib therapy.

The study, sponsored by the University of California, San Diego, was designed based on the strong biological rationale that activation of the cyclin D–CDK4/6–retinoblastoma (Rb) pathway plays a significant role in mediating acquired resistance to EGFR-targeted therapies. In this context, osimertinib functions by irreversibly inhibiting mutant EGFR signaling, thereby suppressing tumor proliferation; however, tumor cells often adapt by activating downstream cell cycle pathways, allowing continued growth despite EGFR blockade. The trial aimed to determine whether the addition of abemaciclib, a selective CDK4/6 inhibitor, could overcome or mitigate this resistance by inducing G1 cell cycle arrest and inhibiting Rb phosphorylation. The study incorporated patients with advanced EGFR-mutated NSCLC who had experienced disease progression following osimertinib treatment, thereby specifically targeting a clinically challenging population with limited therapeutic options. The combination therapy was hypothesized to exert a synergistic effect by simultaneously targeting upstream oncogenic signaling and downstream cell cycle progression. Preliminary findings and the scientific rationale underlying the trial suggest that abemaciclib may enhance the effectiveness of osimertinib by suppressing compensatory proliferative signaling pathways that are activated during resistance. Although complete reversal of established resistance remains difficult, the combination approach is expected to slow tumor progression, stabilize disease, and potentially extend progression-free survival in affected patients.Gomatou G, et al. (2023)[58] comprehensively reviewed the molecular mechanisms underlying resistance to Osimertinib in epidermal growth factor receptor (EGFR)-mutated non-small cell lung cancer (NSCLC) and highlighted emerging therapeutic strategies to overcome this clinical challenge. The authors reported that although osimertinib has significantly improved patient outcomes due to its potent and selective inhibition of both sensitizing EGFR mutations and the T790M resistance mutation, the development of acquired resistance remains inevitable. The mechanisms of resistance are highly heterogeneous and include secondary EGFR mutations such as C797S, activation of bypass signaling pathways including MET amplification, HER2 amplification, and KRAS mutations, as well as phenotypic transformations such as epithelial-to-mesenchymal transition and small cell lung cancer transformation. Importantly, the review emphasized that dysregulation of cell cycle control, particularly activation of the cyclin D–CDK4/6–retinoblastoma (Rb) pathway, plays a crucial role in enabling tumor cells to evade the inhibitory effects of EGFR-targeted therapy. In this context, the authors discussed the therapeutic relevance of combining EGFR inhibitors with cell cycle inhibitors to overcome resistance mechanisms. Abemaciclib, a selective inhibitor of CDK4 and CDK6, was highlighted as a promising agent capable of suppressing tumor cell proliferation by inducing G1 phase arrest through inhibition of Rb phosphorylation. The review pointed out that activation of CDK4/6 signaling is frequently observed in resistant tumors and contributes to continued cancer cell growth despite EGFR inhibition. Therefore, targeting this pathway alongside EGFR inhibition represents a rational strategy to improve treatment outcomes. The authors further noted that preclinical studies have demonstrated synergistic effects when CDK4/6 inhibitors are combined with EGFR tyrosine kinase inhibitors such as osimertinib, resulting in enhanced tumor suppression and delayed onset of resistance.Ishida T, et al. (2023)[59] investigated the role of cyclin-dependent kinase 4 and 6 (CDK4/6) inhibition in enhancing the therapeutic efficacy of Osimertinib in non-small cell lung cancer (NSCLC). The

authors reported that although osimertinib is highly effective in targeting epidermal growth factor receptor (EGFR) mutations, its long-term clinical benefit is significantly limited by the development of acquired resistance. One of the critical mechanisms contributing to this resistance is the activation of the cyclin D–CDK4/6–retinoblastoma (Rb) signaling pathway, which enables cancer cells to bypass EGFR inhibition and continue proliferating. The study demonstrated that increased phosphorylation of the Rb protein is associated with continued tumor growth despite EGFR-targeted therapy, indicating that cell cycle dysregulation plays a central role in resistance mechanisms. In this context, the authors evaluated the effect of CDK4/6 inhibition using Abemaciclib and found that it significantly suppressed tumor cell proliferation by inducing G1 phase arrest and inhibiting Rb phosphorylation. Furthermore, when abemaciclib was combined with osimertinib, a synergistic antitumor effect was observed, characterized by enhanced inhibition of cell growth, increased apoptosis, and reduced tumor viability compared to either agent alone. Importantly, the study highlighted that CDK4/6 inhibition not only improves the initial response to EGFR-targeted therapy but also plays a crucial role in delaying the onset of resistance. The dual targeting of EGFR signaling and cell cycle progression disrupts complementary pathways required for tumor survival, thereby enhancing overall treatment efficacy. The authors concluded that combination therapy involving osimertinib and abemaciclib represents a promising strategy for overcoming resistance and improving clinical outcomes in patients with EGFR-mutated NSCLC.Abebe A, et al. (2014)[60] reported that bilayer tablet technology provides an effective platform for the development of dual-release dosage forms by incorporating both immediate-release and sustained-release layers within a single tablet. The authors emphasized that such systems are particularly advantageous in the management of chronic diseases, as they ensure rapid onset of action followed by prolonged therapeutic effect, thereby maintaining consistent plasma drug concentration. They also highlighted that bilayer tablets can be used to separate incompatible drugs, improving the stability and efficacy of the formulation. Furthermore, the study discussed the use of hydrophilic polymers such as hydroxypropyl methylcellulose and carbopol in sustained-release layers, while superdisintegrants like crospovidone and sodium starch glycolate are employed in immediate-release layers to enhance disintegration and dissolution characteristicsSoni N, et al. (2022)[61] described bilayer tablet technology as a versatile and efficient drug delivery system that allows the administration of combination therapy in a single dosage form. According to their review, this approach significantly improves patient compliance by reducing the number of doses required and simplifying treatment regimens. The authors further explained that bilayer tablets offer flexibility in designing drug release profiles, such as immediate release followed by sustained release or delayed release, depending on therapeutic requirements. They also discussed various manufacturing techniques, including direct compression, wet granulation, and dry granulation, with direct compression being the most preferred due to its simplicity, cost-effectiveness, and suitability for heat-sensitive drugs. However, they also pointed out challenges such as layer separation, weight variation, and the need for specialized equipment, which must be addressed during formulation developmentAulton ME, et al. (2018)[62] explained that bilayer tablets represent a significant advancement in oral drug delivery systems by allowing precise control over drug release kinetics. The authors noted that the design and manufacture of bilayer tablets require careful optimization of formulation variables and

processing parameters to achieve desired therapeutic outcomes. They also highlighted the importance of evaluating parameters such as hardness, friability, thickness, weight variation, and dissolution profile to ensure the quality and consistency of the product. Furthermore, the study emphasized that bilayer tablets have great potential in personalized medicine, where drug release can be tailored according to individual patient needsKamble PR, et al. (2011)[63] highlighted the importance of excipient selection and compatibility in the successful formulation of bilayer tablets. Their study indicated that the physicochemical properties of excipients, such as flowability, compressibility, and particle size, play a crucial role in ensuring uniformity and mechanical strength of the final product. The authors also emphasized that improper formulation can lead to problems such as poor interlayer adhesion, capping, and lamination, which may compromise tablet integrity and performance. Additionally, they discussed the role of polymers in controlling drug release and maintaining the structural integrity of the sustained-release layer.

### 4.1 Brine Shrimp Lethality Bioassay

The extracts, fractions and pure isolated compounds were routinely evaluated in a test for lethality to brine shrimp larvae. Toxicities of compounds were tested at 1, 10, 100, and 1000 ppm in 10 mL sea-water solutions with 1% DMSO (v/v). Ten, nauplii were used in each test and survivors counted after 24 h. Three replications were used for each concentration. The blank control is conducted with Distilled water. The lethal concentration for 50% mortality after 24 h of exposure, the chronic LC50 was determined using the probit method, as the measure of toxicity of the extract or fractions. LC50 values greater than 1000 ppm for plant extracts were considered inactive.[64]The brine shrimp lethality assay (BSLA) is a simple and inexpensive bioassay used for testing the efficacy of phytochemical present in the plant extracts. The present study determined that the extent of lethality was directly proportional to the concentration of the extract. After 24 h of observation all the shrimp were survived in the control. Even though, maximum mortalities were observed upto a concentration of 1000 µg/mL and least mortality at 1 µg/mL concentrations. It was observed that in higher concentration of treatment extracts, the shrimps were start dying only after 8 h and after 24 h all the shrimps died. The lethality concentration (LC50) was calculated by using probit analysis. The LC50 (median lethal concentration) values were calculated by using the regression line obtained by plotting the concentration against the death percentage on a probit scale.In this test, brine shrimp (Artemia salina) eggs were hatched in artificial sea water (38 g/l of sea salt). The brine shrimp test (BST) bioassay experiment was performed according to the procedure described by Meyer (1982). Various concentrations of extracts were prepared (50, 100, 200, 400 µg/ml). Methotrexate and caffeine were used as standard cytotoxic drugs. After 48 hr of incubation, 10 brine shrimps were transferred to each sample vial using Pasteur pipette and artificial sea water was added to make 5 ml. Survivors were counted after 12hr and 24 hr.According to official data from the World Health Organization, cancer is the second leading cause of death in the world population and it is estimated that 9.6 million people died from this disease in 2018. According to the latest issue of the International Agency for Research on Cancer (IARC), September 2018, lung cancer dominates, as the most common form of cancer and is also the most common cause of death among men. It is followed by prostate cancer and colorectal cancers by incidence and liver and stomach cancers by mortality. When it comes to

women, breast cancer is the most commonly diagnosed cancer and is the leading cause of cancer mortality, followed by colorectal and lung cancer by incidence. Cervical cancer ranks fourth in incidence and mortality. Acute lymphoblastic leukemia is the most common hematological cancer in children and adolescents, representing 20% of all cancers diagnosed in persons aged < 20 years in the United States.[65]This assay was first proposed by Michael et al. in 1956. Subsequently, it was further developed by others. This lethality assay has been successively employed as a bioassay guide for active cytotoxic and anti tumor agents in 1982 (Meyer et al., 1982).[68]

### 4.1.1 MATERIALS AND EQUIPMENTS

1. Rectangular glass jar
2. Measuring cylinder (1000 mL)
3. Sea water
4. Spatula
5. Brine shrimp eggs (a few gram)
6. Air pump
7. Analytical balance
8. Pasteur pipette
9. Light source
10. Microtip pipette
11. Test tubes (12 × 100 mm)
12. Microscope
13. Test sample of drug

### 4.1.2 PREPARATION OF REAGENTS

Serial dilution of drug: Clean test tubes were taken and labeled. Standard drug(Methotrexate) of 10 mg was weighed by an analytical balance. Then stock solution was prepared by dissolving 10 mg of test drug in 1 mL of DMSO. Concentrations of 100 µg/ mL, were prepared by serial dilution from the stock solution. Five test tubes were labeled as 1-5. Then 1 mL of prepared solution was taken into the respect test tubes containing 10 nauplii and 1 mL of seawater. The number of dead nauplii was counted after 24 hours.[66]

### 4.1.3 PROTOCOL

Hatching brine shrimp

1. Measure 500 mL of sea water using measuring cylinder and pour into the rectangular jar
2. Mix the water with a spatula Place the tip of an airline from a air pump into the bottom of the jar maintaining proper aeration
3. Add about 15 g of brine shrimp eggs at the top water level of the jar and mix with the water
4. Switch on a light (60-100 Watt bulb) placed a few inches away from the jar
5. After 20-24 hours, the nauplii will hatch Observe the eggs and nauplii
6. Collect the nauplii after the next 24 hours.
7. Hatched nauplii must be separated from the empty egg. It can be done by turn off the air and switch off the lamp. The empty egg will float while the brine shrimp will concentrate in the water column.
8. Transfer 10 nauplii to a test tube using a Pasteur pipette.[67]



**Figure 8: set up of brine shrimp hatching**

Toxicity testing

Expose the nauplii to different concentrations of the drug.

Count the number of survivors and calculate the percentage of death after 24 hours



**Figure 9: brine shrimp microscope view**

### 4.1.4 CALCULATION

The mortality endpoint of this bioassay is defined as the absence of controlled forward motion during 30 sec of observation. The percent of lethality of the nauplii for each concentration and control was calculated. For each tube, count the number of dead and number of live nauplii, and determine the % death,[69,70]

%Death =Number of dead nauplii / Number of dead nauplii + Number of live nauplii x 100

**Table 5: brine shrimp assay result**

| Concentration | Number of live nauplii | Number of live nauplii | % Death |
|---|---|---|---|
| | Day 01 | Day 02 | |
| Sea water | 10 | 10 | 100 |
| STD(Methotrexate)- 100 µg/mL | 10 | 1 | 90 |
| Test(osimertinib)- 100 µg/mL | 10 | 3 | 70 |
| Test(abemaciclib)- 100 µg/mL | 10 | 3 | 70 |
| Test(combination)- 100 µg/mL | 10 | 2 | 80 |

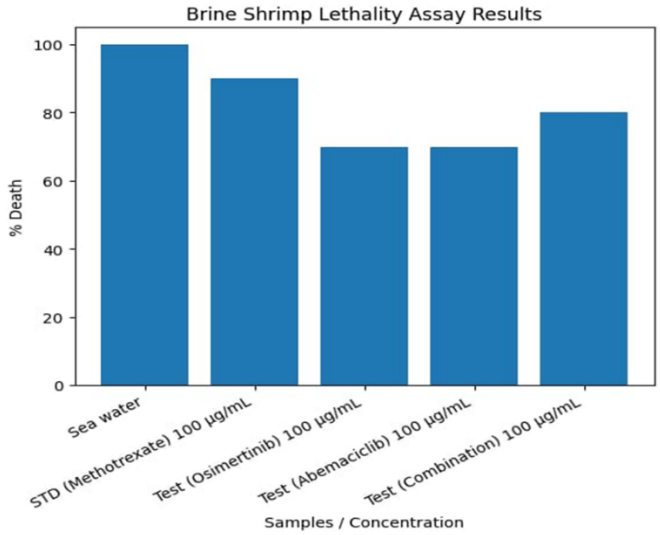



**Figure 10: brine shrimp assay result**

### 4.2 Allium cepa root growth assay

Allium cepa root tip meristems have been widely used for the evaluation of cytotoxicity, anti-mitotic activity, and genotoxicity. Characterized by rather

homogenous meristematic cells, very large chromosomes and only sixteen chromosome numbers (2n = 16), the Allium cepa species (common onion) is ideal for use in bioassays. Onion root meristem cells are very sensitive to genetic damage by chemicals. Allium test could be useful in correlating the antimitotic effect of the plant in Allium cepa with that of mammalian cells as it is reported that Allium test shows good correlation with mammalian test systems. Allium assay has been shown to have correlation with tests in other living systems and serve as an indicator of toxicity of the tested material. The assay also helps to study genotoxicity by observing chromosomal aberrations. Chromosomal aberrations refer to any irregularity or abnormality of chromosome distribution, number, structure, or arrangement. A chromosomal aberration is defined as any abnormality in the structure or the number of chromosomes. Chromosomal aberrations refer to genotoxicity.The onion bulbs used for testing should be of similar size (approximately 1.5–2.0 cm in diameter) and not exposed to herbicide or fungicide treatments. Generally, between three and five onion bulbs are needed for each sample (including the control) to obtain roots. According to the protocol proposed by Tedesco and Laughinghouse as a standard experiment for the Allium test, it is recommended to use five different sets of bulbs: one for the negative control, one for the positive control with a known genotoxic agent, and three groups for different concentrations of the test agent. Some authors initially use a larger number of bulbs to test their germination rate, placing them in water for two or four days. Subsets of three or five bulbs which showed the best root growth are then chosen to be exposed to the test solutions. It is recommended that the bulbs be lightly scraped in the lower area (primary root ring), to favor the emergence of new roots. Many studies report that onion bulbs are initially placed in distilled or tap water (if potable) in narrow glass or plastic containers (50 mL). Only the area where the roots will form is submerged in water. The water should be renewed every day until the roots grow to a certain length. Root growth varies in time (two to four days), depending on the temperature conditions in which the onion bulbs are stored (a room or growth room/chamber at 22 ± 2 °C). If onion bulbs are placed in a growth chamber, a controlled photoperiod (18 h/6 h light/dark) can also be ensured. When the roots reach the appropriate length (0.5–2 cm), the onion bulbs may be transferred to the flasks containing the different test extracts and only the base of each bulb should be immersed/suspended in the extract. The exposure time of onion roots in the tested plant extracts may vary: 24 h, 48 h, 72 h , 96 h. Sabini et al. reported two and five days, as well as two days followed by three days with water (reversion) of exposure.[71,72]

### 4.2.1 Materials & Reagents

- 5 bulbs of allium cepa
- Coupling jars containing test liquid
- 1 N HCL , glacial acetic acid & ethanol
- Water glass, cover slip & slide
- Normal saline water, std. drug, test concentration of drug
- Microscope with 40x lens

### 4.2.2 Procedure for Allium assay:

Healthy and nearly equal sized bulbs, weighing in the range of 30-35 g, of common onion (Allium cepa L.) were chosen for the experiments. For each test compound, the onion bulbs were randomly divided into eight groups, with three onion bulbs in each group. Each onion bulb was kept on a container containing tap water so that only the stem portion is dipped into water. After three days, when most of the roots attained the length of about 20–30 mm, the unhealthy smaller roots were discarded and the three groups of onion root bulb

were transferred to the beakers having three different concentrations of the synthesized compounds. Onions of the fourth group were kept on the containers containing distilled water (control 1). Onions from fifth group were kept on the containers containing the solvent used for preparing dilutions of the synthesized compounds (DMSO). Onions from the sixth, seventh and eighth groups were kept on the containers containing solutions of a Methotrexate, positive controls.[73]To study the effect of synthesized compounds on root growth inhibition, The length of onion roots in different groups were measured daily for seven days. To study the effect of synthesized compounds on mitotic index (MI), the mitotic index (MI) was calculated by counting 1,000 cells per root.The roots thus treated with the above solutions were then cut to separate root tips and the root tips were transferred to the fixing solution {acetic acid (45% v/v) ethanol (95% v/v) in ratio of 1:3 v/v} for 10 15 hrs. After 10-15 hours, these were treated with 1 N hydrochloric acid and warmed in an oven at 50°C for 15 min. These root tips were then washed with distilled water and were stained with 2-3 drops of Carmine stain (LR Central Drug House Pvt Ltd). The slide was then squashed and observed under microscope. The numbers of cells in each stage of cell division were counted in four fields for each group. Mitotic Index was calculated using the formula Number of dividing cells Mitotic Index =Number of dividing cells/Total number of cells x 100 Total number of cells.[74,75,76]



**Figure 11: root growth of onion**







**Table 6: Average growth (in mm) in roots of Allium cepa**

| Concentration | Treatment | Root growth(mm) |
|---|---|---|
| 0 | water | 15.7 |
| 100µg/mL | Methotrexate | 1.3 |
| 100µg/mL | Abemaciclib | 11.4 |
| 100µg/mL | Osimertinib | 7.8 |
| 100µg/mL | Combination | 2.4 |

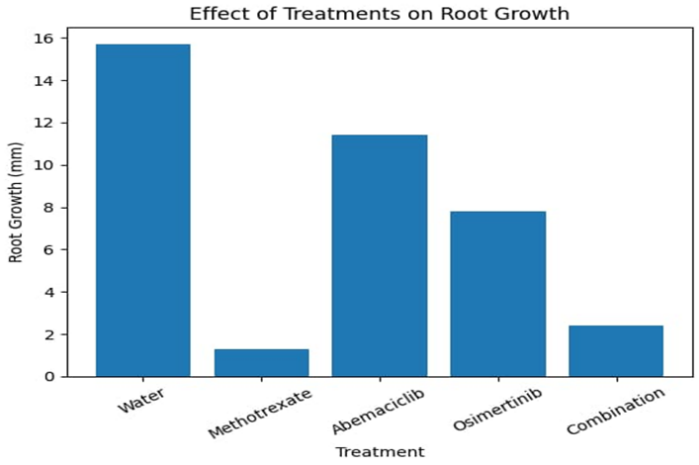



**Figure 12: Effect of treatment on root growth**

**Table 7: Mitotic Index of drugs**

| Concentration | Treatment | Mitotic cell | Total cell | MI% |
|---|---|---|---|---|
| 0 | water | 130 | 1000 | 13 |
| 100µg/mL | Osimertinib | 63 | 1000 | 6.3 |
| 100µg/mL | Abemaciclib | 40 | 1000 | 4 |
| 100µg/mL | Methotrexate | 15 | 1000 | 1.5 |
| 100µg/mL | Combination | 22 | 1000 | 2.2 |

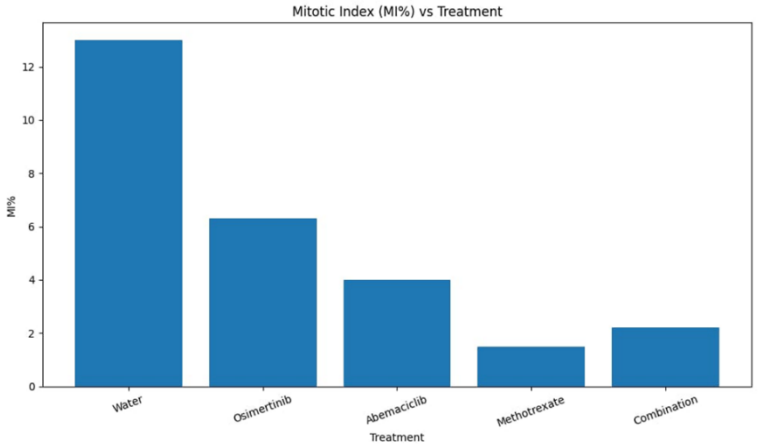



**Figure 13: Mitotic Index vs treatment**

### 4.3 Bilayer tablet

In the last decade, interest in developing a combination of two or more Active Pharmaceutical Ingredients (API) in a single dosage form (bi-layer tablet) has increased in the pharmaceutical industry, promoting patient convenience and compliance. Bi-layer tablets can be a primary option to avoid chemical incompatibilitiesbetween APIS by physical separation, and to enable the development of different drug release profiles (immediate release with extended release).[77]

### 4.3.1 TYPES OF BILAYER TABLET

The term bilayered tablets contains subunits that may be either the same (homogeneous) or different (heterogeneous).

**HOMOGENOUS TYPE**

Bilayer tablets are preferred when the release profiles of the Drugs are different from one another. Bilayer tablets allow for designing and modulating the dissolution and release characteristics. Bilayer tablets are prepared with one layer of drug for immediate release while second layer designed. To release drug, later, either as second dose or in an Extended release manner.

**HETEROGENEOUS TYPE**

Bilayered tablet is suitable for sequential separation two incompatible substance. release of two drugs in combination[78]

**4.3.2 Formulation of bilayer tablet Bilayer tablet is formulated by mainly two methods:-**

Wet granulation method

Direct compression method

**4.3.3 Direct compression method**

Weigh accurately all the excipients and drugs and pass through sieve# 100. Mix all the components in a pestle mortar and compress them in the form of tablet directly

Steps for compression cycle of bilayer tablet

1. Filling of first layer.
2. Compression of first layer.
3. Ejection of upper punch.
4. Filling of second layer.
5. Compression of both the layers together.
6. Ejection of bilayer tablet.

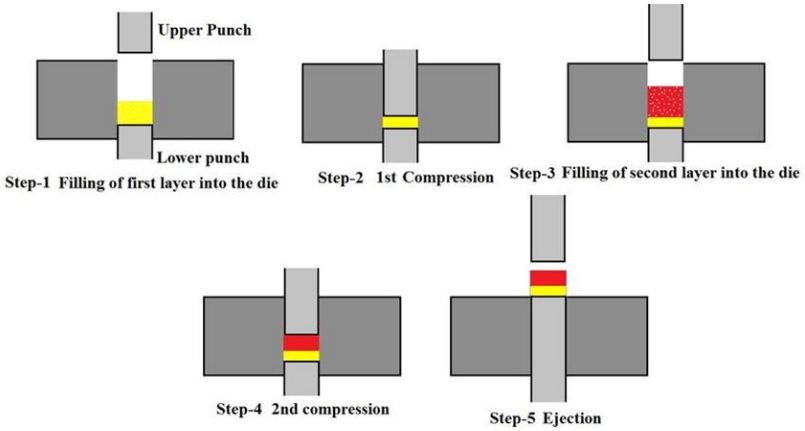



**Figure 14: steps of bilayer tablet**

**4.3.4 Formulation Tables with Ingredient Uses**

**Formulation table 8: Osimertinib layer**

| Sr. No. | Ingredient | Qty (mg) | Batch Qty | Use |
|---|---|---|---|---|
| 1 | Osimertinib | 47.7 | 238.5 | API |
| 2 | Mannitol | 110 | 550 | Diluent |
| 3 | MCC | 55 | 275 | Binder |
| 4 | Crospovidone | 18 | 90 | Disintegrant |
| 5 | SLS | 4 | 20 | Surfactant |
| 6 | Colloidal Silicon Dioxide | 5 | 25 | Glidant |
| 7 | Magnesium Stearate | 10.3 | 51.5 | Lubricant |

**Formulation table 9: Abemaciclib layer**

| Sr. No. | Ingredient | Qty (mg) | Batch Qty | Use |
|---|---|---|---|---|
| 1 | Abemaciclib | 150 | 750 | API |
| 2 | MCC | 120 | 600 | Diluent |
| 3 | Lactose Monohydrate | 55 | 275 | Diluent |
| 4 | Sodium starch glycolate | 20 | 100 | Disintegrant |
| 5 | Colloidal Silicon Dioxide | 8 | 40 | Glidant |
| 6 | Magnesium Stearate | 17 | 85 | Lubricant |

**Total 370 mg**



**Figure 15: Bilayer tablet**

### 4.3.5 Characterization of bilayer tablet

1. Appearance
2. Weight variation
3. Thickness
4. Hardness
5. Friability
6. Disintegration time
7. Dissolution time[79]

## 5: Evaluation

### 5.1 Weight variation

Weigh 20 tablets accurately. Determine average weight of tablets. The individual weight of each tablet was compared with average tablet weight.[80]

**Table 10: Weight variation**

| Tablet | Weight of tablets (mg) |
|---|---|
| 1 | 622 |
| 2 | 621 |
| 3 | 622 |
| 4 | 623 |
| 5 | 624 |
| 6 | 625 |
| 7 | 624 |
| 8 | 624 |
| 9 | 623 |
| 10 | 622 |
| 11 | 624 |
| 12 | 624 |
| 13 | 623 |
| 14 | 624 |
| 15 | 622 |
| 16 | 623 |
| 17 | 624 |
| 18 | 624 |
| 19 | 624 |
| 20 | 622 |
| Average | 623.2 |

### 5.2 Hardness:

The tablet crushing strength was tested by commonly used Pfizer tablet hardness tester. A tablet is placed between the anvils and the crushing strength, which causes the tablet to break, is recorded. Hardness Showing Tablet Crushing Strength which causes the tablet to break, is recorded.

**Table 11: Hardness**

| No. of Tablet | Hardness(Kg/cm$^2$) | Avg. |
|---|---|---|
| 1 | 4.5 | |
| 2 | 4.6 | 4.533 |
| 3 | 4.5 | |

### 5.3 Friability:

Tablet strength was tested by Roche friabilator. Pre weighed tablets were given 100 revolutions in 4 min and were dedusted. The percentage weight loss was calculated by reweighing the tablets.Add 11 bilayer tablets approximately 6820 mg in friabilator.

% Friability = [(Initial Weight - Final Weight) / Initial Weight] × 100

= 6820-6800/ 6820 x 100

=20/6820 x 100

= 0.293%

### 5.4 Thickness

Randomly tablet was selected and its thickness was measured by using vernier caliper scale.

Table 12: Thickness

| No. of Tablet | Thickness(mm) | Avg. |
|---|---|---|
| 1 | 5.5 | |
| 2 | 5.5 | 5.466 mm |
| 3 | 5.4 | |

### 5.5 Disintegration time

6 tablets are taken in disintegration apparatus with acetate buffer pH 4 medium at 370C. Calculate time at which tablet gets converted to soluble particles. Disintegration time for immediate release tablets and bilayer tablets was determined. Disintegration time for immediate release tablets should not be more than 15 minutes.[81]

**Table 13: Disintegration time**

| No. of Tablet | Dt time (min) | Avg. |
|---|---|---|
| 1 | 14.3 | |
| 2 | 14.3 | 14.26 min |
| 3 | 14,2 | |

### 5.6 Dissolution time

Dissolution profile is evaluated with the help of USP paddle apparatus. 900ml of suitable dissolution mediums are taken in vessel maintained at 370C at 75rpm. The dissolution was carried out for about 12 hrs. 5ml of sample was withdrawn at regular time intervals, and 5ml of fresh medium is inserted in vessel. Absorbance is recorded for each sample at specific lambda maxima for the combination drugs.

#### 5.6.1 Preparation of Osimertinib calibration curve

Weigh 100 mg Osimertinib API (accurate to 0.1 mg). Transfer to 100 mL volumetric flask. Add 2-3 mL DMSO → sonicate 10 min. Dilute to 100 mL mark with acetate buffer pH 4. Sonicate 15 min. Pipette 10.0 mL stock solution (1 mg/mL) into 100 mL volumetric flask. Dilute to 100 mL mark with acetate buffer pH 4. Mix well.

1. Label six 10 mL volumetric flasks (blank + 5 standards).
2. Blank: Add 10 mL acetate buffer pH 4.
3. For each standard: Pipette exact volume of 10 µg/mL working solution
4. Add acetate buffer pH 4 to 10 mL mark
5. Mix thoroughly by inversion
6. Scan 200-400 nm vs. blank → confirm $\lambda_{max} \approx 267$ nm
7. Measure absorbance at 267 nm for all standards
8. Plot: Absorbance (Y-axis) vs. Concentration (X-axis)
9. Equation: $y = mx + c$ ($R^2 \geq 0.999$)

**Table 14: Calibration curve For Osimertinib**

| Concentration | Absorbance |
|---|---|
| 0.1 | 0.0394 |
| 0.2 | 0.0708 |
| 0.3 | 0.0951 |
| 0.4 | 0.1201 |
| 0.5 | 0.1501 |

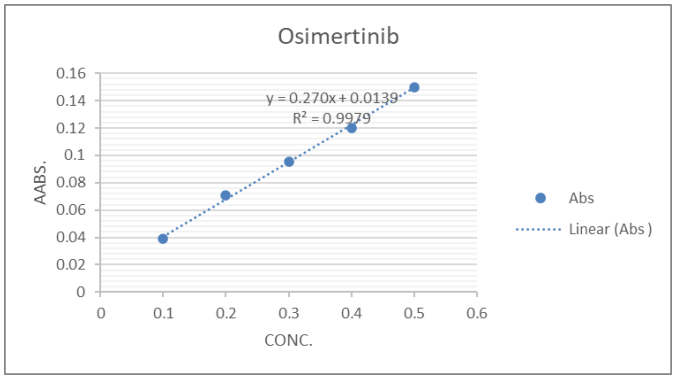



**Table 15: calibration curve for Abemaciclib**

| Concentration | Absorbance |
|---|---|
| 0.1 | 0.0729 |
| 0.2 | 0.0957 |
| 0.3 | 0.1142 |
| 0.4 | 0.1374 |
| 0.5 | 0.1524 |

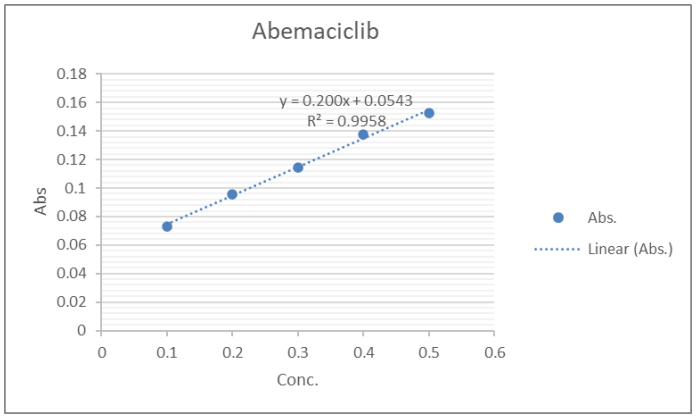


**Table 16: %DR of Osimertinib (267 nm)**

| Time (min) | Abs | Conc. | Df | Actual conc. | In mg/ 5 mL | Conc./900ml | Cumulative | %DR |
|---|---|---|---|---|---|---|---|---|
| 5 | 0.3972 | 1.7085 | 20 | 34.170 | 0.034170 | 30.753 | 30.753 | 12.30 |
| 10 | 0.4561 | 2.0019 | 20 | 40.038 | 0.040038 | 36.0342 | 66.7872 | 26.71 |
| 15 | 0.5435 | 2.4374 | 20 | 48.748 | 0.048748 | 43.8732 | 110.6604 | 44.26 |
| 20 | 0.5981 | 2.7095 | 20 | 54.190 | 0.054190 | 48.771 | 159.4314 | 63.77 |
| 25 | 0.6678 | 3.0568 | 20 | 61.136 | 0.061136 | 55.0224 | 214.4538 | 85.78 |

**Table 17: %DR of Abemaciclib(298 nm)**

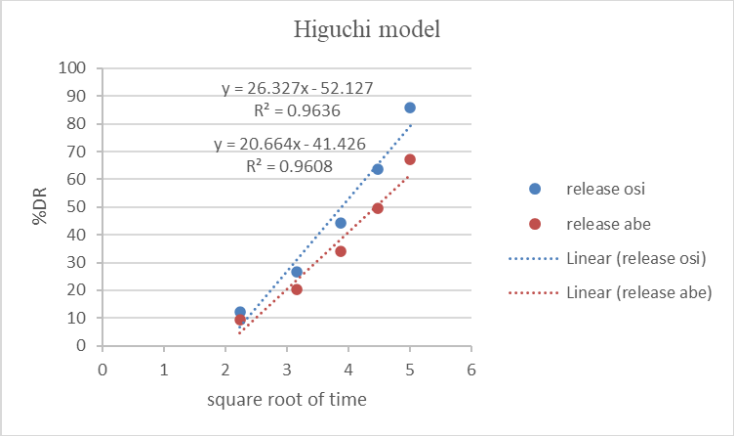
| Time (min) | Abs | Conc. | Df | Actual conc. | In mg/ 5 mL | Conc./900ml | Cumulative | %DR |
|---|---|---|---|---|---|---|---|---|
| 5 | 0.5296 | 1.9050 | 20 | 38.1 | 0.0381 | 34.29 | 34.29 | 9.267 |
| 10 | 0.6358 | 2.2973 | 20 | 45.946 | 0.045946 | 41.35 | 75.64 | 20.44 |
| 15 | 0.7681 | 2.7861 | 20 | 55.722 | 0.055722 | 50.149 | 125.78 | 33.994 |
| 20 | 0.8727 | 3.1725 | 20 | 63.45 | 0.06345 | 57.105 | 182.88 | 49.427 |
| 25 | 0.9812 | 3.6246 | 20 | 72.492 | 0.072492 | 65.242 | 248.12 | 67.05 |

**Higuchi plot**

## 5.7 ANALYSIS OF RELEASE PROFILES

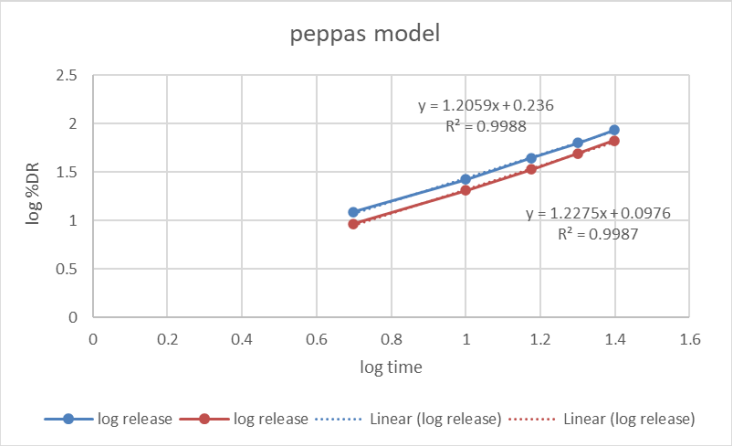
The rate and mechanism of release of bilayer tablet was analyzed by kinetic model such as[82]

**Graph no: 01 % drug release v/s square root of time**



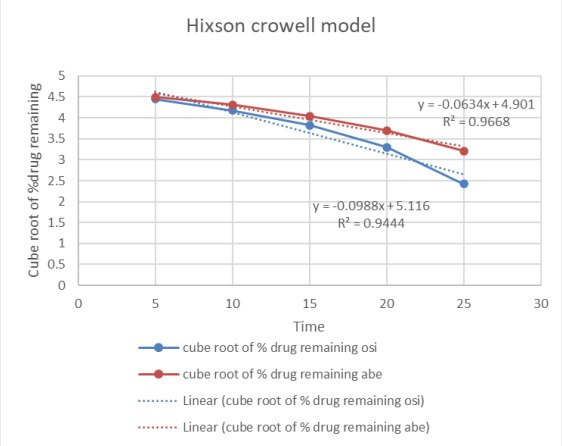

**Peppas Model**

**Graph no: 02 log % drug release v/s log time**




**Hixson Crowell cube rout model**

**Graph no: 03 cube root of % drug remaining v/s time**




## 6 RESULT AND DISCUSSION

### 6.1) Brine shrimp lithality test

Both Osimertinib and Abemaciclib possess moderate cytotoxic activity individually.
The combination therapy exhibits higher cytotoxicity (80%), suggesting a possible synergistic or additive effect.
The activity of the combination is slightly lower than the standard Methotrexate but significantly higher than individual treatments.

### 6.2) Allium cepa root growth test

The effect of different treatments on Allium cepa root growth was assessed by measuring the average root length (mm) after exposure to 100 µg/mL concentrations of test compounds.
The control group (water) showed the highest root growth, with an average length of 15.7 mm, indicating normal cell division and growth. In contrast, all treated groups exhibited a reduction in root growth compared to the control.

**Among the individual treatments:**

Methotrexate showed the strongest inhibitory effect, with a drastic reduction in root growth to 1.3 mm, suggesting high cytotoxicity.

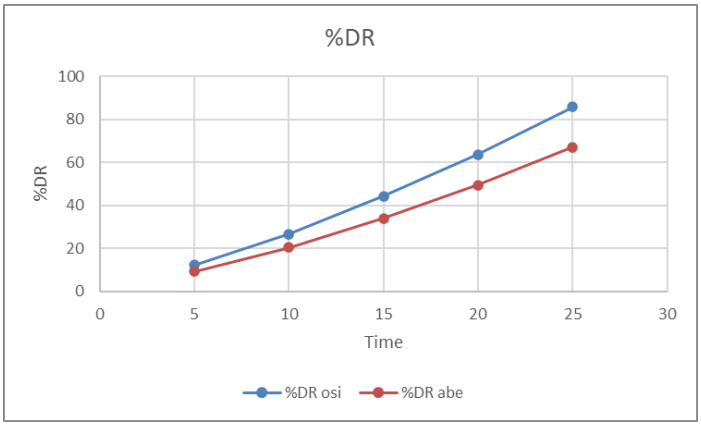
Abemaciclib resulted in moderate inhibition, with an average root length of 11.4 mm, indicating partial suppression of cell division.

Osimertinib showed greater inhibition than Abemaciclib, reducing root growth to 7.8 mm.

The combination treatment exhibited a strong inhibitory effect, with root growth reduced to 2.4 mm, which is significantly lower than most single-drug treatments but slightly higher than Methotrexate alone.

### 6.3) Bilayer tablet evaluation result

| Sr.no. | Parameter | Result |
|---|---|---|
| 1 | Hardness | 4.533 |
| 2 | Friability | 0.293% |
| 3 | Thickness | 5.466 mm |
| 4 | Disintegration time | 14.26 min |
| 5 | %DR | |
| | Osimertinib | 85.78% |
| | Abemaciclib | 67.05% |




### 6.4) Regression analysis

| Sr. no | Linear regression analysis | | |
|---|---|---|---|
| | Higuchi drug release | Korsemeyer peppas drug release | Hixson crowell drug release |
| 1- Osimertinib | 0.963 | 0.998 | 0.966 |
| 2- Abemaciclib | 0.960 | 0.998 | 0.944 |

From above kinetic model Peppas model was best fitted for release kinetics of bilayer tablet.

## CONCLUSION

The present study was designed to explore a dual-targeted strategy for lung cancer by combining EGFR inhibition with CDK4/6 blockade and to integrate this combination into a patient-friendly oral dosage form. Preliminary biological screening using brine shrimp lethality and Allium cepa root assays showed that both osimertinib and abemaciclib possess measurable cytotoxic and antiproliferative activity when used alone, while the combination produced higher lethality and stronger suppression of root growth and mitotic index than either single drug. Although the combination remained slightly less potent than the standard methotrexate in both models, the data indicate a meaningful enhancement of biological

activity, suggesting a synergistic or at least additive effect when EGFR and CDK4/6 pathways are inhibited simultaneously.From a formulation perspective, an immediate-release bilayer tablet containing an osimertinib layer and an abemaciclib layer was successfully developed using direct compression and pharmaceutically acceptable excipients. The finished tablets complied with standard quality attributes, including acceptable weight variation, adequate mechanical strength (hardness around 4.53 kg/cm²), low friability (0.293%), suitable thickness (5.466 mm) and disintegration within 15 minutes, confirming their robustness and suitability for oral administration. In vitro dissolution studies demonstrated rapid and substantial drug release (approximately 85.78% for osimertinib and 67.05% for abemaciclib), and regression analysis indicated that the Korsmeyer–Peppas model best described the release kinetics for both drugs, reflecting a diffusion-controlled mechanism from the bilayer matrix.Taken together, these findings support the hypothesis that concurrent EGFR and CDK4/6 inhibition through the combination of osimertinib and abemaciclib can offer superior preliminary cytotoxic and antiproliferative effects compared with monotherapy, and that an IR–IR bilayer tablet is a feasible platform for simultaneous delivery of both agents. However, the bioassays employed are simple screening models and do not fully replicate the complexity of human lung cancer, indicating that further work is required in cancer cell lines, animal models, stability studies and eventual clinical evaluation to confirm the therapeutic benefit, safety and pharmacokinetic performance of this combination dosage form.

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