Role of prostanoid receptor EP1 in the progression of Non-Small Cell Lung Cancer (NSCLC)

Inaugural Dissertation
Submitted to the
Faculty of Medicine
in partial fulfilment of the requirements
for the degree of Doctor of Human Biology
in the Faculty of Medicine
of the Justus Liebig University of Giessen
by

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Giessen 2012
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Date of Doctoral Defense: 07-05-2013

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LIST OF ABBREVIATIONS

AC	Adenylate cyclase
APS	Ammonium persulphate
bp	Base pairs
BSA	Bovine serum albumin
cAMP	Cyclic 3’5’-adenosine monophosphate
cDNA	Complementarz deoxzribonucleic acid
cGMP	Cyclic 3’5’-guanosine monophosphate
COX1	Cyclooxygenase 1
COX2	Cyclooxygenase 2
Cpm	Counts per minute
CREB	cAMP response element binding
Ct	Threshhold cycle
ΔΔCt	Delta delta Ct
^{o}C	Centigrade
Da	Dalton
DAPI	4’,6-diamidino-2-phenylindole
DEPC	Diethyl-pyrocarbonate
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylendinitrilo-N,N,N’,N’ tetra acetate
EP1	Prostaglandin E Receptor 1
EP2	Prostaglandin E Receptor 2
EP3	Prostaglandin E Receptor 3
EP4	Prostaglandin E Receptor 4
et al	et al (others)
FBS	Fetal bovine serum
FITC	Fluorescene-5-isothiocyanate
gm	Grams
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H	Hour(s)
HBSS	Hank’s balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horse radish peroxidase
IBMX	3-isobutyl-1-methyl xanthine
Kb	Kilo basepairs
Kda	Kilo dalton
M	Molar
Mg	Milligram
Min	Minute(s)
Ml	Millilitre
mM	Millimolar
mRNA	messenger ribonucleic acid
µCi	Microcurie
µg	Microgram
µl	Microlitre
µm	Micrometer

ABBREVIATIONS	
µM	Micromolar
nM	Nanomolar
PAGE	Poly acrylamide gel electrophoresis
PBGD	Porphobilinogen deaminase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
PGH2	Prostaglandin H2
PKG	Protein kinase G
PKA	Protein kinase A
PMSF	Phenyl methyl sulfonyl fluoride
P/S	Penicillin/Streptomycin
qRT-PCR	Quantitative real time polymerase chain reaction
rpm	Rotations per minute
RT-PCR	Reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Room temperature
SDS	Sodium dodecyl sulphate
Sec	Second(s)
TAE	Tris-acetate EDTA
TBST	Tris-buffered saline buffer+0.1% Tween 20
TCA	Trichloro acetic acid
TEMED	N,N’,N’-tetra methyl-ethane-1,2-diamine
Tris	Tris-(hydroxyl methyl)-amino methane
UV	Ultraviolet
V	Volt

1 SUMMARY

Increasing evidence indicates that Prostaglandin E2 (PGE2) promotes lung tumor growth by stimulating prostaglandin E receptor type 4 (EP4) signalling with subsequent enhancement of cellular proliferation, metastasis and suppression of immune responses. However, the role of other EP receptors in cancer is still not known. We hypothesized that prostanoid receptors other than the EP4 receptor might be involved in progression of Lung cancer.

We analyzed the expression profile of EP receptors in lung adenocarcinoma cell lines A549 and H1299 by using RT-PCR, western blotting and immunofluorescence. All four EP receptors were found to be expressed in both cell lines. While EP1 and EP2 receptors were localized on both cell and nuclear membrane, expression of EP3 and EP4 receptors was limited to the cell membrane.

The EP4 receptor antagonist, L-161982 and the EP1 receptor antagonist SC-51322 inhibited proliferation of both NSCLC cell lines. EP2 and EP3 receptor blockers did not inhibit proliferation. SC-51322 did not show any effect on cAMP levels in A549. Interestingly, it led to a decrease in intracellular calcium levels suggesting a contribution of calcium signaling to proliferation of cancer cells.

The anti-proliferative effects of EP1 receptor inhibition was proved to be largely due to a decrease in intracellular calcium levels that may subsequently alter downstream signaling events such as phosphorylation of the Extra cellular Regulated Kinase (ERK). In our investigation, we found that inhibition of EP1 receptor by employing the selective inhibitor SC-51322, did not elicit an increase in intracellular calcium, reduced ERK phosphorylation and attenuated proliferation of A549 and H1299 cells. Furthermore, SC-51322 inhibited migration of A549 cells in presence of FCS and the EP1 receptor specific agonist 17-P-T-PGE2.

Taken together, this study supports a central role of EP1 receptor in progression and migration of A549 cells. Investigating the signaling pathway downstream to EP1 receptor will provide us with a better understanding of the contribution of this pathway in lung cancer and a novel therapeutic opportunity for the treatment of NSCLC.

2 ZUSAMMENFASSUNG

Zunehmende Hinweise zeigen, dass Prostaglandin E2 (PGE2) Lungentumorwachstum fördert, indem es Prostaglandin E-Rezeptor Typ 4 (EP4) stimuliert, was eine anschließende Erhöhung der zellulären Proliferation, Metastasierung und Unterdrückung von Immunantworten zur Folge hat. Jedoch ist die Rolle der anderen EP-Rezeptoren in Krebszellen noch unbekannt. Wir stellten die Hypothese auf, dass abgesehen vom EP4-Rezeptor noch andere Prostanoidrezeptoren im Fortschreiten von Lungenkrebs beteiligt sein könnten.

Wir untersuchten die Expression von EP-Rezeptoren in den aus Lungen stammenden Adenokarzinom-Zelllinien A549 und H1299 mit RT-PCR, Western Blot und Immunfluoreszenz. Es konnte nachgewiesen werden, daß alle vier EP-Rezeptoren in beiden Zelllinien exprimiert werden. Während EP1 und EP2-Rezeptoren auf Zell- und Kernmembran gefunden werden konnten, war die Expression von EP3 und EP4-Rezeptoren auf die Zellmembran beschränkt.

Der EP4-Rezeptor-Antagonist, L-161982 als auch der EP1-Rezeptor-Antagonist SC-51322 inhibierte die Proliferation von beiden NSCLC-Zelllinien. EP2 und EP3-Rezeptor-Blocker hingegen konnten die Proliferation nicht hemmen. SC-51322 zeigte keinen Einfluss auf den cAMP-Level in A549 Zellen. Interessanterweise führte dies zu einer Abnahme des intrazellulären Kalziumspiegels, was auf eine Beteiligung des Calcium-Signalwegs bei der Proliferation von Krebszellen schließen läßt.

Die antiproliferative Wirkung von EP1-Rezeptor-Hemmung erwies sich als Folge der Abnahme des intrazellulären Calcium-Spiegels, die nachgeschaltete Signalwege, wie die Phosphorylierung der „Extrazellular Regulated Kinase“ (ERK) verändern können. In unserer Untersuchung haben wir festgestellt, dass die Inhibition des EP1-Rezeptors durch die Verwendung des selektiven Inhibitors SC-51322, keine Erhöhung des intrazellulären Calciumlevels hervorruft, auch die ERK-Konzentration verringert und die Proliferation von A549 und H1299-Zellen hemmt. Darüber hinaus inhibiert SC-51322 die Migration von A549-Zellen in Gegenwart von FCS und dem EP1-rezeptorspezifischen Agonisten 17-P-T-PGE2.

Zusammengefasst unterstützt diese Studie eine zentrale Rolle des EP1-Rezeptors in der Progression und der Migration von A549-Zellen. Die Untersuchung des dem EP1-Rezeptor nachgeschalteten Signalwegs wird uns ein besseres Verständnis der Rolle dieses Signalwegs bei Lungenkrebs und eine neue therapeutische Möglichkeit zur Behandlung von NSCLC bieten.

3 INTRODUCTION

Lung cancer is the leading cause of cancer deaths for both men and women worldwide. The 5 year survival rate is ~14%, primarily due to inability to detect the disease early [1]. Lung cancer is the most common cancer in the world. Approximately 1.61 million cases, representing 12.7 % of all new cancers and 1.38 million deaths (18.2 % of the total) are from lung cancer. Lung cancer is now the fourth most frequent cancer of women with 8.5% of all cancers and the second most common cause of death from cancer with 12.8% of the total [2]. More than 70% of people diagnosed with lung cancer are the people above 65 years making it a disease of elderly while less than 3% of lung cancers occur in people less than 45 years of age [3].

Majority of cases now occur in developing countries. Lung cancer incidences are at high rates in Central-eastern and Southern Europe, Northern America and Eastern Asia. Very low rates are still estimated in Middle and Western Africa [4]. The highest incidence rate is observed in Northern America (where lung cancer is now the second most frequent cancer in women), and the lowest in Middle Africa (15th most frequent cancer) [5]. Lung cancer is expected to rise in those countries with high incidence of tobacco smoking though recent clinical advances like low-dose computed tomography screening and advancements in surgery and radiation may change this outcome [6].

The increase in fall of lung cancer incidences can be observed in many developing countries following public education about the dangers of smoking and introduction of effective smoking cessation programs.

According to 2009 estimates, lung cancer is the most commonly diagnosed cancer world wide among all the other cancers (Figure 1).

3.1 Etiology of lung cancer

3.1.1 Smoking

The most documented causal relationship in biomedical research has been the role of cigarette smoking in causing lung cancer, accounting for about 90% of lung cancer cases [7].

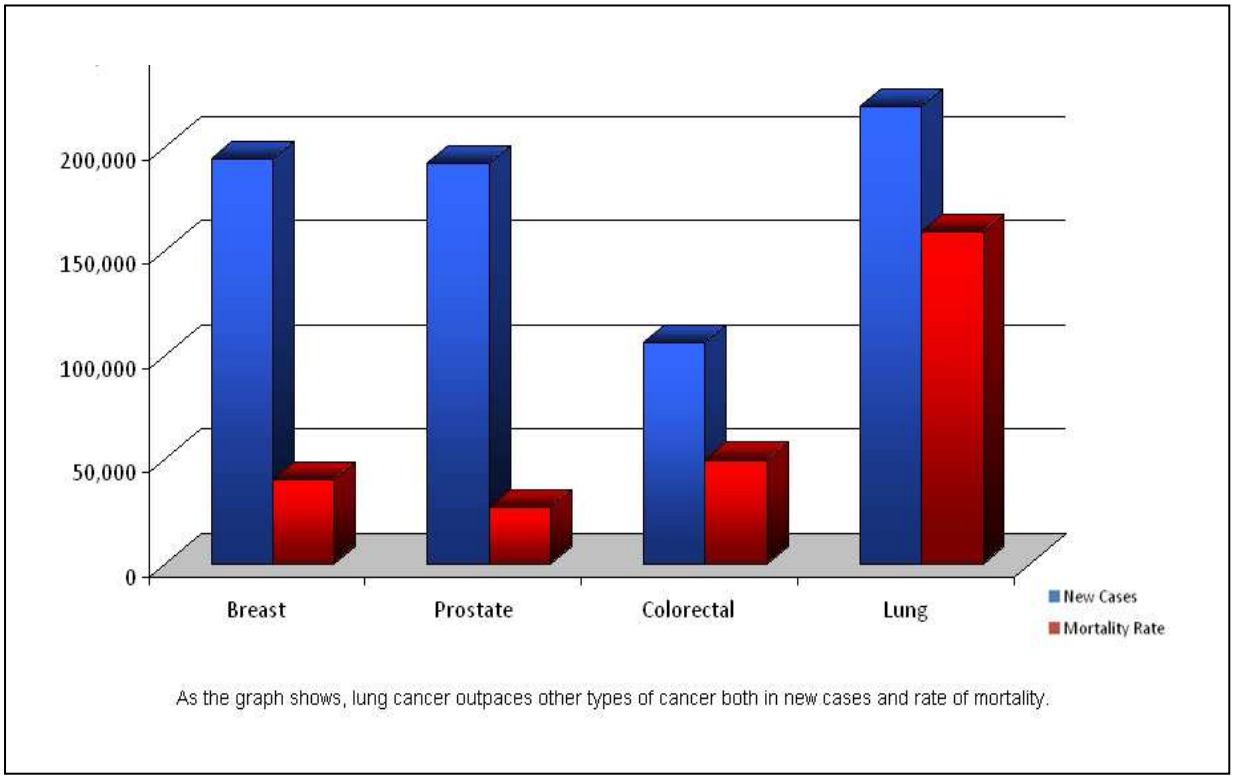


Figure 1: The American Cancer Society estimates 2009

Cigarette smokers smoking one pack of cigarettes per day are considered to have a 25 times higher risk of developing lung cancer than a non-smoker. Whereas, pipe and cigar smokers have 5 times higher risk than that of a non-smoker. Earliest links between smoking and lung cancer were reported by german scientists [6]. Epidemiologic studies in Britain and the United States using the case control methods had demonstrated that cigarettes smoking was strongly associated with the risk of lung cancer [8], [9] and these results were further, corroborated by American Cancer Society.

3.1.2 Passive smoking

Involuntary inhalation of tobacco smoke by nonsmokers, has also been found to cause lung cancer. Two studies published in 1981 ascribed that the inhalation of second hand smoke or environmental tobacco smoke led to an increase in the risk of lung cancer in passive smokers [6], [10]. Additionally, a review by the National Research Council has concluded that there is a chance of developing lung cancer by 30% in spouses who are married to cigarette smokers than spouses of non-smokers [6].

3.1.3 Asbestos fibers

Retrospective cohort studies by Doll et al in 1955 [11] have established asbestos also as one of the leading causes of lung cancer. Increased exposure to asbestos is directly proportional to increased risk of lung cancer [12]. The mechanism of how asbestos exposure leads to cancer whether by acting as a carcinogen itself or by causing chronic inflammation is to be studied. Though asbestos and cigarette smoking are independent causes of lung cancer, in combination, they act synergistically to increase the risk of lung cancer. Cigarette smoking increases the risk of cancer associated with asbestos exposure probably by retention of asbestos fibres [6], [13].

3.1.4 Radon gas

Radon is an inert gas that is produced naturally from radium in the decay series of uranium. Two of the decay products of radon emit alpha particles that, by virtue of their high energy and mass, can cause damage to the DNA of cells of the respiratory epithelium. Radon gas is a well-known cause of lung cancer, with an estimated 12% of lung-cancer deaths attributable to radon gas. On average, indoor exposures to radon for the general population are much less than those received by occupational groups such as uranium miners. Cigarette smoking and radon decay products synergistically influence lung cancer risk in a supra additive manner [6].

3.1.5 Familial predisposition

Numerous studies have shown that relatives of both non-smoking and smoking people are prone to lung cancer. Recent studies have identified that human chromosome 15 contains genes coding for proteins that interact with nicotine and other tobacco toxins [6].

3.1.6 DNA repair

DNA repair capacity has now been examined as a specific risk factor for lung cancer. The lower the DNA repair capacity, the greater the risk of lung cancer from smoking [14]. Although much research remains to be done to delineate the link between those two, the evidence suggests that this is a promising lead.

3.1.7 Lung diseases

Substantial body of evidence shows that acquired lung diseases like Chronic Obstructive Pulmonary Disease (COPD) and fibrotic disorders increase the susceptibility of lung cancer by four to six fold [6].

3.1.8 Prior history of lung cancer

Survivors of lung cancer have a greater risk of developing a second lung cancer than the general population. Survivors of non-small cell lung cancers (NSCLCs) have an additive risk of 1% -2 % per year where as 6% per year for the survivors of small cell lung cancers (SCLCs) [6], [15].

3.1.9 Air pollution

The percentage of lung cancer deaths because of air pollution is as low as 1% of all cancer deaths, experts believe that it may get equivalent to death percentage of passive smoking upon prolonged exposure to air pollutants [6].

3.2 Lung cancer classification

Carcinomas of the lung, also known as primary lung tumors, are classified based on their histological appearance and tissue of origin or alternatively according to the predominant cell type. The histo-pathological type is connected with tumor behaviour and patients' prognosis [16].

The two main histological groups imply small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [16], [17].

These two types of cancers grow and spread in different ways and may have different treatment options. So, a distinction between these two types is important. SCLC comprises about 20% of lung cancers and is the most aggressive, rapidly growing of all lung cancers. SCLCs are strongly related to cigarette smoking, with only 1% of these tumors occurring in non-smokers. SCLCs have a greater metastatic potential and are most often discovered after they have spread extensively.

Table 1 Classification of Lung tumors

1 Epithelial Tumors
- 1.1. Benign
  - 1.1.1. Papillomas
    - 1.1.1.1. Squamous cell papilloma Exophytic Inverted
    - 1.1.1.2. Glandular papilloma
    - 1.1.1.3. Mixed squamous cell and glandular apilloma
  - 1.1.2. Adenomas
    - 1.1.2.1. Alveolar adenoma
    - 1.1.2.2. Papillary adenoma
    - 1.1.2.3. Adenomas of salivary-gland type Mucous gland adenoma Pleomorphic adenoma Others
    - 1.1.2.4. Mucinous cystadenoma
    - 1.1.2.5. Others
- 1.2. Preinvasive lesions
  - 1.2.1. Squamous dysplasia/Carcinoma in situ
  - 1.2.2. Atypical adenomatous hyperplasia
  - 1.2.3. Diffuse idiopathic pulmonary neuroendocrine cell hyperplasia
- 1.3. Malignant
  - 1.3.1. Squamous cell carcinoma Variants
    - 1.3.1.1. Papillary
    - 1.3.1.2. Clear cell
    - 1.3.1.3. Small cell
    - 1.3.1.4. Basaloid
  - 1.3.2. Small cell carcinoma Variant
    - 1.3.2.1. Combined small cell carcinoma
  - 1.3.3. Adenocarcinoma
    - 1.3.3.1. Acinar
    - 1.3.3.2. Papillary
    - 1.3.3.3. Bronchioloalveolar carcinoma
      - 1.3.3.3.1. Non-mucinous (Clara/pneumocyte type II)
      - 1.3.3.3.2. Mucinous
      - 1.3.3.3.3. Mixed mucinous and non-mucinous or intermediate cell type
    - 1.3.3.4. Solid adenocarcinoma with mucin
    - 1.3.3.5. Adenocarcinoma with mixed subtypes
    - 1.3.3.6. Variants
      - 1.3.3.6.1. Well-differentiated fetal adenocarcinoma
      - 1.3.3.6.2. Mucinous ("colloid") adenocarcinoma
      - 1.3.3.6.3. Mucinous cystadenocarcinoma
      - 1.3.3.6.4. Signet-ring adenocarcinoma
      - 1.3.3.6.5. Clear cell adernocarcinoma
  - 1.3.4. Large cell carcinoma Variants
    - 1.3.4.1. Large cell neuroendocrine carcinoma
      - 1.3.4.1.1. Combined large cell neuroendocrine carcinoma
      - 1.3.4.2. Basaloid carcinoma
      - 1.3.4.3. Lymphoepithelioma-like carcinoma
      - 1.3.4.4. Clear cell carcinoma
      - 1.3.4.5. Large cell carcinoma with rhabdoid phenotype
  - 1.3.5. Adenosquamous carcinoma
  - 1.3.6. Carcinomas with pleomorphic, sarcomatoid or sarcomatous elements

Table 1 continued

- 1.3.6.1. Carcinomas with spindle and/or giant cells
  - 1.3.6.1.1. Pleomorphic carcinoma
  - 1.3.6.1.2. Spindle cell carcinoma
  - 1.3.6.1.3. Giant cell carcinoma
- 1.3.6.2. Carcinosarcoma
- 1.3.6.3. Pulmonary blastoma
- 1.3.6.4. Others

1.3.7. Carcinoid tumor
- 1.3.7.1. Typical carcinoid
- 1.3.7.2. Atypical carcinoid

1.3.8. Carcinomas of salivary-gland type
- 1.3.8.1. Mucoepidermoid carcinoma
- 1.3.8.2. Adenoid cystic carcinoma
- 1.3.8.3. Others

1.3.9. Unclassified carcinoma

2 Soft Tissue Tumors
- 2.1 Localized fibrous tumor
- 2.2 Epithelioid hemangioendothelioma
- 2.3 Pleuropulmonary blastoma
- 2.4 Chondroma
- 2.5 Calcifying fibrous pseudotumor of the pleura
- 2.6 Congenital peribronchial myofibroblastic tumor
- 2.7 Diffuse pulmonary lymphangiomatosis
- 2.8 Desmoplastic small round cell tumor
- 2.9 Other

3 Mesothelial Tumors
- 3.1 Benign
  - 3.1.1 Adenomatoid tumor
- 3.2 Malignant
  - 3.2.1 Epithelioid mesothelioma
  - 3.2.2 Sarcomatoid mesothelioma
    - 3.2.2.1 Desmoplastic mesothelioma
  - 3.2.3 Biphasic mesothelioma
  - 3.2.4 Other

4 Miscellaneous Tumors
- 4.1 Hamartoma
- 4.2 Sclerosing hemangioma
- 4.3 Clear cell tumor
- 4.4 Germ cell neoplasms
  - 4.4.1 Teratoma, mature or immature
  - 4.4.2 Malignant germ cell tumor
- 4.5 Thymona
- 4.6 Melanoma
- 4.7 Others

5 Lymphoproliferative Disease
- 5.1 Lymphoid interstitial pneumonia
- 5.2 Nodular lymphoid hyperplasia
- 5.3 Low-grade marginal zone B-cell lymphoma of the mucosa-associated lymphoid tissue
- 5.4 Lymphomatoid granulomatosis

Table 1 continued

6 Secondary Tumors
7 Unclassified Tumors
8 Tumor-like Lesions
  8.1 Tumorlet
  8.2 Multiple meningothelioid nodules
  8.3 Langerhans cell histiocytosis
  8.4 Inflammatory pseudotumor (Inflammatory myofibroblastic tumor)
  8.5 Organizing pneumonia
  8.6 Amyloid tumor
  8.7 Hyalinizing granuloma
  8.8 Lymphangioleiomyomatosis
  8.9 Multifocal micronodular pneumocyte hyperplasia
  8.10 Endometriosis
  8.11 Bronchial inflammatory polyp
  8.12 Others

The new World Health Organization classification of lung tumors. E. Brambilla, W.D. Travis, T.V. Colby, B. Corrin, Y. Shimosato. ERS Journals Ltd 2001.

NSCLC are the most common lung cancers, accounting for about 80% of all lung cancers. NSCLC can be divided into three main types that are named based upon the type of cells found in the tumor.

3.2.1 Adenocarcinomas

Adenocarcinoma is the predominant histological subtype of lung carcinoma comprising 50% of lung cancers. It is the most prevalent form of lung cancer in younger males (< 50 yrs old), in women of all ages, in never smokers, and in former smokers. Adenocarcinomas are histologically heterogenous, making them difficult to sub-classify. Only a minority of cases like acinar, papillary and solid adenocarcinoma show a pure histological pattern. Most adenocarcinomas arise in the outer, or peripheral, areas of the lungs. Bronchioloalveolar carcinoma is a subtype of adenocarcinoma that frequently develops at multiple sites in the lungs and spreads along the preexisting alveolar walls [16], [18].

3.2.2 Squamous cell carcinomas

Squamous cell carcinomas are next most prevalent to adenocarcinomas. This type is mainly located centrally, with common segmental or lobar collapse, and tumors are often larger than 4cm in diameter. This histological type, correlated with a smoking history, is more prevalent in men than women. At present, they account for about 30% of NSCLC. They are also known as epidermoid carcinomas [18].

3.2.3 Large cell carcinoma

Large cell carcinomas, sometimes referred to as undifferentiated carcinomas, are the least common type of NSCLC with early metastasis to the mediastinum and brain. It is a fast-growing form that grows near the surface of the lung. They are found in approximately 10%-15% of lung cancer cases.

Other rare types of cancer may be found, although they represent a very small percentage (less than 5%) of the total number of lung cancer cases, which includes adenosquamous carcinoma, sarcomatoid carcinoma, carcinoid tumor, and salivary gland tumors [19], [20].

3.3 Lung cancer symptoms and signs

Symptoms of lung cancer are not always easy to identify and sometimes there may not be any symtoms. Symptoms of lung cancer vary depending upon the severity of it. The following kinds of symptoms can be observed in lung cancer patients –

No symptoms: In up to 25% of patients with lung cancer, it is first discovered on a routine chest X-ray or CT scan as a solitary small mass sometimes often called a coin lesion, as in a two-dimensional X-ray or CT scan, the round tumor resembles a coin. These patients with coin lesions often report no symptoms at the time the cancer is discovered.

Symptoms related to the cancer: Breathing problems like cough, shortness of breath, wheezing leading to chest pain and coughing up blood result due to the growth and invasion of cancers in to the surrounding tissues. Pancoast's syndrome (shoulder pain etc) or paralysis of the vocal cords occurs when cancer invades nerves. Invasion of oesophagus leads to dysphagia and invasion of airways leads to the collapse of that particular portion of lung leading to infections like pneumonia and abscesses [19].

Symptoms related to metastasis: Metastatic cancers when they are in bones produce excruciating pains in joints and the places where there is an involvement of bone. When it spreads to brain, it may lead to loss of sensation to parts of body along with symptoms like blurred vision, seizures and symptoms of stroke [19].

Paraneoplastic symptoms: Lung cancers are frequently accompanied by symptoms that result from production of hormone-like substances by the tumor cells. Paraneoplastic syndromes seen in SCLC are over secretion of cortisol by adrenal glands leading to cushing's syndrome. Where as in NSCLC, it leads to elevated levels of calcium in the bloodstream [20].

Nonspecific symptoms: Nonspecific symptoms as observed with many cancers are also seen in lung cancers. They include weight loss, weakness, and fatigue. Psychological symptoms such as depression and mood changes are also common [20].

3.4 Staging of lung cancer

Methods to diagnose and stage the disease in the individual patient are essential for accurate prognosis and planning the optimal treatment. It helps to standardize a general language for researchers and clinicians and is used to stratify patients in treatment categories and to determine their prognosis.

Staging of lung cancer is mainly based on the lesion size, local and regional lymph node spread as well as presence or absence of metastases (Kumar et al 2007 Robbins basic pathology, 8th edition). Staging is mainly done on the results of pulmonary X-rays, CT-scans of the thorax and upper abdomen, bronchoscopy, and, in elective cases, mediastinoscopy basing on the recommendations from Union Internationale Contre le Cancer (UICC). The classification is based on the size, spread and spread of the tumor in relation to main bronchus or thoracic wall, node involvement and metastasis [21], [22] and the identification method is called the TNM system. The TNM system is used to determine the stage (numbered 0 to IV) of the tumor. The four stages, based on the TNM classification described below, provide a consistent and reproducible classification for describing the extent of disease [16], [23].

Since the small cell lung cancer (SCLC) is overt metastatic at diagnosis, the TNM system is not recommended for its management.

Hence, these cancers are broadly classified into two categories, extensive and limited stage disease. Limited stage disease is defined as cancer confined to the hemithorax of origin, the mediastinum, and the supraclavicular nodes. With extensive stage disease, the

tumor is widespread; patients with distant metastases are always included in this group [16].

Lung Cancer Staging				
Stage	**Tumor**	**Lymph Nodes**	**Metastasis**	**Definition**
0	Tis	N0	M0	Tis: Carcinoma In situ N0: No regional lymph node metastasis M0: No distant metastasis
IA	T1a	N0	M0	T1: Tumor ≤ 3 cm, surrounded by lung or visceral pleura, Without invasion more proximal than the lobar bronchus T1a: Tumor ≤ 2 cm
	T1b	N0	M0	T1b: Tumor > 2cm but ≤ 3 cm
IB	T2a	N0	M0	T2: Tumor > 3 cm but ≤ 7 cm or tumor involves main bronchus, ≥ 2 cm distal to carnia; invadees visceral pleura; associated with atelectasis or obstructive pneumonitis extending to hilar region but not entire lung T2a: Tumor > 3 cm but ≤ 7 cm
IIA	T2b	N0	M0	T2b: Tumor > 5 cm but ≤ 7 cm
	T1a	N1	M0	N1 : Metastasis in ipsilateral peribronchial and/or hilar lymph nodes and intrapulmonary nodes by direct extension
	T1b	N1	M0	
	T2a	N1	M0	
IIB	T2b	N1	M0	
	T3	N0	M0	T3: Tumor > 7 cm or directly invades parietal pleural, chest wall,diaphram,phrenic nerve,mediastinal pleura,parietal pericardium; or main bronchus < 2 cm distal to cornia; or associated atelectasis or obstructive pneumonitis of entire lung or seperate tumor nodule(s) in same lobe
IIIA	T1a	N2	M0	N2: Metastasis in ipsilateral mediastinal and/or subcarnial lymphnode(s)
	T1b	N2	M0	
	T2a	N2	M0	
	T2b	N2	M0	
	T3	N1	M0	
	T4	N2	M0	T4: Tumor of any size invades mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, esophagus, vertebral body, carnia, seperate tumor nodule(s) in different ipsilateral lobe
	T4	N1	M0	
IIIB	T1a	N3	M0	N3: Metastasis in contralateral lymphnode(s), or

				supraclavicular lymph node(s)
	T1b	N3	M0	
	T2a	N3	M0	
	T2b	N3	M0	
Stage	**Tumor**	**Lymph Nodes**	**Metastasis**	**Definition**
	T4	N2	M0	
	T4	N3	M0	
IV	Any T	Any N	M1a	M1: Distant metastasis M1a: Separate tumor nodule(s) in a contra lateral lobe; tumor with pleural nodules or malignant pleural (or pericardial) effusion
	Any T	Any N	M1b	M1b: Distant metastasis (in extra thoracic organs)

Table 2: Lung Cancer Staging System.

Developed by the American Joint Committee on Cancer, 7th edition published 2009, table revised from Savai, 2006.

SCLC are staged using a two-tiered system:

- Limited-stage (LS) SCLC refers to cancer that is confined to its area of origin in the chest.

- In extensive-stage (ES) SCLC, the cancer has spread beyond the chest to other parts of the body.

3.5 Treatment for lung cancer

The aim of treatment is to cure and prolong life. Furthermore, metastasis is an important part in determining curative intention or palliative treatment to reduce the suffering of the patients. Palliative therapy, to improve quality of patient's life, is the primary concern for advanced disease [24].

Treatment of lung cancer can involve surgery, chemotherapy, or radiation therapy, as well as combinations of all these treatments depending on the location of the tumor and severity of the cancer.

3.5.1 Surgery:

Though there are chances of remission, surgery remains to be one of the main cures for about one third of patients with NSCLC (stages I, II, and a limited group of patients with stage IIIA) who have surgically resectable disease. In SCLC, however, surgery is not feasible, since most patients have either locally advanced disease or distant metastases.

There are three surgical procedures commonly used to treat lung cancer: wedge resection, or segmentectomy (in which a small section of the lung is removed); lobectomy (in which an entire section is removed); pneumonectomy in which entire lung is removed [25], [26].

3.5.2 Radiation:

Radiation therapy can be given as a curative, palliative or as an adjuvant therapy in combination with surgery or chemotherapy. In this therapy, dividing cancer cells are killed using high energy radiation (eg: X-rays etc). Radiation therapy may be employed as a treatment for both NSCLC and SCLC.

Brachytherapy is a term used to describe the use of a small pellet of radioactive material placed directly into the cancer or into the airway next to the cancer. This is usually done through a bronchoscope. Radiotherapy elicits responses in about 90% of patients with SCLC and in about 50% of those with NSCLC [27], [28].

3.5.3 Chemotherapy:

Drugs are used to stop the growth of cancer cells either by killing them or preventing their cell division. Both NSCLC and SCLC can be treated by chemotherapy. Chemotherapy may be given alone, as an adjuvant to surgical therapy, or in combination with radiotherapy. So far, the platinum-based drugs have been the most effective in treatment of lung cancers. For most SCLCs, chemotherapy is the treatment of choice where as for NSCLCs, chemotherapy prolongs the survival in many cases [29].

New drugs like taxanes (paclitaxel and docetaxel), camptothecins (topotecan and irinotecan), new anti-metabolites (such as gemcitabine and difluorodeoxycytidine) and anti-tubulin agents (vinorelbine) have shown promise in the management of NSCLC [30], [31], [32].

3.5.4 Targeted therapy:

Targeted therapy drugs more specifically target cancer cells, resulting in less damage to normal cells than general chemotherapeutic agents. Erlotinib and gefitinib target a protein called the epidermal growth factor receptor (EGFR) that is important in promoting the division of cells. This protein is found at abnormally high levels on the surface of some types of cancer cells, including many cases of non-small cell lung cancer. Additionally,, patients with NSCLC can be treated with Cetuximab as an antibody based targeted therapy which binds specifically to EGFRs [33].

3.6 Prostanoid Signaling

Prostanoids are the cyclooxygenase metabolites of arachidonic acid. The cyclooxygenases COX-1 and COX-2 are the rate limiting enzymes in the synthesis of all prostanoids from arachidonic acid [34]. While COX-1 is expressed constitutively in a subset of cell types, COX-2 is highly regulated by transcriptional and post-translational mechanisms in response to a plethora of stimuli. Induction of COX-2 triggers the synthesis of different prostanoids that play essential roles in many physiological processes and responses, such as inflammation, pain, fever, and platelet aggregation. Cyclooxygenases catalyze a two-step reaction that converts arachidonic acid to prostaglandin H2 (PGH2) which in turn serves as the precursor for the synthesis of all biologically active prostanoids: PGD2, PGE2, PGF2, prostacyclin (PGI2), 15-deoxy-D12, 14-PGJ2, and thromboxane A2 [35].

In general, basing on the type of G-protein activated by different receptors, prostanoid receptors can be grouped into three categories. The first category includes the relaxant receptors, IP, EP2, EP4, and DP receptors activating Gs which stimulates cAMP production by adenylate cyclase. The second category includes the contractile type of prostanoid receptors TP, EP1, and FP activate the G-protein Gq, mediating enhanced intracellular Ca^{2+} levels by influencing phosphatidylinositol turnover. Finally, the last group contains a lone receptor EP3, which inhibits adenylate cyclase acting via G-protein Gi.

A comprehensive work of the past decade substantiated by targeted molecular designs derived from preclinical *in vitro* and *in vivo* studies, has proved that COX-2 and the sub-set of prostanoids and their receptors playing role in oncogenic pathways.

Receptor type		Kd, nM (radio ligand)	Rank order of binding affinity[a]	Signaling	Gene locus[c] mouse / human	Alternatively spliced isoforms[d]
EP	EP1	21 [^{3}H]PGE_2	PGE_2 > iloprost > PGE_1	$[Ca^{2+}]\uparrow$	chr 8 / 19p13.1	2 (rat)[b]
	EP2	27 [^{3}H]PGE_2	PGE_2 = PGE_1 > butaprost	cAMP↑	–/ 14q22	None
	EP3	3 [^{3}H]PGE_2	PGE_2 = PGE_1 > iloprost	cAMP↓ $[Ca^{2+}]\uparrow$	chr 3 / 1p31.2	3 (mouse)[b] 7 (human) 4 (bovine)[b]
	EP4	11 [^{3}H]PGE_2	PGE_2 = PGE_1	cAMP↑	chr 15 / 5p13.1	None
DP		40 [^{3}H]PGD_2	PGD_2 > BW245C	cAMP↑	chr 14 /–	None
FP		1.3 [^{3}H]$PGF_{2\alpha}$	PGF2α > PGD_2	$[Ca^{2+}]\uparrow$	chr 3 / 1p31.1	2 (ovine)
IP		4.5 [^{3}H]iloprost	cicaprost > iloprost > PGE_1	cAMP↑ $[Ca^{2+}]\uparrow$	chr 7 / 19q13.3	None
TP		3.3 [^{3}H]S-145	S-145 > STA_2 > U46619	$[Ca^{2+}]\uparrow$ cAMP↓	chr 10 / 19p13.3	2 (human)[b]

Y. Sugimoto et al., Progress in Lipid Research 2000

Table 3: Properties of mouse prostanoid receptors

In general physiology, the TXA2 prostanoid receptor designated as 'TP' has an important role in hemostasis, platelet aggregation and smooth muscle contraction. TP is also implicated in the pathogenesis of athrosclerosis and myocardial infarcts. Prostaglandin $F_{2\alpha}$ binds to the receptor FP and plays role in hypertrophic cell growth, interleukin synthesis and uterine contraction where as prostaglandin D_2 binds to the receptor DP and inhibits platelet aggregation and causes relaxation of both vascular and non-vascular smooth muscle cells similar to IP (Prostaglandin I_2 receptor). PGE2 and its receptor regulation are discussed in a separate section.

3.7 Prostanoid signaling in Cancer

COX2 induced tumorigenesis was first reported by Liu et al [36] using mouse models. They observed hyperplasia and carcinoma in epithelial cells, higher expression of COX-2 protein along with increased PGE2 levels, when they inserted COX-2 gene downstream to murine mammary tumor promoter. This is the only study demonstrating a clear role of COX-2 in tumor initiation. Transgenic studies indicate that COX-2 over expression is insufficient for tumor induction but sufficient to transform epidermis in to an auto promoted state causing sensitization of genotoxic carcinogens in it. These studies support the role of COX-2 in tumor promotion rather than initiation [37].

The role of COX-2 in tumor progression is further supported by Oshima et al [38] in colorectal tumor models. In their studies, they observed a decrease in the number and size of the intestinal polyps when there is a prostaglandin synthase 2 (Ptgs2) null mutation. These results provide direct genetic evidence to the role of COX-2 in tumorigenesis and indicate that COX-2-selective inhibitors can be a novel class of therapeutic agents for colorectal polyposis and cancer [38].

Several studies related to the downstream molecules of COX-2 also support its role in carcinogenesis. It has been observed that, PGE2 being most important downstream effector of COX-2, gets up regulated in cancers.

Synergistic effects of Non-Steroidal Anti Inflammatory Drugs (NSAIDs) and conventional chemotherapeutic agents on tumor growth and development has been shown by Teicher et al [39]. In these studies, authors used Lewis lung carcinoma model as a model of NSCLC. Combinations of a COX inhibitor with chemotherapeutic agents such as cisplatin, carmustine, melphalan and cyclophosphamide resulted in tumor growth delay and a decreased lung metastases [39].

In the past few years, the role of COX-2 has been reported in numerous human solid tumors showing that over-expression of COX-2 is not limited to colon cancer but can be considered as a common feature in various other epithelial tumors [40].

3.8 PGE2 regulation and its receptors

Steady state cellular levels of PGE2 depend on the relative rates of COX-2/PGE synthase dependent biosynthesis and 15-hydroxyprostaglandin dehydrogenase (15-PGDH) dependent degradation. For example, cytosolic or microsomal PGE2 synthases can convert PGH2 to PGE2. Two cytosolic PGE2 synthases called cytosolic glutathione transferases (GSTM2-2 and GSTM3-3) catalyse the conversion of PGH2 to PGE2 in the human brain. [41] The two microsomal PGE2 synthases characterised to date are mPGES1 and mPGES2. mPGES1 exhibits a higher catalytic activity than other PGES isomerases, indicating that it probably plays a key role in the synthesis of PGE2 from PGH2. 15-PGDH, a prostaglandin degrading enzyme, catalyses oxidisation of the 15(S)-hydroxyl group of PGE2 to yield an inactive 15-keto PGE2 [40].

PGE2 is a common ligand for a group of membrane receptors EP1, EP2, EP3 and EP4 belonging to the family of seven transmembrane G-protein coupled receptors showing a tissue specific expression and distribution [42], [43].

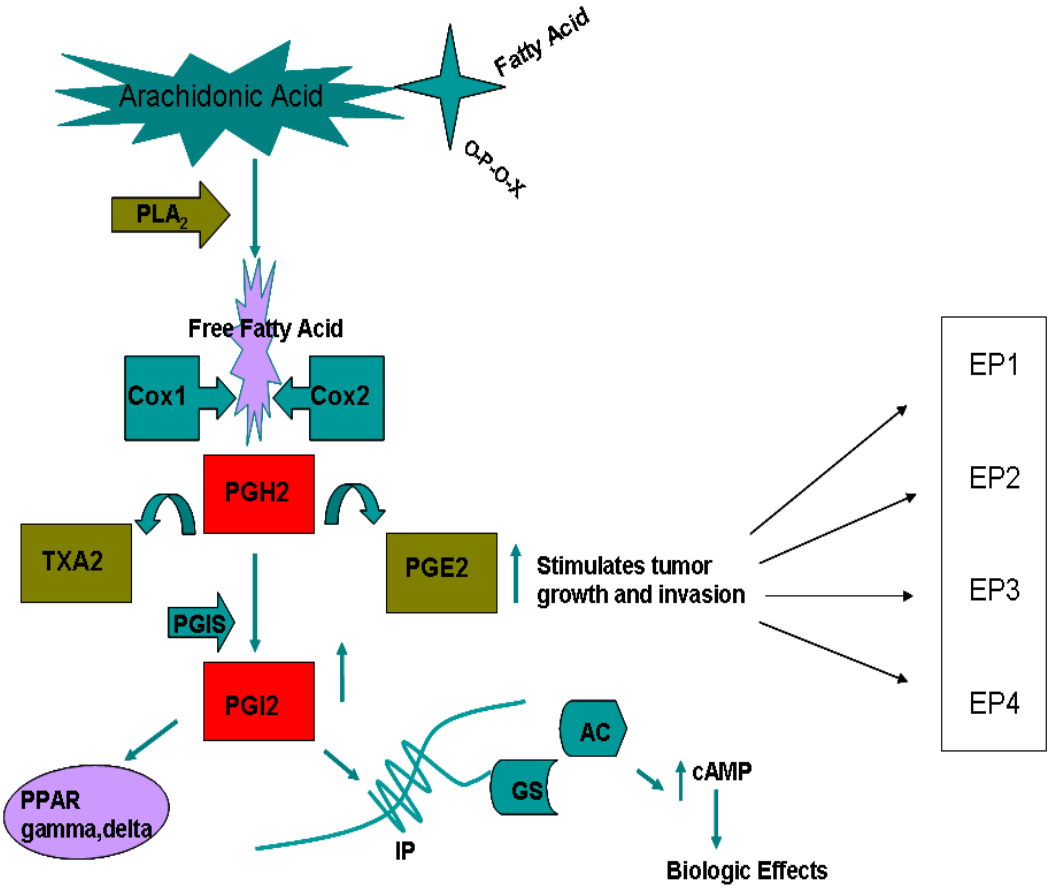
3.8.1 EP1 Receptor

EP1 receptor with a molecular weight of 43 KDa contains 405 amino acids. EP1 receptor expression profile in mice ranges various tissues like epidermis of the skin, sebaceous gland, kidney, bladder, blood vessel, and in the lung. On ligand induced activation, EPI induces an influx of extracellular calcium through voltage independent, Mn^{2+} impermeable calcium channel along with release of calcium from intracellular stores in a PLC dependent manner (Figure 3).

Interestingly in a study by Katoh & Watabe et al [44], EP1 receptor is desensitized with PKC phosphorylation by dissociating with G-protein. A lowered EP1 mRNA expression was observed Chinese hamster ovary cells over expressing EP1 on treatment with TPA (a potent PKC activator) for 24hours. However, Funk [45] observed an induction of EP1 mRNA two days after TPA treatment.

The role of Src/EGFR/Stat3/Akt downstream to EP1 receptor, resulting in PGE2 dependent cell growth and invasion of human cholangiocarcinoma has been reported by Han & Wu [46], [47]. EP1 mediated ERK activation in squamous cell carcinoma of NSCLC has been reported by Krysan et al [48].

In normal physiology, EP1 is very important in the brain. When EP1 receptor was antagonised, mice were protected against the brain injury [49]. A reduced social interaction and an increased aggressiveness was observed in EP1 receptor deficient mice [50].

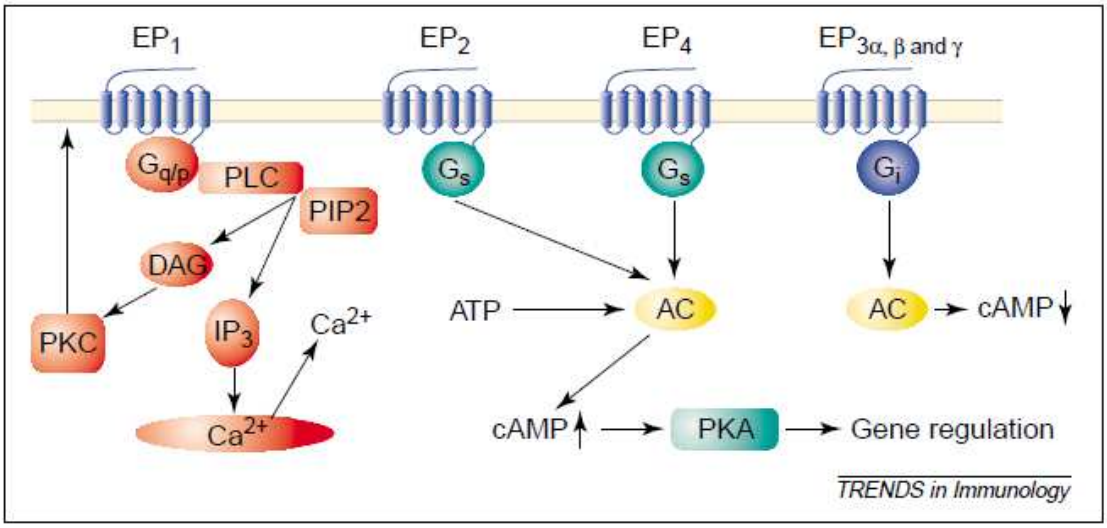


modified from Tennis et al., Translational Research, February 2010

Figure 2: Prostanoid Signaling Pathway

Involvement of EP1 in mediating pain response/sensitivity is also suggested by Stock et al [51] not only in the acetic acid–induced writhing test, but also in the 2-phenyl-1, 4-benzoquinone–induced (PBQ-induced) stretch assays, as reduced responses were observed in their $EP1^{-/-}$ mice. Their pain sensitivity responses were reduced by approximately 50%, and this reduction in the perception of pain was virtually identical to that in WT mice using piroxicam, a COX inhibitor. $EP_1^{-/-}$ mice showed a hyperalgesic action in the hot plate test and the hyperalgesia was alleviated by PGE_2 in a dose-dependent manner [52].

EP1 receptor propagates neurotoxicity and hence, selective blockade could be considered as a potential preventive and/or therapeutic tool against ischemic/hypoxic neurological conditions [53].



Harris et. al., Trends in immunology, 2002

Figure 3: Signaling through EP receptors

EP-Receptors are rhodopsin-type receptors with seven transmembrane-spanning domains. The four main subtypes of EP-R (EP1–EP4) are coupled to different G proteins and use different second messenger signaling pathways. EP1 is coupled to Gq/p and ligand binding results in an increase in the level of intracellular calcium. EP2 and EP4 are coupled to Gs proteins and induce the expression of cAMP, which leads to gene regulation. The three isoforms of EP3 (α, β and γ) are coupled primarily to Gi and are most often inhibitory to cAMP. (There is some evidence that additional signaling cascades might be activated by EP3 binding.) Abbreviations: AC, adenylate cyclase; DAG, diacylglycerol; IP3, inositol triphosphate; PIP2, phosphatidylinositol diphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C.

PGE2-EP1 signaling has been demonstrated to play crucial roles in suppression of myoelectrical activity of gastric smooth muscles and inhibition of gastric emptying, putting forward EP1 as an obvious target for drugs that control gastric emptying [54].

EP1 deficient mice have reduced systolic blood pressure [51], develop insensitivity to pain [55]. Important role of EP1 in inflammation makes it a suitable target for both inflammation and cancer. In stomach, PGE2 failed to induce bicarbonate ion in EP1 deficient mice [56] where as in Leydig cell progenitors, EP1 mediates IL-1β expression [57].

3.8.2 EP2 Receptor

EP2 receptor couples to Gαs to increase intracellular cAMP levels which binds to regulatory subunits of cAMP dependent protein kinase (PKA) leading to phosphorylation of PKA substrates like cAMP-response element binding protein (CREB) at Ser 133 which ultimately results in the translocation of CREB co-activators from cytosol to nucleus [58].

In normal physiology, EP2 receptor is known to play a role in female reproductive system. A reduced number of ovulation and a lower fertilization rate have been observed in EP2 deficient female mice [59]. Kennedy et al found EP2 -/- female mice have consistently fewer pups than WT and detected slightly impaired ovulation and a reduction in fertilization concluding the failure in COX-2 deficient mice is due to dysfunction of the EP2 receptor [60]. Whether polymorphisms in the EP2 receptor are associated with infertility in humans remains to be determined.

In the lung, EP2 receptor mediates broncho-dilating actions of PGE2 [61]. In mice lacking EP2 receptor, salt sensitive hypertension develops [60] and lowering of blood pressure by PGE2 is impaired [62].

For instance, upon PGE2 or butaprost (EP2 receptor specific PGE2 analog) administration intravenously, only wildtype but not EP2 -/- mice became hypotensive. Surprisingly, EP2 -/- mice became hypertensive with PGE2 indicating the absence of EP2 abolishes the ability of the vasculature to dilate in response to PGE2 and unmasks the contractile response *via* vasoconstrictor EP receptor(s) [60]. In addition, PGE2- EP2 signaling is also reported to contribute to the spinal inflammatory hyperalgesia in the zymosan, a peripheral inflammation model [63]. Yeast extract injected subcutaneously into the paw induces inflammatory hyperalgesia. Although thermal and mechanical sensitization in EP2 -/- mice is similar to WT mice at 2 hours post injection, from 4 hours onward EP2 -/- mice recovered faster from hyperalgesia. These results suggest a dominant role for PGE2-EP2 signaling in the generation of inflammatory pain.

3.8.3 EP3 Receptor

EP3 receptor has three isoforms (EP3α, EP3β and EP3γ) in mouse and seven isoforms in human. EP3 receptors are coupled to Gαi and decrease intracellular cAMP levels by inhibiting adenylate cyclase. Activation of EP3 also increases intracellular calcium levels by activation of phospholipase C (PLC) through the Gβγ subunit [64].

Mice with targeted deletion of the EP3 receptor exhibit an impaired febrile response to PGE2, suggesting that EP3 receptor antagonists could be effective antipyretic agents [65]. EP3 deficient mice were also used to determine the role of PGE2 in pain perception. EP3 and IP were found to be the major prostaglandin receptors mediating the enhanced pain

response in mice pre-treated with LPS to induce COX-2 expression [66]. EP3 deficient mice also demonstrated that the PGE2 -EP3 pathway is an important negative modulator of allergic reactions. Only EP3 deficient mice were shown to develop an allergic inflammation that was much more pronounced than that in wtmice or mice deficient in other EP receptor subtypes. Conversely, an EP3-selective agonist suppressed the inflammation [65].

EP3 deficient mice exhibit increased frequency of feeding during the light cycle of the day and develop an obese phenotype under a normal fat diet fed *ad libitum*. Although EP3 deficient mice showed an increased motor activity, this did not compensate sufficiently to offset the increased body weight. These findings further indicate that there is a link between inflammatory signaling and obesity.

Additionally, EP3 deficient mice were found to have impaired duodenal bicarbonate secretion and mucosal integrity. The presence of EP3 receptors were found to be essential for maintaining duodenal acid secretion and maintaining the mucosal integrity against luminal acid [67]. EP3 is also known to maintain blood pressure [68] and urine concentrations [69].

3.8.4 EP4 Receptor

Similar to EP2 receptor signalling, EP4 also couples to Gαs and increases cAMP formation resulting in activation of PKA. Activated PKA phosphorylates CREB, GSK-3 and further leads to activation of β-catenin. EP4 also couples to Gαi decreasing cAMP and activating PI3K/AKT pathway. EP4 is expressed in human and mouse heart, lung, thymus, spleen, ileum and skin [70],[61]. In mice, PGE2 enhances bone resorption through EP4 [71].

EP4 receptor activation has been shown to have important vasodilator effects in venous and arterial beds [72]. When examined in the mouse gastrointestinal tract, EP4 mRNA was found to be highly expressed in the gland of the gastric antrum suggesting this subtype is involved in PGE2-mediated mucus secretion. Additionally, EP4 has found to be expressed in epithelial cells of the intestinal villi [73]. Since EP4 increases cAMP and PGE2 stimulates chloride secretion and inhibits salt absorption *via* production of cAMP, taken together, the above findings suggest that EP4 is involved in these processes and consequently PGE2-induced diarrhoea [74].

Mice with targeted disruption of the EP4 receptor gene have shown a disturbed perinatal closure of the pulmonary ductus arteriosus [75]. Disruption of the EP4 gene results in death of most homozygous EP4 -/- neonates within 3 days of birth due to pulmonary congestion and heart failure [75]. As noted earlier, EP4 mRNA is present in the ductus and works in the dilation of the vessel. EP4 -/- neonates showed full patency of the ductus after birth indicating a critical role of EP4 in the ductus. The EP4 receptor ligands may prove useful in promoting closure or maintaining patency of the ductus arteriosus in newborns with congenital heart disease. Additionally, EP4 deficient mice have shown that PGE2-EP4 signalling facilitates initiation of skin immune responses by promoting the migration and maturation of Langerhans cells. While PGE2 is produced substantially in skin exposed to antigen, its role was unclear. Although Langerhans cells express all four EP subtypes, their migration to regional lymph nodes was decreased only in EP4-deficient mice and in wtmice treated with an EP4 antagonist [76]. Lastly, EP4 deficiency impaired mucosal barrier function and induced epithelial loss, crypt damage, and aggregation of neutrophils and lymphocytes in the colon. Among the eight prostanoid receptor-deficient mice tested, only EP4-deficient mice developed severe colitis with 3% dextran sodium sulfate treatment, which induced only marginal colitis in wildtype mice. These studies conclude that EP4 is important for maintainence of intestinal homeostasis by protecting mucosal integrity and downregulating immune response [77].

3.9 Cross talk of PGE2 signaling with MAPK pathway

Activation of an oncogene Ras has been found in a wide variety of malignancies which induces cell survival, proliferation and transformation by triggering downstream signalling pathways such as Raf/MEK/ERKs and PI3K/AKT pathways. The Ras-MAP kinase cascade is one of the major intracellular signalling pathways responsible for cell proliferation. Constitutively active Ras or MEK upregulates COX-2 expression resulting in increased cell proliferation in a variety of cell culture models [78], [79], [80], [81]. It has been shown that, NSAIDs and COX-2 selective inhibitors act via Ras-MAPK signalling pathway in inhibiting cell proliferation [82], [83], [84]. Many findings support the notion of upregulation of COX-2 derived PGE2 promoting human cancer cell growth by autoregulation which depends primarily on PGE2 induced activation of Ras-MAPK pathway.

Though NSAIDs and COX-2 selective inhibitors are promising, concern over their safety over cardio vascular side effects has prompted researchers to look for more effective

chemopreventive agents with minimal toxicity. Understanding the molecular mechanisms of COX-2 and its downstream targets will help to identify specific molecular targets for developing saferagents targeting this pathway. Significant progress has been made in the elucidation of PGE2 downstream signalling pathways which mediate the chemopreventive effect of NSAIDs [85], [86]. The combined therapy involving multiple agents may allow for a lower dose of drug. Taken together, efforts to develop novel chemopreventive agents targeting PGE2 pathway with minimal toxicity and to design strategies for combinations of different agents targeting multiple pathways may yield significant benefits for cancer patients.

In the future, it is important to carefully determine the EP receptor profile in human cancers. Taken together, these findings may provide a rationale for the development of EP receptor antagonists which may offer an alternative to COX-2 selective inhibitors.

4 AIM OF THE STUDY

Available clinical data has well-established the protective effect of COX-2 inhibition on human cancer progression. Despite these encouraging outcomes, the appearance of unwanted side effects remains a major hurdle for the general application of COX-2 inhibitors as effective cancer drugs. Hence, a better understanding of the molecular signals downstream of COX-2 is needed for the elucidation of drug targets that may improve cancer therapy.

Antagonising the prostaglandin receptors have been shown to be effective in inhibiting different cancers. An increasingly large body of evidence indicates that PGE2 promotes tumor growth by stimulating EP receptor signalling with subsequent enhancement of cellular proliferation, promotion of angiogenesis, inhibition of apoptosis, stimulation of invasion/motility, and suppression of immune responses. These findings prompted us to elucidate PGE2 signalling pathways and identify PGE2 downstream targets that are involved in promoting tumor growth.

Here we aim to show that the COX-2 product prostaglandin E2 (PGE2) acts on cognate receptor EP1 to promote the proliferation and migration of NSCLC lung cancer cells. Our aims are to establish/check -

- Gene expression profile of EP receptors both at mRNA and protein level in NSCLC cells.
- *In vitro* effects of pharmacological inhibition of the EP receptors in thymidine incorporation assay.
- *In vitro* effects of pharmacological inhibition of the EP1 receptor in intra cellular calcium and cAMP measurement assay.
- *In vitro* effects of pharmacological inhibition of the EP1 receptor in transwell migration assay.
- The downstream pathway to EP1 receptor involved in the proliferation and migration of NSCLC cells.

5 MATERIALS AND METHODS

5.1 Materials

Table 4 Materials

Product	Company	Country
Bovine Serum Albumin Powder	Serva	Germany
Bovine Serum Albumin solution (2mg/ml)	Bio-Rad	USA
cAMP EIA kit	Cayman Europe	Estonia
DAPI	Dakocytomation	USA
Dc protein assay kit	Bio-Rad	USA
DEPC water	Roth	Germany
Digest All 2 (trypsin)	Vector	USA
Enhanced Chemiluminiscence (ECL) Kit	Amersham	USA
Fluorescent mounting medium	Dakocytomation	USA
Go Taq PCR Core system I	Promega	USA
ImProm-II Reverse transcription system	Promega	USA
Milk powder	Roth	Germany
N,N'-Methylene-bis-Acrylamide solution, Rotiphorese gel 30	Roth	USA
Protein rainbow marker	Amersham	USA
RIPA buffer	Santacruz	USA
RNase Away	Molecular Bioproducts	USA
Scintillation solution (Rotiszint eco plus)	Roth	Germany
SDS solution, 10% w/v	Promega	USA
SYBR Green ER qPCR Supermixes Universal kit	Invitrogen	USA
[^{3}H]-thymidine (1mCi/ml)	Amersham	USA
Tris-HCl 0.5M, pH 6.8	Amresco SOLON	USA
Tris-HCl 1.5M, pH 8.8	Amresco SOLON	USA
Trizol	Invitrogen	USA
Ultra Pure water	Cayman Europe	Estonia

Table 5 Cell culture materials

Cell Culture materials	Company	Country
DMEM F12 OptiMEM	Invitrogen	USA
HBSS	Invitrogen	USA
Fetal Bovine Serum	Biowest	Germany
Phosphate Buffer Saline (PBS)	PAN	Germany
L-Glutamine	PAN	Germany
Penicillin-Streptomycin	PAN	Germany
Trypsin EDTA	PAN	Germany

Table 6 Antibodies

Antibodies	**Company**	**Country**
Mouse anti-GAPDH monoclonal	Sigma-Aldrich	USA
Rabbit anti-EP1 polyclonal antibody	Cayman Chemicals	USA
Rabbit anti-EP2 polyclonal antibody	Cayman Chemicals	USA
Rabbit anti-EP3 polyclonal antibody	Cayman Chemicals	USA
Rabbit anti-EP4 polyclonal antibody	Cayman Chemicals	USA
Rabbit anti-pERK polyclonal antibody	Santacruz	USA
Rabbit anti-ERK polyclonal antibody	Santacruz	USA
Rabbit anti-p21 polyclonal antibody	Santacruz	USA
Rabbit anti-p27 polyclonal antibody	Santacruz	USA
Rabbit anti-mouse IgG antibody	Sigma-Aldrich	USA
Goat anti-rabbit IgG antibody	Pierce	USA
Alexa Fluor 488 goat anti-rabbit IgG antibody	Invitrogen	USA
FITC conjugated goat anti-rabbit IgG antibody	Invitrogen	USA

Table 7 Oligonucleotides

Oligonucleotides		
EP1 (151bp)	Forward	5’ CTTGTCGGGTATCATGGTGGT 3’
	Reverse	5’ AAGGGTCCAGGATCTGGTT 3’
EP2 (187bp)	Forward	5’ CCTCATTCTCCTGGCTATCA 3’
	Reverse	5’ TCTCAGAACAGGAGGCCTAA 3’
EP3 (169bp)	Forward	5’ GAGACGGCCATTCAGCTTAT 3’
	Reverse	5’ CCAGGCGAACAGCTATTAAG 3’
EP4 (160bp)	Forward	5’ TCTTACTCATTGCCACCTCC 3’
	Reverse	5’ TCTAGGATGGGGTTCACAGA 3’
IP (186bp)	Forward	5’ CACAGACCGACACAGGCAGC 3’
	Reverse	5’ CACCGGCCACGAACATCAGG 3’
TP (174bp)	Forward	5’ GAGTCCGAGGTGGAGATGAT 3’
	Reverse	5’ GCGCAAGTAGATGAGCAGC 3’
PBGD	Forward	5’ CAGGAGTCAGACTGTAGGACGAC 3’
	Reverse	5’ ACTCTCATCTTTGGGCTGTTTTC 3’

Table 8 Table of Euipments

Equipments		
Equipment	**Company**	**Country**
BioDoc Analyzer	Biometra	USA
Cell culture incubator	Hera Cell Heraeus	Germany
Electrophoresis Chamber	Biometra	USA
Fluorescence microscope	Leica	Germany
Freezer (+4 ^{0}C, -20 ^{0}C, -80 ^{0}C)	Bosch	Germany
Infinite 200 microplate reader	Tecan	Switzerland
Inolab pH meter	WTW	Germany
Light microscope	Hund	Germany
Liquid Scintillation Counter	Beckmann	USA
Multifuge centrifuge	Heraeus	Germany
Mx3000P QPCR system machine	Stratagene	USA
Spectrophotometer	Nanodrop Technologies	USA
PCR - thermocycler	Biometra	Germany
Pipetboy and pipettes	Eppendorf	USA
Power supply units	Biometra	USA
Precellys 24 homogenizer	Bertin Technologies	France
Shaker	Biometra	USA
Waterbath for cell culture	Medingen	Germany
Waterbath for tubes	HLC	Germany
Westernblot Unit	Biometra	USA
Vortex machine	VWR	Germany

Table 9 Other materials

Othermaterials	**Company**	**Country**
Falcon tubes	Greiner Bio-One	Germany
PCR tubes	Greiner Bio-One	Germany
Glass pipettes	Greiner Bio-One	Germany
Cell culture dishes and plates	Greiner Bio-One	Germany
96 well microplate	Corning	USA
AGFA cronex 5 medical X-ray film	AGFA	Belgium
Film cassette Kodak	Kodak	USA
Filter tips (10,100, 1000 µL)	Nerbe plus	Germany
Gel blotting paper	Whatman	USA
Nitrocellulose membrane	Pall Corporation	USA
Osmotic minipump (2ml)	Durect Corporation	USA
Radiographic film hypersensitive	Amersham	USA
Tips (10,100,1000 µl)	Eppendorf	USA
Reaction tube	Sarstedt	Germany
Real time tube	Thermo Fisher	USA
Tissue culture chamber slide	BD Falcon	USA

5.2 Methods

5.2.1 Cell culture

A549 cells were maintained in DMEM F/12 medium supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (0.1 mg/ml), 1% MEM vitamin solution, 2 mM glutamate and 1% non-essential amino acids at 37 ^{0}C in humidified atmosphere containing 5% CO2.

H1299 cells were maintained in DME M F/12medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) at 37 ^{0}C in humidified atmosphere containing 5% CO2.

5.2.2 RNA isolation

Total RNA from tissues or cells was extracted using Trizol reagent according to the manufacturer's instructions. 2×10^6 A549 cells were collected in 1 ml Trizol. Trizol lysates were kept at RT for 5 min to dissociate the RNA from histone proteins. Then 0.2 ml of chloroform was added and mixed vigorously for 15 sec and centrifuged under 12000 rpm at 4 ^{0}C for 30 min. After that, the transparent upper layer was carefully transferred to a new tube and gently mixed with 0.5 ml 2-propanol. After 15 min the mixture was centrifuged at 12000 rpm at 4 ^{0}C for 15 min and the RNA pellet was washed with 1 ml 75% ethanol and dried in the air. RNA was dissolved in DEPC-water and stored at -80 ^{0}C. The concentration and quality of RNA were estimated by Nano Drop spectrophotometer.

5.2.3 Reverse transcription-PCR (RT-PCR)

cDNA was synthesized by a two-step RT-PCR using ImProm-II™ reverse transcription system according to the manufacturer’s instructions. 1 µg RNA in 5 µl reaction A was denatured at 70 ^{0}C for 5 min, followed by a quick chill for 5 min and addition of 15 µl reaction B. The reverse transcription reactions were subjected to cDNA synthesis by firstly, annealing at 25 ^{0}C for 5 min and incubating at 42 ^{0}C for 60 min, followed by thermal inactivation of reverse transcriptase at 70 ^{0}C for 15 min. The cDNA was stored at -20 ^{0}C.

5.2.3.1 Reaction A component Volume Final concentration

Table 10 Reaction A component Volume Final concentration

Ingredient	Stock Concentrations	Final Concentrations	Volume
Total RNA	1 µg/ µl	1 µg/20 µl	1 µl
Oligo(dT)$_{15}$ primer	0.5 µg/ µl	1 µg/20 µl	2 µl
Nuclease Free Water			2 µl

5.2.3.2 Reaction B component Volume Final concentration

Table 11 Reaction B component Volume Final concentration

Ingredient	Stock Concentrations	Final Concentrations	Volume
ImProm-II™ 5X reaction buffer		1X	4 µl
$MgCl_2$	25 mM	2.5 mM	2 µl
dNTPmix	40 mM	2 mM	1 µl
RNasin® ribonuclease inhibitor		20 u/20 µl	1 µl
ImProm-II™ reverse transcriptase		0.5 u/20 µl	1 µl
Nuclease-free			6 µl
Total			15 µl

5.2.4 Quantitative realtime- PCR (qRT-PCR)

The intron-spanning primer pairs were designed using the Primer3 program and are shown in Table 3. Primers were cross checked to insure the specificity by blasting to the whole genome. The product size was controlled within the range of 80 bp-150 bp.

5.2.4.1 qRT-PCR reaction component Volume Final concentration

Table 12 qRT-PCR reaction component Volume Final concentration

Ingredient	Stock Concentrations	Final Concentrations	Volume
cDNA		0.2 µg/25 µl	2 µl
$MgCl_2$	25 mM	1 mM	1 µl

ROX	100 µM	25 µM	0.1 µl
Upstream Primer	10 µM	0.2 µM	0.5 µl
Downstream primer	10 µM	0.2 µM	0.5 µl
2 X SYBR® GreenER™ SuperMix Universal buffer		1X	12.5 µl
Nuclease-free water			8.4 µl
Total			25 µl

qRT-PCR was performed on a Mx3000P® QPCR system using SYBR® GreenER™ qPCR SuperMixes Universal kits according to manufacturer's instructions. For the negative control, the cDNA was omitted. The annealing temperature for every gene was standardised to 58 ^{0}C. By using the MxPro™ QPCR software, a dissociation curve was generated for each gene to ensure a single product amplification and the threshold cycle (Ct values) for each gene was determined. The comparative $2^{-\Delta\Delta Ct}$ method was used to analysis mRNA fold changes between treated and untreated, which was calculated as Ratio = $2^{-(\Delta Ctcontrol-\Delta Ct\ treated)}$ where Ct is the cycle threshold, and ΔCt ($Ct_{target} - Ct_{reference}$) is the Ct value normalized to the reference gene Porphobilinogen Deaminase (PBGD) obtained for the same cDNA sample. Each reaction was run in duplicate and repeated three times independently. The calculated $2^{-\Delta\Delta Ct}$ was transformed into a percentage using the control as 100% to show the mRNA expression difference.

5.2.4.2 qRT-PCR programm Temperature Time Cycle

Table 13 qRT-PCR programm Temperature Time Cycle

Step	Temperature	Time	Cycles
Activation	95 ^{0}C	10 min	1
Denaturation	95 ^{0}C	30 Sec	40
Annealing	58 ^{0}C	30 Sec	
Extension	72 ^{0}C	30 Sec	
Denaturating	95 ^{0}C	60 Sec	1
Dissociation curve	55-95 ^{0}C	indefinite	1
Soak	4 ^{0}C	Indefinite	1

5.2.5 Western blotting

5.2.5.1 Protein isolation

Total protein was extracted in RIPA buffer containing 1XTBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide. PMSF, proteinase inhibitor cocktail and sodium orthovanadate (10µl each in 1 ml RIPA) were added to RIPA buffer freshly before use. 250-300 µl of RIPA buffer cocktail was added to 2×10^6 A549 cells in a petridish on ice and cells are scraped out using a scraper and the mixture is taken in to a tube. The tube is centrifuged under 12000 rpm for 30 min at 4 ^{0}C and the supernatants were stored at -80 ^{0}C.

5.2.5.2 Protein concentration analysis

A series of bovine serum albumin (BSA) solution from 0.2-1.6 mg/ml were used as standard. The protein samples were pre-diluted into the range of the standard and the concentration of each sample was double estimated by D_c protein assay kit based on the method of Bradford using a microplate reader.

5.2.6 SDS-polyacrylamide (SDS-PAGE) gel electrophoresis

Protein samples of the same concentration were mixed with 5X SDS gelloading buffer at a ratio of 4:1 (v/v) and denatured at 100 ^{0}C for 5 min. Protein samples (30 µg for pERK, ERK, p21, p27 and 15 µg for GAPDH) or rainbow marker were loaded in the wells of 10% SDS-PAGE gel and run at 100-130 v for 1-2 hours to separate. Buffers are listed as follows.

5.2.6.1 5×SDS gel-loading buffer component Final concentration

Table 14 5×SDS gel-loading buffer component Final concentration

Ingredient	**Final Concentration in mM or Final Percentage in V / V or W / V**
Tris-Cl (2 M, pH 6.8)	375 mM
SDS	10% (w/v)
Glycerol	50% (v/v)
β-Mercaptoethanol	12.5% (v/v)
Bromophenol blue	0.02% (w/v)

5.2.6.2 Running buffer component Final concentration

Table 15 Running buffer component Final concentration

Ingredient	Final Concentration in mM or Final Percentage in V / V or W / V
Tris-HCl	25 mM
Glycine	192 mM
SDS	10% (w/v) or 0.1% (w/v)

5.2.6.3 Resolving gel (10%) component Volume Final concentration

Table 16 Resolving gel (10%) component Volume Final concentration

Ingredient	Final Concentration in mM or Final Percentage in V / V or W / V	Volume
Tris-Cl (1.5 M, pH 8.8)	375 mM	1.5 ml
Acrylamid	30% (w/v) or 10% (w/v)	2 ml
SDS	10% (w/v) or 0.1% (w/v)	60 µl
APS	10% (w/v) or 0.05% (w/v)	30 µl
TEMED	0.1 %	6 µl
Double distilled Water		2.4 µl
Total		6 ml

5.2.6.4 Stacking gel (6%) component Volume Final concentration

Table 17 Stacking gel (6%) component Volume Final concentration

Ingredient	Final Concentration in mM or Final Percentage in V / V or W / V	Volume
Tris-Cl (0.5 M, pH 6.8)	125 mM	0.625 ml
Acrylamid	30 % (w/v) or 6 % (w/v)	0.5 ml
SDS	10% (w/v) or 0.1% (w/v)	25 µl
APS	10% (w/v) or 0.05% (w/v)	12.5 µl
TEMED	0.1 %	2.5 µl
Double distilled Water		1.34 µl
Total		2.5 ml

5.2.7 Immunoblotting

The proteins separated on the SDS-PAGE were transferred to nitrocellulose membrane using an electrophoretic transfer machine. After being soaked in blocking buffer for 1 h at RT, membranes were probed with specific primary antibodies (rabbit polyclonal anti- EP1 antibody,1:1000; rabbit polyclonal anti-EP2 antibody, 1:000; rabbit polyclonal anti-EP3 antibody; rabbit polyclonal anti-EP4 antibody, 1:000; rabbit polyclonal anti-phospho-ERK antibody, 1:000; rabbit polyclonal anti-ERK antibody, 1:000; mouse monoclonal anti-GAPDH antibody, 1:5000) overnight at 4 ^{0}C. After wash with TBST for 3 times, horse radish peroxidase (HRP) conjugated secondary antibodies (anti-rabbit, 1:50000; anti-mouse, 1:50000) were applied to the membranes respectively for 1 h at RT. After washing, the blots were developed with an enhanced chemiluminescence (ECL) kit for 5 min and chemiluminescence signal was captured on an X-ray film. Each blot was repeated 3 times or more independently with representative blots shown.

5.2.7.1 Blotting buffer Final concentration

Table 18 Blotting buffer Final concentration

Ingredient	**Final Concentration in mM or Final Percentage in V / V or W / V**
Tris-HCl	50 mM
Glycine	40 mM
Methanol	20% (v/v)

5.2.7.2 TBST buffer (pH 7.6) component Final concentration

Table 19 TBST buffer (pH 7.6) component Final concentration

Ingredient	**Final Concentration in mM or Final Percentage in V / V or W / V**
Tris-HCl	20 mM
NaCl	137 mM
Tween	0.1% (v/v)

5.2.7.3 Blocking buffer component Final concentration

Non-fat milk 5% (w/v) in TBST

5.2.8 Immunocytochemistry

A549 cells were grown on 8-well chamber slides 24 hours. Cells are fixed with the ice-chilled acetone-methanol mixture (1:1) for 10 min at 4 ^{0}C. After washing with PBS, the fixed cells were sequentially incubated with blocking buffer (3% BSA in PBS） for 1 h at RT, and then the primary antibody against EP1, EP2, EP3 and EP4 (1:300 in blocking buffer) for 1 h at RT or overnight at 4 ^{0}C. After the primary antibody incubation, cells were washed 5 times with PBS and subjected to FITC-conjugated anti-mouse or Alexa Fluor® 488 anti-rabbit secondary antibody (1:1000 in PBS) for 1 h at RT in dark. Then cells were washed 5 times with PBS and counterstained for nuclei with DAPI (500 ng/ml in blocking buffer) for 3 min. After washing with PBS, the upper chamber was removed and the slide was covered with a cover slide using the fluorescent mounting medium. The staining was visualized using a Leica DMLA fluorescence microscope and Leica QWin imaging software. The wells without primary antibodies were negative controls.

5.2.8.1 EP receptor inhibitors

SC-51322, AH-6809, L-798106 and L-161982 were used as selective EP inhibitors for EP 1, EP2, EP3 and EP4 respectively.

5.2.9 Intracellular Calcium Measurement Assay

Intracellular Ca^{2+} concentrations were measured by determining the ratio of the fluorescence of fura 2 after excitation at 340 nm and 380 nm (Grvnkiewicz et al.. 1985). Cells on glass coverslips were loaded with the dye by incubation in Hepes Ringer solution containing fura 2-AM (5 µM) and bovine serum albumin (0.1%) for 60 min at 37 ^{0}C. After incubation, cells were washed twice with Hepes Ringer solution and then mounted in a cell chamber (36 ^{0}C) before starting fluorescence measurements in presence and absence of EP1 receptor specific agonists and antagonists. Experiments were performed using a system from Till Photonics (Martinsried, Germany) with monochromator (Polychrome V), camera (PCO Sensi Cam), and software (TILLvisION v3.3) as well as microscope from Olympus (IX70 WI, Olympus, Hamburg, Germany).

The cells formed a confluent monolayer and a fixed square diaphragm was used to limit the fluorescence measurement to a field of view which typically contained 60-80 cells. The cells were excited at 340 nm and 380 nm every second, and emitted fluorescence signal were

detected. Cells were marked and ratio of fluorescence after excitation at 340 nm and 380 nm were calculated after background subtraction.

Solutions: The Herpes Ringer solution used during fluorescence measurements contained (mM): NaCl (136.4), KCl (5.6), $CaCl_2$ (2.2), MgCl2 (1): Glucose (11), HEPES (10) (pH 7.4). Agonists and antagonists were added to Hepes Ringer solution. Stock solutions of both agonists and antagonists were frozen until use.

5.2.10 cAMP enzyme immunoassay (EIA)

Intracellular cAMP content of A549 cells was determined by a competitive non-acetylated EIA, using a specific cAMP EIA Kit according to the manufacturer's instructions. At the end of culture, cells were washed twice with PBS and lysed in 0.1 M HCl at RT for 20 min. The lysates were collected and centrifuged under 12000 rpm for 30min at 4 ^{0}C. The supernatant was transferred to a new tube and stored at -80 ^{0}C. The protein concentration was estimated by the D_c protein assay as described before and equalized to 0.3 µg/ µl for use. 50 µl protein samples or standard solutions were incubated in dark with 50 µl tracer and 50 µl antibody overnight at 4 ^{0}C. After washing 5 times, the plate was incubated with Ellman's solution for 90-120 min at RT with gentle shaking. The plate was measured at a wavelength of 405 nm and the concentration was calculated by the ready-made Cayman EIA Double workbook. The standard curve was made as a plot of the %B/B_0 value (%Bound/Maximum Bound) *vs* concentration of a series of known standards using a linear (y) and a log (x) axis. Using the 4- parameter logistic equation obtained from the standard curve, the cAMP concentration of samples was determined and given as nmol/mg protein. Each sample was performed in duplicate and repeated twice.

5.2.11 Proliferation assay

A549 cell proliferation was achieved by [^{3}H]-thymidine incorporation assay. A549 cells (around 1×10^4 cells/well) were seed on 48- well plates and the following day the medium was substituted with DMEM/F12 containing 0.1% FBS with or without EP inhibitors to render the cells quiescent. After serum starvation, cells were induced to cell cycle re-entry by 10% FBS together with different EP inhibitors for 24 h. The concentration of EP inhibitors used for proliferation assay was pre-proved for EP selectivity according to the previous experiments. During the last 4 h of agonist stimulation, 10 µl [^{3}H]-thymidine (0.01 µCi/ ml) was added to 250 µl medium to incorporate into the DNA. Cells were then washed

twice with 500 µl chilled HBSS, fixed with 250 µl ice-cold methanol for 15 min at 4 ^{0}C and then precipitated by 250 µl 10% trichloroacetic acid (TCA) for 15 min at 4 ^{0}C. After washing with water, samples were finally lysed in 0.1 M NaOH, transferred into 4 ml scintillation solution and counted by a beta counter giving a CPM value. All labeling was performed on quadruplicate cultures and repeated twice independently. The proliferation of A549 cells under agonist stimulation is shown as a percentage taking the CPM of unstimulated A549 cells under 0.1% FBS as 100%.

5.2.12 Transwell Invitro Migration Assay

We determined the ability of SC-51322, an EP1 receptor specific inhibitor to inhibit the migration of A549 cells *in vitro* by Transwell migration assay. A549 cells were starved for 24 h before seeding 20,000 cells per well in the upper chamber of the transwell containing serum free DMEM F-12 medium, where as the lower chambers contain three different subsets of DMEM F-12 medium with 10% FCS, 10% FCS+10 µM PGE2, 10% FCS+10 µM PGE2 + SC-51322. The transwell chamber was incubated at 37 ^{0}C for 6 h to allow the migration of cells through the membrane into the lower chamber. Media was removed using a gentle suction. Cells were then washed with 1X PBS and fixed to the membrane using methanol wash for 2-3 minutes. After the fixation, cells were stained for 1-2 minutes with haematoxylin which gives purple color to the cells. After the staining, the transwell was cleaned from inside with a cotton swab to remove the unmigrated cells and then we quantified the number of migrated cells that reached the lower part of the transwell filter membrane using a phase contrast microscope counting in six random fields.

5.2.13 MTT cytotoxicity assay

Cell viability/cytotoxicity were assessed by the MTT assay using a CellTiter 96AQ kit according to the manufacturer's instructions. Briefly, the cells were plated in 96-well plates and allowed to attach for 6 h, and then cultured under serum-free conditions with various concentrations of SC 51322 for 48 h. The number of surviving cells was determined by measuring the absorbance at 560 nm of the dissolved formazan product after addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt for 1 h. All of the experiments were carried out in triplicate.

5.2.14 Statistical analysis

Data are expressed as mean and standard error of mean (SEM). All statistical analysis was performed with Student's *t*-test between two groups or with oneway ANOVA and Newman-Keuls post-hoc test for multiple comparisions, as appropriate. Difference between groups is considered significant when $P < 0.05$.

6 RESULTS

6.1 Expression profile of EP receptors in A549 cells

Expression profile of EP receptors in A549 cells was done by quantitative RT-PCR and by western blotting. EP1 and EP3 receptors are less expressed compared to EP2 and EP4 in A549 cells at mRNA level (Figure 4a) and also at protein level (Figure 4b, 4c)

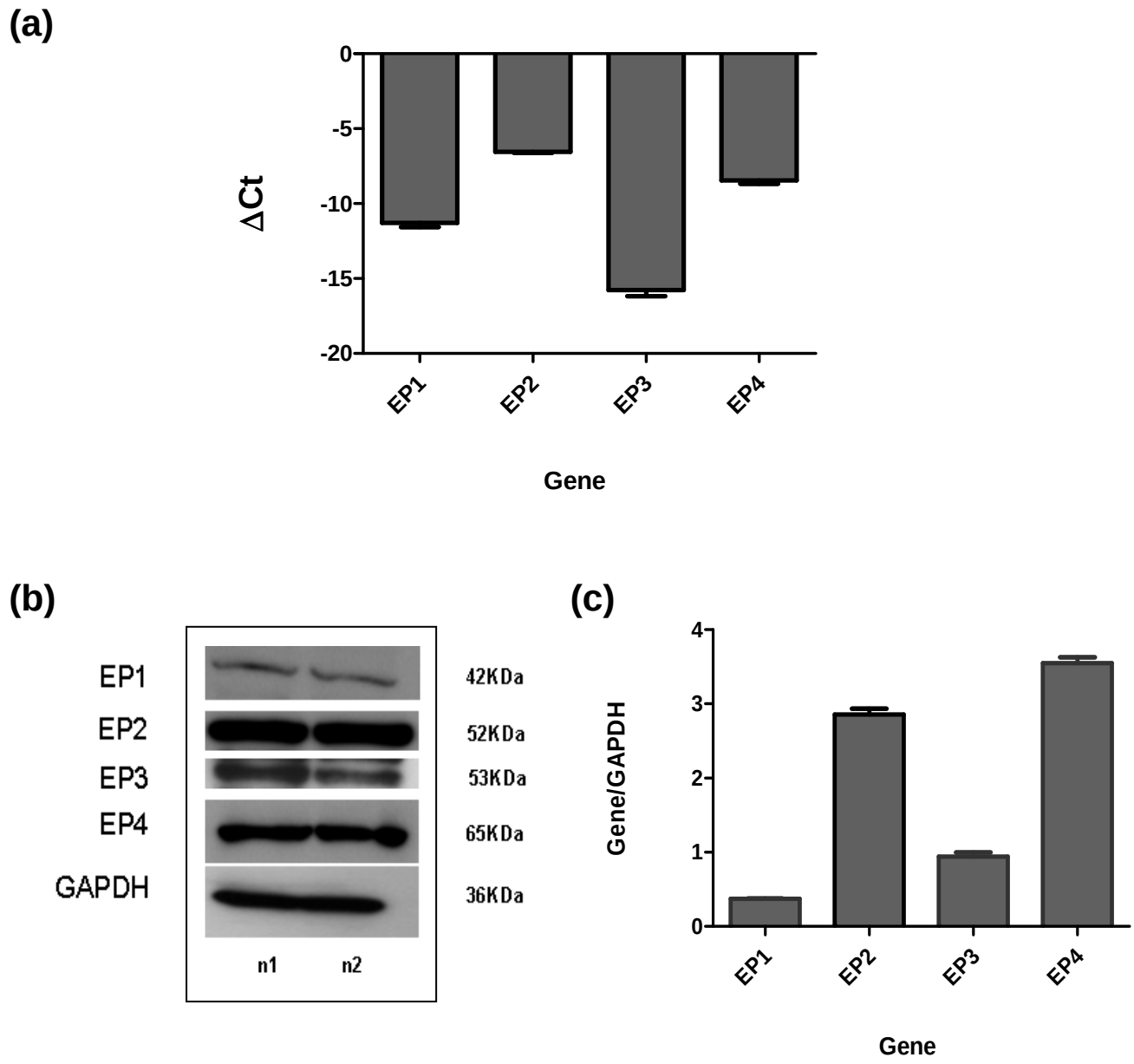


Figure 4: **mRNA and Protein expression profile of EP receptors in A549 cells**

(a) m-RNA expression profile of EP receptors in A549 cells (b) Representative immunoblots against EP receptors with GAPDH as a loading control (C) Densitometric quantification of EP receptors' expression in A549 cells is shown as a ratio by normalization to GAPDH in a bar graph.

6.2 Cellular localisation of EP receptors in A549 cells

The cellular localisation of all EP receptors in A549 cells by using immunocytochemistry. Immunofluorescence staining showed a predominant expression of EP1 and EP2 receptors both on cell membrane and nucleus where as EP3 and EP4 receptors are confined mostly to cytoplasm (Figure 5).

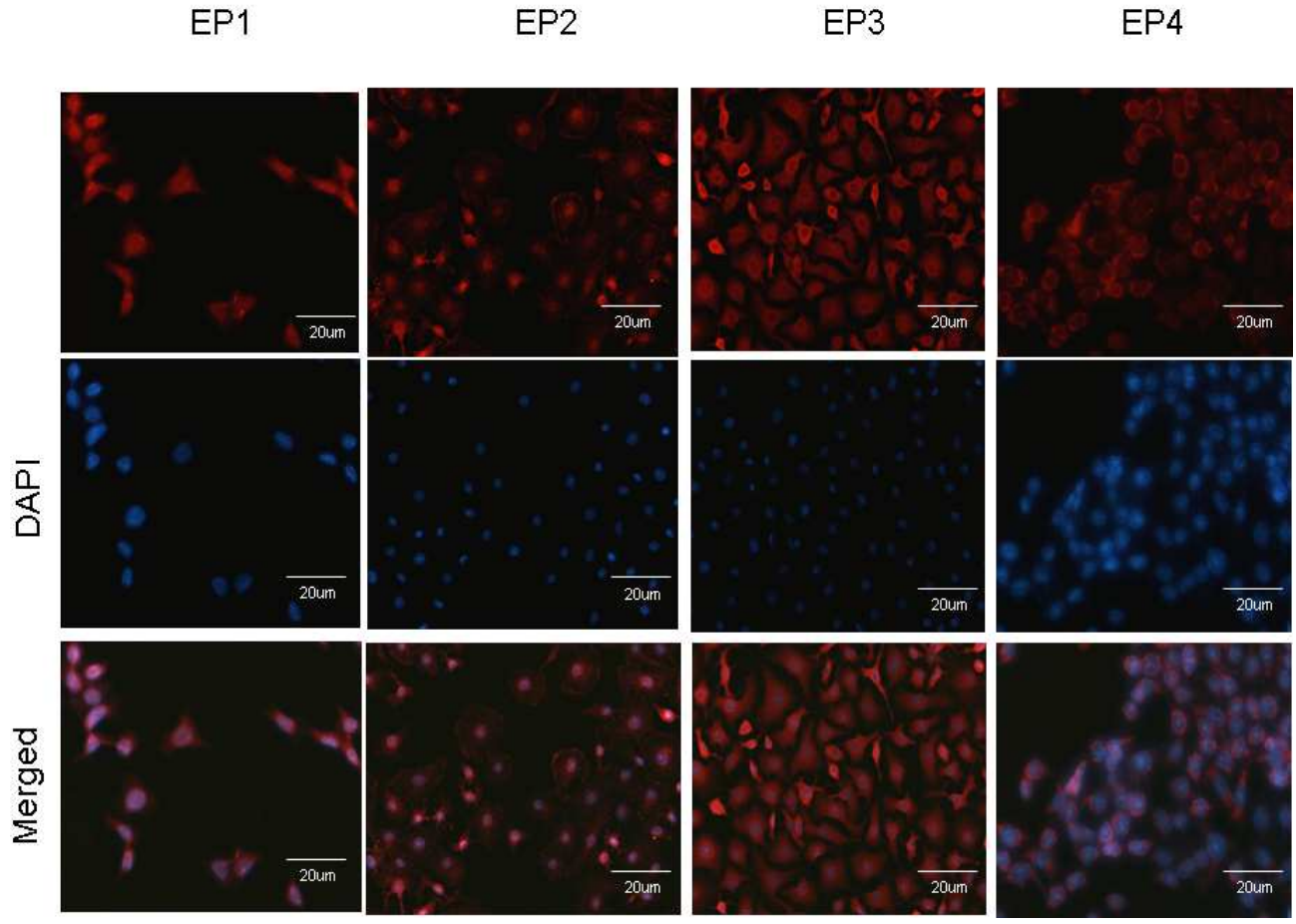


Figure 5: **Immunocytochemical staining of EP receptors in A549 cells.**

Cellular localization of EP1, EP2, EP3 and EP4 receptors in A549 cells, shown by representative immunofluorescence. Red (EP1-4, FITC-conjugated); Blue (nuclei, DAPI). Scale 20 µm.

6.3 Anti proliferative effect of EP inhibitors on A549 cells

We further tested the contribution of all EP receptors to the proliferation of A549 cells by using receptor specific antagonists. A549 cell proliferation was reduced significantly by EP1 receptor inhibitor SC-51322 (1, 5 & 10 μM) (Figure 6a) and EP4 receptor inhibitors, L-161,982 (5 & 10 μM) (Figure 6d) where as EP2 and EP3 receptor inhibitors, AH-6809 and L -798106 respectively (Figure 6b & 6c) have no affect on proliferation of A549 cells. Basing on these experiments we concluded that among the EP receptors, only EP1 and EP4 receptors, contribute to the proliferation of A549 cells.

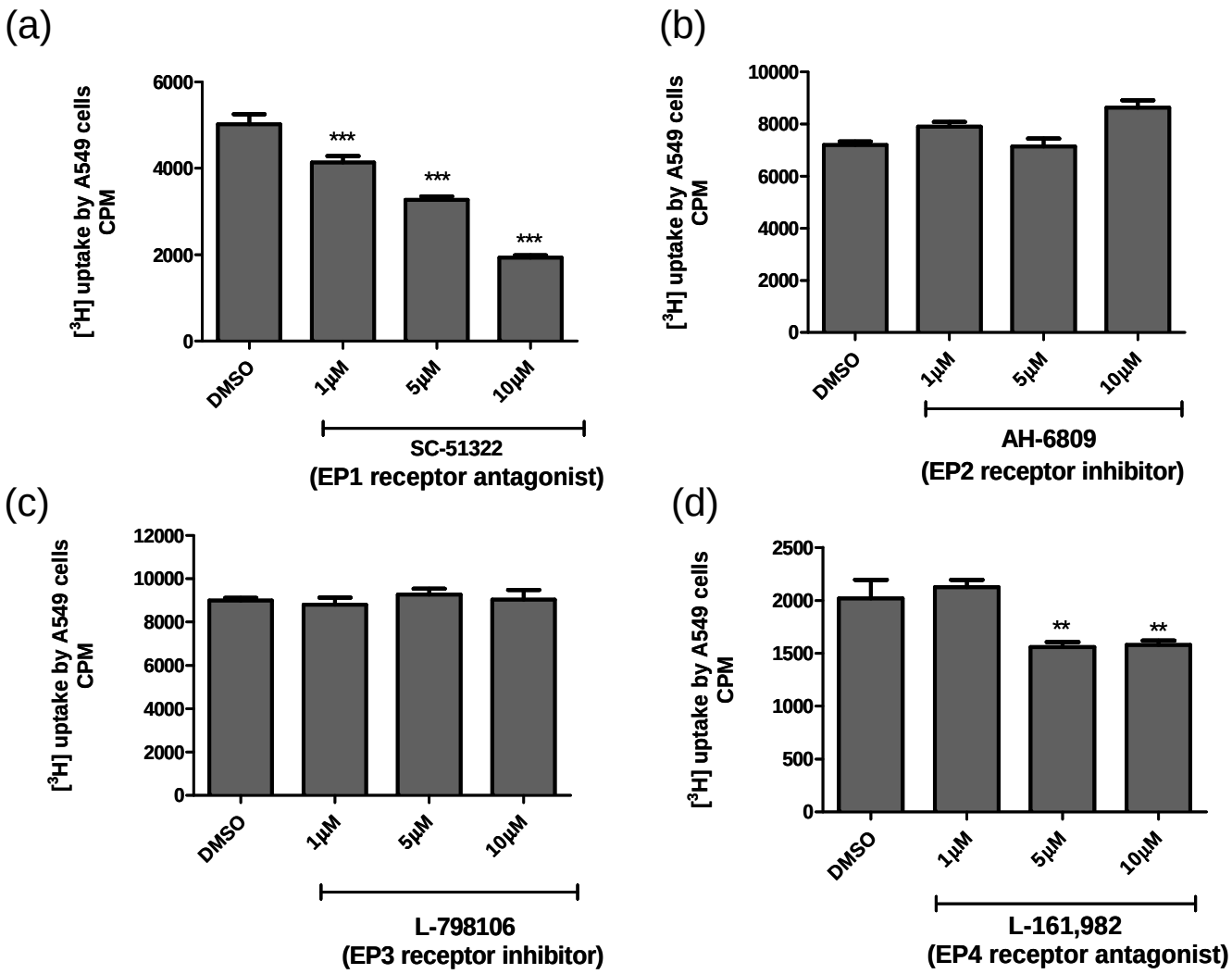


Figure 6: Anti-proliferative effect of EP receptor inhibitors on A549 cells

A549 cells were starved for 24 h and EP receptor specific inhibitors (SC-51322, EP1 inhibitor; AH-6809, EP2 inhibitor; L-798106, EP3 inhibitor; L-161982, EP4 inhibitor) were applied respectively for 24 hours. [3H]-thymidine incorporation was performed in the last 4 h of experiment. Results represent the mean ± SEM for n=3 experiments done in duplicate; **p ≤ 0.01 and *** p ≤ 0.001 compared with control.

6.4 Expression profile of EP receptors in H1299 cells

In order to substantiate the findings from A549 cell line, Expression profile of EP receptors in H1299 cells was done by western blotting. EP1 and EP2 receptors exhibited low expression as compared to EP3 and EP4 in H1299 cells as observed by western blotting(Figure 7a), followed by densitometric analysis (Figure 7b).

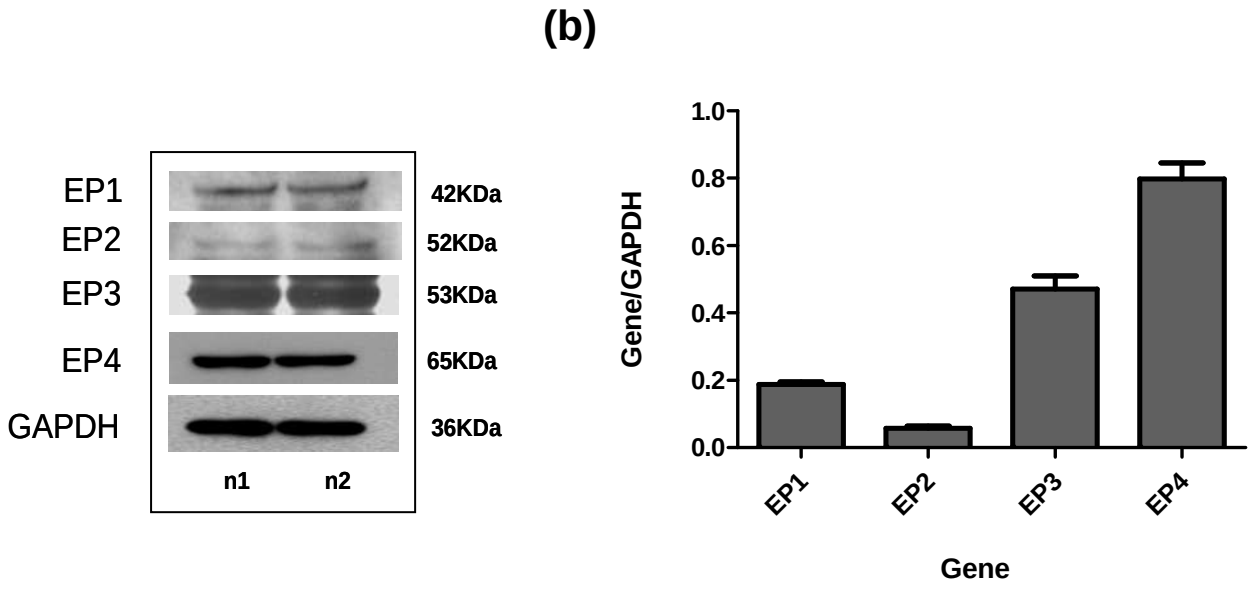


Figure 7: EP receptor expression profile in H1299 cells

Relative protein levels of EP receptors in H1299 cells and (a) representative immunoblots against EP receptors with GAPDH as a loading control (b) Densitometric quantification of EP receptors' expression in H1299 cells is shown as a ratio by normalization to GAPDH in a bar graph.

6.5 Cellular localisation of EP receptors in H1299 cells

We further confirmed our western blot data by investigating the cellular localisation of all EP receptors in H1299 cells by using immunocytochemistry. Immunofluorescence staining showed a predominant expression of EP1 and EP2 receptors both on cell membrane and nucleus where as EP3 and EP4 receptors were confined mostly to cell membrane (Figure 8).

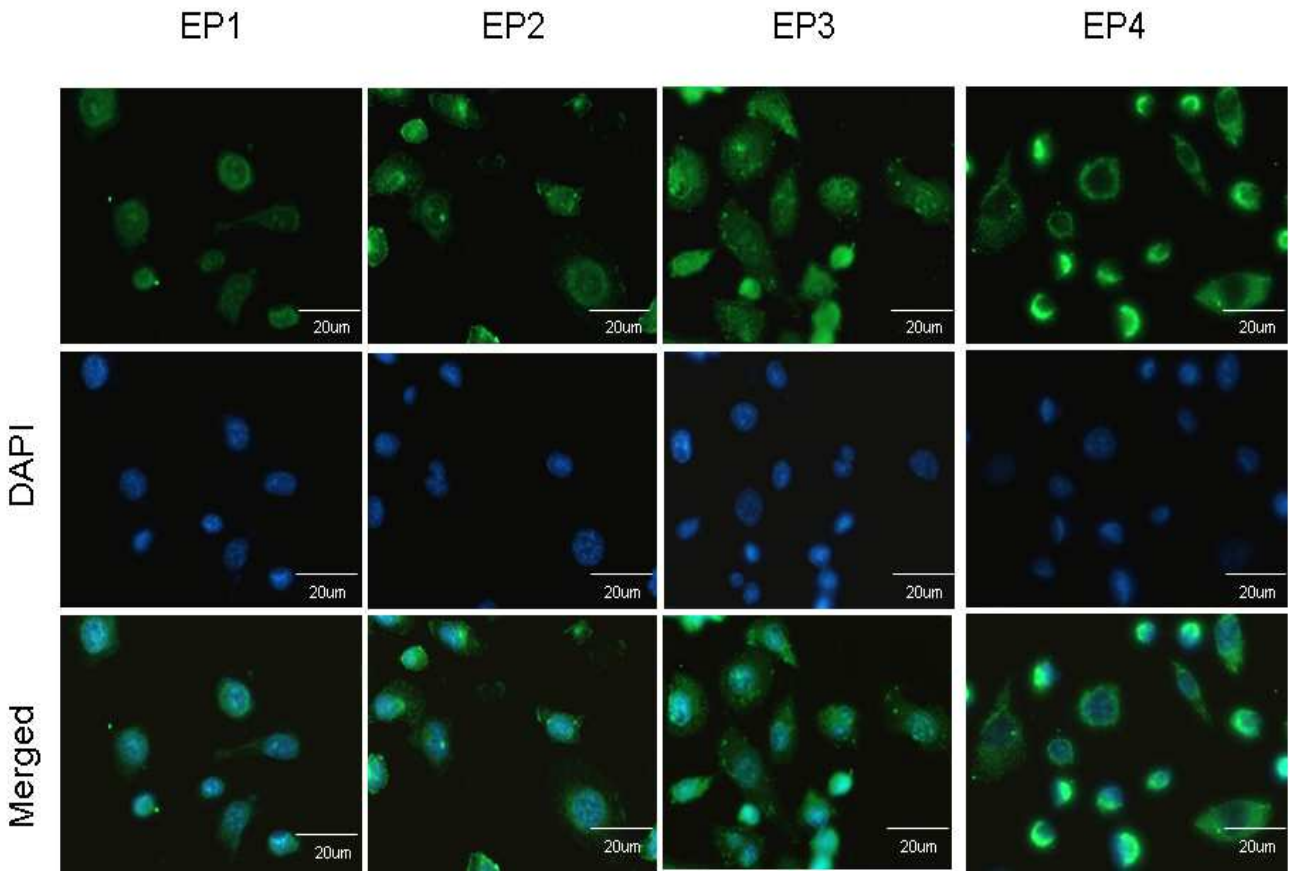


Figure 8: **Immunocytochemical staining of EP receptors in H1299 cells.**

Cellular localization of EP1, EP2, EP3 and EP4 in H1299 cells, shown by representative immunofluorescence. Green (EP1-4, Alexa 488/555 - conjugated); Blue (nuclei, DAPI). Staining is shown in a 400×magnification.

6.6 Anti proliferative effect of EP1 inhibitor on H1299 cells

Similar to our studies in A549 cell line, we investigated the contribution of EP receptors to the proliferation of H1299 cells by using EP receptor specific antagonists. H1299 cell proliferation was reduced significantly by EP1 receptor inhibitor, SC-51322 (Figure 9) and EP4 receptor inhibitors, L-161,982 at 10 μM concentrations. Where as EP2 and EP3 receptor inhibitors, AH-6809 and L -798106 respectively have no affect on proliferation of H1299 cells that EP1 and EP4 receptors contribute the most to proliferation of H1299 cells.

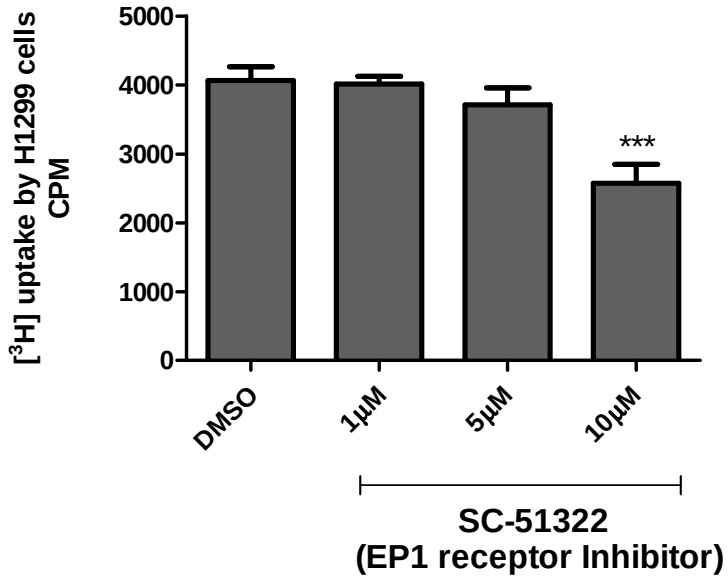


Figure 9: Anti-proliferative effect of EP receptor inhibitors on H1299 cells.

H1299 cells were starved for 24 hours and EP receptor specific inhibitors (SC-51322, EP1 inhibitor; AH-6809, EP2 inhibitor; L-798106, EP3 inhibitor; L-161982, EP4 inhibitor) were applied respectively for 24 hours. [3H]-thymidine incorporation was performed in the last 4 h of experiment. Results represent the mean ± SEM for n=3 experiments done in duplicate; *** $p \leq 0.001$ compared with control.

6.7 Inhibiton of EP1 receptor does not modulate intra cellular cAMP accumulation

To determine the secondary messenger downstream to EP1 receptor, we measured intracellular cAMP levels in presence and absence of SC-51322 (EP1 receptor inhibitor).

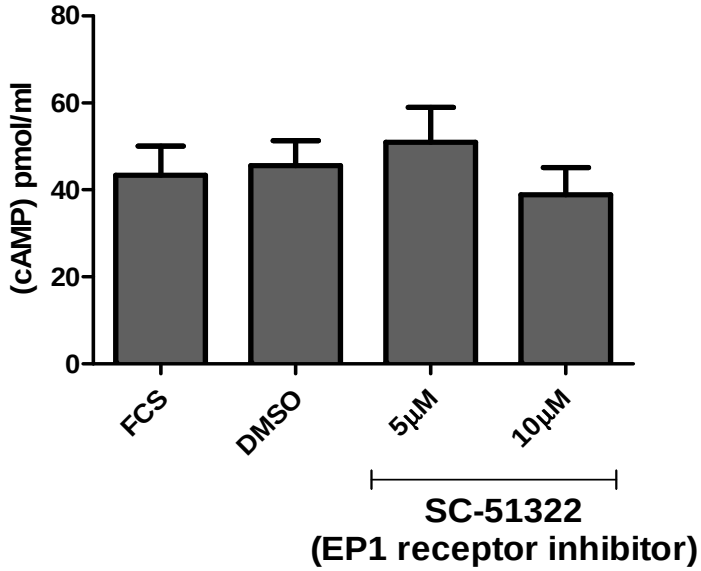


Figure 10: Pharmacological inhibition of EP1 receptor does not alter intracellular cAMP levels

A549 cells were starved for 24 h and treated with 10% FCS. After the treatment, Intra cellular cAMP levels of A549 cells were measured in presence of SC-51322 using Tecan spectrophoto meter. SC-51322 could not alter the intracellular cAMP levels at 5 µM & 10 µM.

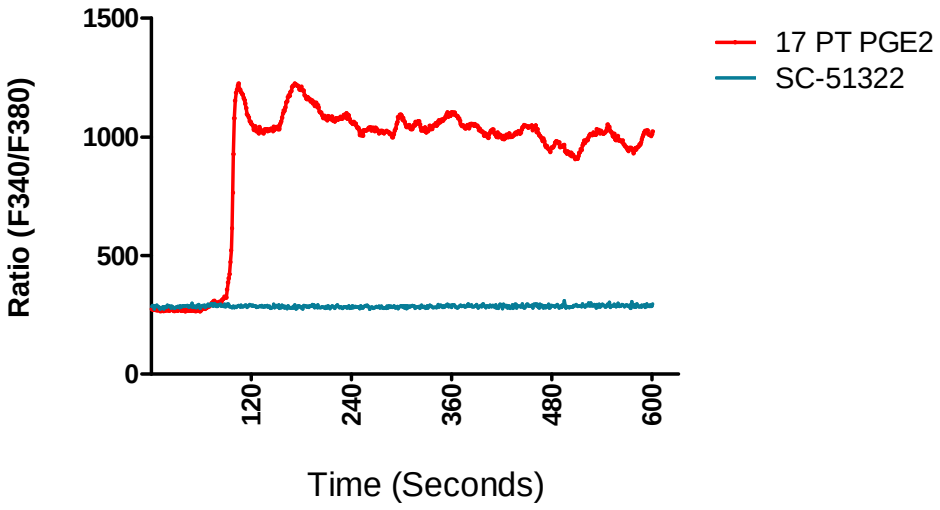
Intracellular cAMP levels of A549 cells did not show any significant changes after inhibiting with SC-51322 (Figure 10).

6.8 Inhibiton of EP1 receptor modulates intra cellular calcium levels

The EP1 receptor agonist, 17-PGE2 induced changes in Ca^{2+} in cultured A549 cells. Figure 9 shows the effects of a maintained bath application of 17-PGE2 on intra cellular Ca^{2+}, in A549 confluent cultures. The cells were superfused twice, first with a normal Hepes Ringer solution twice to yield a steady baseline in the resting Ca^{2+}, then to one with agonists added. The responses shown are for the agonists from different coverslips of cells of the same passage. The shape of the responses for PGE2 and 17-PGE2 is similar; there is an initial large intra cellular Ca^{2+} spike (raising the intra cellular Ca^{2+}, by about 300-400 nM), followed by a decaying oscillatory phase. This phase consists of a series of spikes with diminishing amplitudes and a periodicity initially of 20-30 set, but increasing to 40- 100 set as the response gradually is damped. About 66% of experiments gave an oscillatory response to PGE2 and 17-PGE2 during sustained agonist application.

While an initial rapid spike was observed consistently in all experiments, the number of oscillations which followed showed some variations; In-addition, in a few experiments with 17-PGE2, oscillations did not appear to be damped out entirely but continued on until the end of the experiment (10 min). In contrast to PGE2 and 17-PGE2, 10 µM of SC-51322 (EP1 antagonist) elicited virtually no effect on intra cellular Ca^{2+}.

(a)



(b)

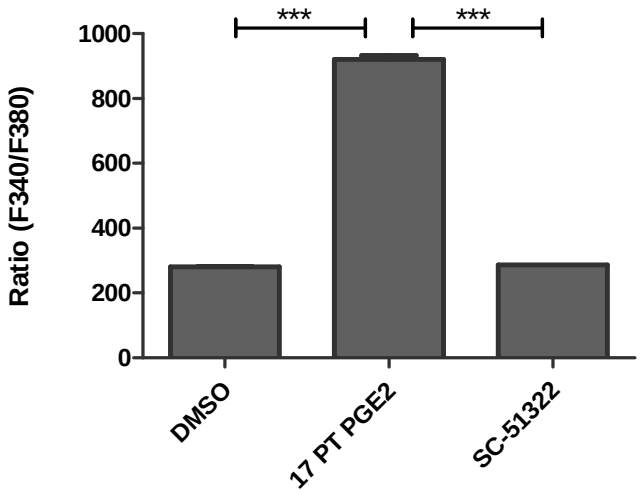


Figure 11: Inhibiton of EP1 receptor modulates intra cellular calcium levels of A549 cells

A549 cells were treated with 500µM of 17-P-T-PGE2, increased intracellular calcium was observed (Figure 11a&b) where as no increased intracellular calcium was observed when A549 cells were treated with 10 µM concentrations of EP1 inhibitor, SC-51322 (Figure 11a&b) and DMSO control has shown no effect on intra cellular calcium levels. Results represent the mean ± SEM for n=3 experiments done in duplicate; *** $p \leq 0.001$ compared with DMSO control**.**

6.9 Pharmacological Inhibiton of EP1 receptor modulates ERK phosphorylation

A549 cells were serum starved for 24 h and treated with the EP1 inhibitor, SC-51322 for 30 minutes and 24 h. SC-51322 reduced the activation of ERK in A549 cells when treated for 30 minutes but showed no significant effect when A549 cells were treated for 24 h (Figure 12).

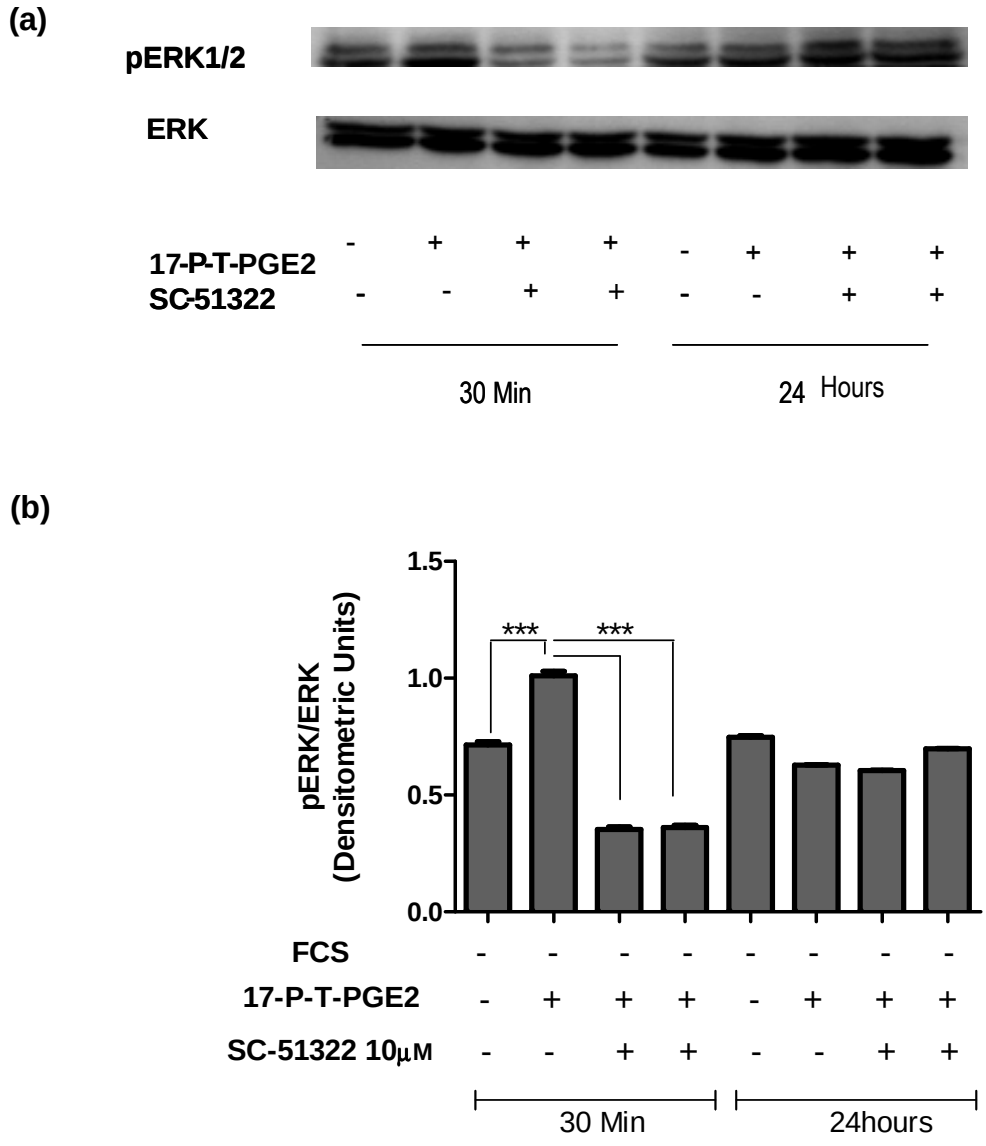
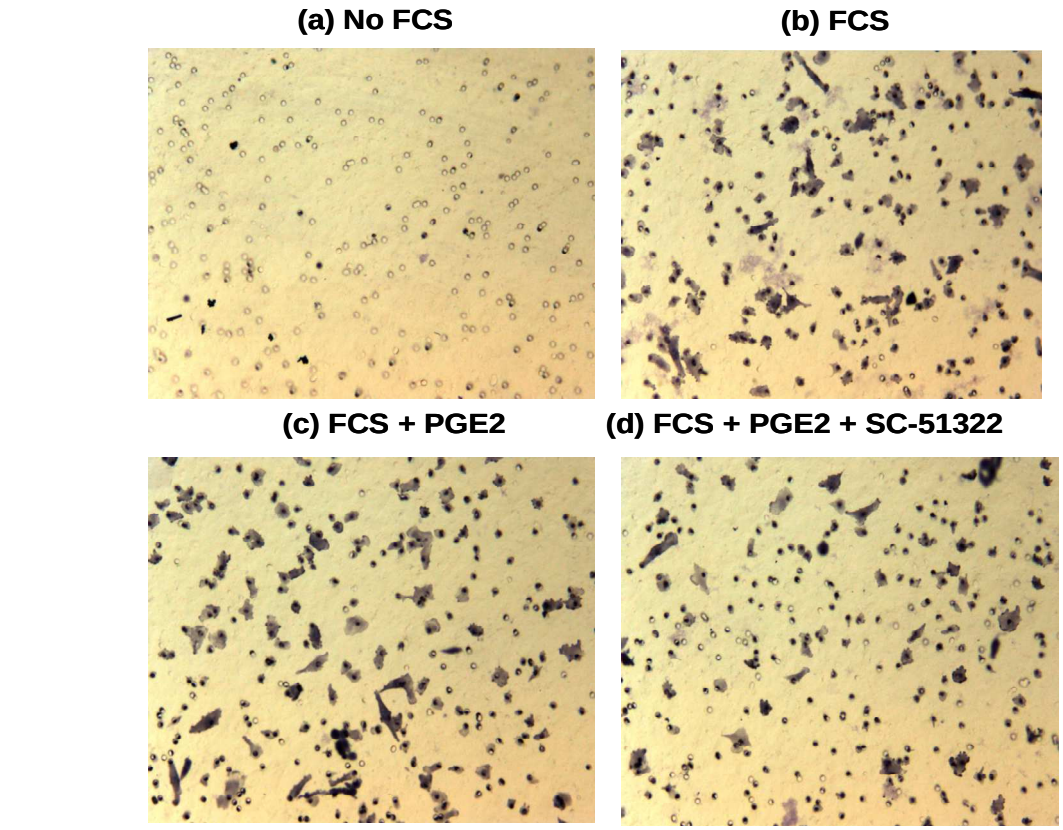


Figure 12: Inhibition of phosphorylation of ERK by EP1 receptor inhibitor.

A549 cells were starved for 24 hours and treated with EP1 inhibitor, SC-51322 for 30 m and 24 h. Proteins were isolated from the treated A549 cells and immunoblotted against phospho-ERK and total ERK, with GAPDH as a loading control. Results represent the mean ± SEM for n=3 experiments done in duplicate; *** $p \leq 0.001$ compared with control**.**

6.10 Pharmacological inhibition of EP1 receptor inhibits A549 cell migration.

A549 cells were serum starved for 24hours and then stimulated with 10% FCS in presence and absence of 10μM PGE2 and SC-51322 (EP1 receptor inhibitor) in a transwell migration chamber.



(e)

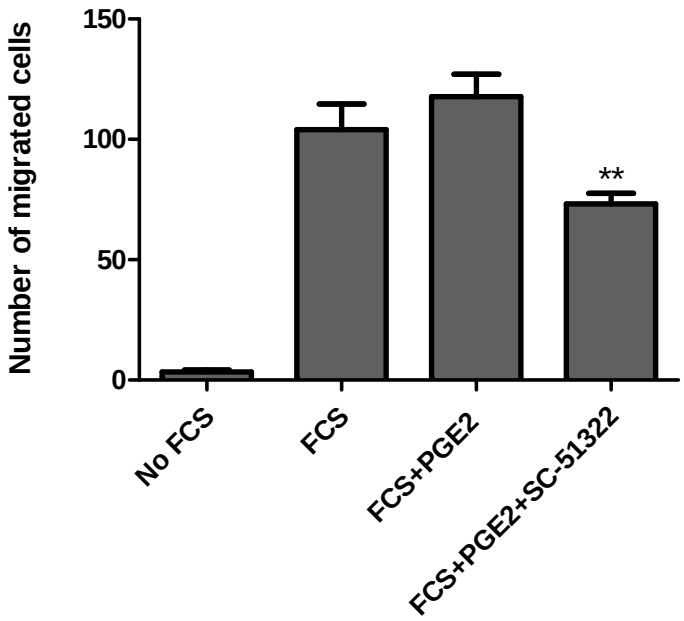


Figure 13: EP1 receptor antagonist inhibits A549 cell migration.

Migration of A549 cells towards 10% FCS was observed (Figure 13 b). 10 µM PGE2 along with 10% FCS attracted more number of cells compared to 10% FCS alone (Figure 13 c) where as, very less number of migrated cells were observed when cells were pre-treated with 10 µM of SC-51322 (Figure 13 d). DMSO/No FCS control can be seen with almost no migrated cells (Figure 13 a) and a representative graph can be seen in Figure 13e. Results represent the mean ± SEM for n=3 experiments done in duplicate; *** $p \leq 0.001$ compared with control.

6.11 EP1 inhibitor has no cytotoxicity on A549 cells

Cell viability/cytotoxicity were assessed by the MTT assay using a CellTiter 96AQ kit according to the manufacturer's instructions. Briefly, the cells were plated in 96-well plates and allowed to attach for 6 h, and then cultured under serum-free conditions with various concentrations of SC 51322 for 48 h. The number of surviving cells was determined by measuring the absorbance at 560 nm of the dissolved formazan product after addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt for 1 h.

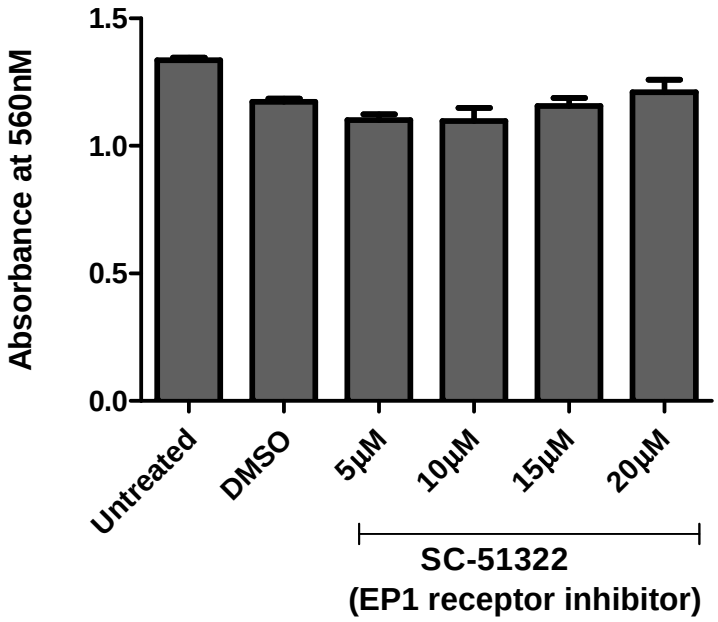


Figure 14: Cytotoxicity of EP1 inhibitor on A549 cells

No cytotoxic effects were observed when A549 cells were treated with 5 µM, 10 µM, 15 µM and 20 µM of EP1 receptor specific inhibitor SC-51322.

7 DISCUSSION

A very high expression of COX-2 is frequently found in many types of cancer, including lung cancers and is usually associated with poor prognosis and short survival [87]. Identification of four subtypes of the PGE receptors (EP1, EP2, EP3 & EP4) has made it possible to analyze their effects on human cancer cells. Cancer cells express multiple PGE receptor subtypes and that each subtype may be linked to different actions of PGE2. So, it is important to know the role of each receptor individually to identify them as potential targets for cancer treatments.

Here, we examined the expression pattern at mRNA and protein level of all four EP receptors in A549 and H1299 cells (adenocarcinoma cell lines) using qRT-PCR and western blots. All four receptors exhibited strong mRNA expression in the cell line (Figure 4a). In line with this result, all the EP receptors showed protein expression (Figure 4b, 4c & 7a, 7b). Additionally, immuno-cytochemical stainings showed EP3 and EP4 receptors to be confined mostly to cell membrane, where as EP1 and EP2 receptors were found to be expressed in both cell and nuclear membrane (Figure 5 & 8). Finding these receptors on nuclear membranes might have a role in transferring message received from cell membrane to the nucleus, and would be a subject for investigation in future.

The importance of EP1 receptors in carcinogenesis has been studied using diverse models. In EP1 deficient mice, tumor incidence, tumor multiplicity and tumor volume were decreased in the AOM-induced colon carcinogenesis model [88]. Antagonisisng EP1 receptor through diet reduced abberant crypt foci formation in mouse [89], [90]. Increase in EP1 positive cells was observed in mice skin after prolonged UV treatment [91]. In UVB-induced mouse papillomas and SCCs and human SCCs, EP1 mRNA expression and protein levels were increased 2 ~ 10 fold [70], [92]. In glioma cells, SC51089 and AH6809 (EP1 receptor antagonists) suppressed cell proliferation by 50% [93]. Though EP1 is not expressed in normal mouse mammary glands, an increase in EP1 expression is observed in mammary tumors [94]. In rat hepatocytes an increase in DNA synthesis was observed in a PLC dependent manner when they were treated with 17-phenyl-trinor-PGE2 and sulprostone, EP1 agonists [95]. In human breast cancer cells, EP1 receptor antagonist treatment significantly suppressed VEGF-C protein production [96]. PGE2 mediated upregulation was inhibited by EP1 receptor siRNA in colon tumor cells [97].

In lymphangiogenesis, EP1 is also known to play an important role via VEGF-C. Treatment with SC19220, an EP1 antagonist or an EP1 antisense oligonucleotide inhibited PGE2 mediated VEGF-C mRNA and protein induction effectively. Su, Shih et al in 2004 have proved that EP1 mediated VEGF-C induction is mediated through Src and Her2/Neu activation [98].

The EP2 receptor was shown to play a significant role in the protumorigenic action of PGE2 in skin tumor development. The EP2 knockout mice produced significantly fewer tumors and reduced tumor incidence compared with wildtype mice in a two-stage skin carcinogenesis model [99]. Furthermore, UV-irradiated mice lacking EP2 receptors exhibit decreased proliferation and a poor capacity for epidermal hypertrophy in response to UV injury. Interestingly, in a chronic irradiation model, these animals were protected from tumor formation, developing 50% fewer tumors than wildtype controls. Despite this capacity to protect against tumorigenesis, animals lacking EP2 receptors grew tumors that were larger in size, with a more aggressive phenotype. This was found to be associated with greater quantities of active matrix metalloproteinases than keratinocytes expressing the EP2 receptor, thereby enhancing the invasive potential of EP2-/- cells.

Furthermore, one study showed that disruption of EP2 decreases the size and number of intestinal polyps in $APC^{\Delta 716}$ mice [100]. The lack of EP2 in *MMTV-COX-2* transgenic mice strongly suppressed COX-2–induced effects such as precocious development of the mammary glands. The development of mammary hyperplasia in multiparous female mice suggests disruption of EP2 signalling as a chemopreventive approach to breast cancer [101].

The EP3 receptor is the one EP receptor that has consistently *not* been directly involved in tumor production. Examination of mRNA expression levels in colon tissue of mice, rats and human showed a marked reduction in the EP3 receptor in colon cancer compared to normal mucosa [102-103]. Expression of EP3 mRNA was detected in only one of eleven colon cancer cell lines tested and treatment of that one cell line with an EP3 agonist decreased viable cell numbers by 30%. Immunohistochemical staining demonstrated rat EP3 protein expressed in normal epithelium and some parts of small carcinomas but absent in large carcinomas of the colon, suggesting EP3 is downregulated during colon cancer progression. While deficiency in EP3 had no effect on ACF development, an

increase in AOM-induced colon carcinoma was found in EP3 knockout mice suggesting EP3 may not impact early stages as greatly as later stages of colon carcinogenesis. As noted, EP2 and EP4 stimulate adenylate cyclase, whereas EP3 inhibits cAMP formation, suggesting a possible suppressive role against colon carcinogenesis.

In addition to CRC, EP1, EP2 and EP4 receptors were elevated, whereas EP3 receptor levels were decreased in mammary tumors from *COX-2/MMTV* mice [104]. In contrast to these findings, a recent study reported EP3 receptor signaling to play an important role in tumor-associated angiogenesis [105]. In this context, implanted tumor growth (sarcoma-180, Lewis lung carcinoma) and tumor-associated angiogenesis were markedly reduced in EP3 null mice. Moreover, EP3 antagonists reduced tumor size in wildtype but had no effect on EP3 null mice. Using the sponge implantation model, it was shown that VEGF expression is significantly reduced in EP3 null mice compared to wildtype mice.

There is a growing appreciation for the EP4 receptor as an important transducer of PGE_2 signaling, leading to cell invasion and motility during tumorigenesis. The EP4 receptor is over expressed in several different cancers including colon and rectal cancers [106]. Interestingly, constitutive expression of EP4 receptor promotes proliferation and anchorage-independent growth, demonstrating that the EP4 receptor may also be a key regulator of tumor progression [107]. This receptor has been shown to have the highest affinity towards pro-tumorigenic PGE_2 ligand in ligand binding assays ([108-109]. EP4 receptor signaling also appears to be important for cell movement and motility during development. Studies in the zebra fish show that the EP4 receptor transduces PGE_2 signals to regulate appropriate speed of cell migration during gastrulation, demonstrating that regulation of cell motility by EP4 receptor signaling is evolutionarily conserved [110].

Signaling mediated through the EP4 receptor in colorectal carcinogenesis is constantly being appreciated. PGE_2 was shown to stimulate the proliferation and motility of LS174T adenocarcinoma cells through the EP4 receptor dependent activation of PI3K/AKT signaling [111]. Whereas PGE_2 inhibits apoptosis in human Caco-2 adenocarcinoma cells through an EP4 dependent pathway [112]. Furthermore, premalignant ACF formation in EP4 deficient mice following AOM treatment is suppressed as compared to those in EP4 wild type mice. This study also showed a reduction in colon adenomatous polyp formation in mice wild-type for the EP4 receptor but treated with the EP4 receptor antagonist ONO-

AE2-227 [113]. Treatment with another EP4 antagonist, ONO-AE3-208, decreased liver metastases after intrasplenic injection of MC26 colon cancer cells [114]. In addition, *in vitro* studies indicate that PGE_2/EP4 receptor signaling via ERK activation promotes tumorigenic behavior in colon cancer cells [115]. Another avenue that PGE_2/EP4 signaling has been shown to contribute towards carcinogenesis is the Wnt signaling pathway. Interestingly, PGE_2 stimulated EP4 receptor can transcriptionally activate a transcription factor Tcf/Lef (T Cell Factor/Lymphoid Enhancer Factor) via a PI3K mediated pathway. This transcription factor regulates the expression of Cyclin D1 which is a key protein in cell cycle progression in colon carcinogenesis [116], [117]. In addition, the EP4 receptor was determined to be a genetic risk factor in both ulcerative colitis as well as Crohn's disease in a study that used genome-wide associations to understand molecular pathways leading to Inflammatory Bowel Disease (IBD) [118].

A reduced number of azoxymethane (AOM)-induced aberrant crypt foci were observed in EP4 receptor deficient mice and antagonizing EP4 receptor also resulted in a similar manner in Min mice [113]. In one of the lung cancer models, Lewis lung carcinoma (LLC) cells, a reduced metastasis has been observed when they were treated with an EP4 receptor antagonist [114]. Mouse mammary tumor cells treated with EP4 antagonists also had reduced metastasis in vivo [119].

Cancer cells divide uncontrollably increasing both in size and number. So, pharmacological interventions to prevent the rate of proliferation of cancer cells have always been of great interest. PGE2 signaling through the EP4 receptor has previously been associated with NSCLC. Prostaglandin E2 stimulates human lung carcinoma cell growth through induction of integrin-linked kinase with the involvement of EP4 receptor and Sp1 protein [120]. Ritzenthaler JD et al [121] in 2009 have shown that NSCLC cells express PPARbeta/delta protein and that treatment with a selective PPARbeta/delta agonist, GW501516, stimulated the expression of EP4 and induced NSCLC cell proliferation. In agreement to previous studies, L-161982 an EP4 receptor specific inhibitor prevented the A549 and H1299 cells from proliferation in thymidine incorporation assays (Figure 6 & 9).

Though In lung cancer, especially in NSCLC, prostanoid receptor EP4 is known to play role in growth and metastasis, no other EP receptor has been shown to be involved in pathology of NSCLC.

Here, we studied the effect of EP receptor specific inhibitors on the rate of proliferation of both A549 and H1299 cells. SC-51322 an EP1 receptor specific inhibitor showed inhibitory effects at 1 µM, 5 µM and 10 µM on A549 cells (Figure 6) where as, it showed inhibitory effects at 10 µM on H1299 cells (Figure 9). Both AH-6809 (EP2 receptor specific inhibitor) and L-798106 (EP3 receptor specific inhibitor) showed no inhibitory effect on A549 and H1299 cell proliferations (Figure 6 & 9). So, from the above results it is a new finding to know the role of EP1 receptor in the proliferation of NSCLC cell lines besides an already known role of EP4 receptor. The cytotoxicity of SC-51322 on proliferation of A549 cells has been established using MTT assay. MTT assay shows no cytotoxic affects on A549 cells when they were treated with different concentrations of SC-51322 (Figure 14). From this MTT assay we can conclude that reduced proliferation values of A549 cells, is truly EP1 receptor mediated and not by formation of dead cells due to its cytotoxicity.

PGE2 signals through four G-protein-coupled cell surface receptors, termed as EP1, EP2, EP3, and EP4. The EP1 receptors are coupled to Gαq protein and increase cytosolic Ca^{2+} levels in response to PGE2 [109]. Both EP2 and EP4 receptors increase intracellular cyclic AMP (cAMP) levels by activating adenylyl cyclise by coupling to Gαs, whereas Gαi-coupled EP3 receptors inhibit cAMP formation [109]. Thus, the effects of PGE2 on cell proliferation appear to be mediated by its overall second messenger response [122]. To further decipher the role of EP1 receptor in proliferation of A549 cells, they were treated with 500 µM of 17-P-T-PGE2 which elicits in an increase in intra cellular calcium where as no increase of calcium was observed when cells were pre-treated with 10 µM of SC-51322 for 30 min (Figure 11a & b). This experiment establishes the role of calcium in EP1 mediated proliferation of A549 cells.

Involvement of ERK pathway and prostanoid signaling in the progression and metastasis of many types of cancers has been well documented previously [123], [124]. Recent literature suggests a crosstalk of these two pathways in the malignant cancers [125]. In the present study, we demonstrated a concentration and time-dependent activation of ERK in A549 cells in presence of 17-P-T-PGE2 (EP1 receptor specific agonist) at various concentrations. A selective EP1 receptor antagonist, SC-51322 suppresses 17-P-T-PGE2 induced ERK phosphorylation after 30 min of incubation. In this context, our results suggest that selective targeting of EP1 receptor with EP1 receptor antagonists such as SC-51322 may be

beneficial to block PGE2 effects on cell proliferation of human lung cancer cells. We plan to evaluate the efficacy of SC-51322 analogs *in vivo*.

Cancer cells divide uncontrollably and start to invade other parts of the body by a process called metastasis. It is a very important phase of cancer development both in terms of treatment and survival of patients. Once cancer gets metastasized, it becomes hard to treat them. Mortality in patients with cancer principally results from the metastatic spread of cancer cells to distant organs [126].

PGE2 has been reported to stimulate the motility of A549 cells via EP4-dependent stimulation of betaArrestin1, and c-Src which mediates cancer cell migration [127]. It has also been shown that, PGE2 enhances the migration of chondrosarcoma cells by increasing alpha2beta1 integrin expression through the EP1/PLC/PKCalpha/c-Src/NF-kappaB signal transduction pathway [128]. In oral cancer cells, prostaglandin E2/EP1 signaling pathway enhances intercellular adhesion molecule 1 (ICAM-1) expression and cell motility and a cross-talk between the COX-2/PGE(2)/EP(1) and EGFR/c-Met signaling pathways has been reported which coordinately regulate human HCC cell invasion [129-130]. These findings led us to investigate the contribution of EP signaling in mediating migration of A549 cells.

A549 cells, after 24 hours of serum starvation, significantly started moving towards 10% FCS in a transwell migration chamber (Figure 13b). The number of cells moving towards 10% FCS had increased in presence of 10 µM of 17-P-T-PGE2 in the media (Figure 13e). Where as, SC-51322 had significantly reduced the number of migrating cells towards a stimulus of 10% FCS and 10 µM 17-P-T-PGE2 (Figure 13d & e). Though, SC-51322 inhibited migration of A549 cells in transwell migration assay, it needs to be further investigated *in vivo* to completely establish a role of EP1 receptor in metastasis of NSCLC.

Conclusion:

Our studies, which are in agreement with several other reports, indicate an important role for EP1 receptor in proliferation of NSCLC for the following reasons:

1. Reduced proliferation of A549 & H1299 cells in response to the SC-51322

2. Pre-treatment of A549 cells with AH-6809 (EP2 antagonist) and L-798106 (EP3 antagonist) did not have any effect on PGE2-induced ERK phosphorylation.

3. 17-phenyltrinor PGE2 (a selective EP1 agonist), induced ERK phosphorylation.

4. L-161,982 (EP4 receptor antagonist) and SC-51322 (EP1 receptor antagonist) at concentrations of 10μM abrogated 17-P-T-PGE2-induced ERK phosphorylation.

5. SC-51322 has also inhibited release of intracellular calcium when stimulated with 17-P-T-PGE2 establishing a EP1/PLC/ERK pathway in proliferation of A549 cells. SC-51322 has not decreased cAMP levels in A549 (Figure 10) which is in agreement to our speculation.

In summary, the data presented in these studies strengthen an important role for the EP1 receptor in NSCLC. Since PGE2 is frequently elevated in NSCLC, signalling mediated by the EP1 receptor could represent an important step in the clonal evolution of lung cancer epithelial cells during the adenoma-carcinoma sequence and contribute to malignancy. Finally, in light of the recent side-effects associated with the use of COX-2 inhibitors, our studies support the notion that the inhibition of EP1 receptor signalling events may represent an alternative therapeutic target for the prevention and treatment of NSCLC.

Proposed pathway of prostanoid signalling in NSCLC proliferation

1. 17-P-T-PGE2 binds specifically to cell membrane bound prostanoid receptor EP1.
2. EP1 receptor upon binding to 17-P-T-PGE2, elicits an increase in intra cellular calcium.
3. Increased intra cellular calcium activates ERK resulting in an increase of pERK which up on translocation to nucleus results in cellular proliferation, metastasis and angiogenesis.

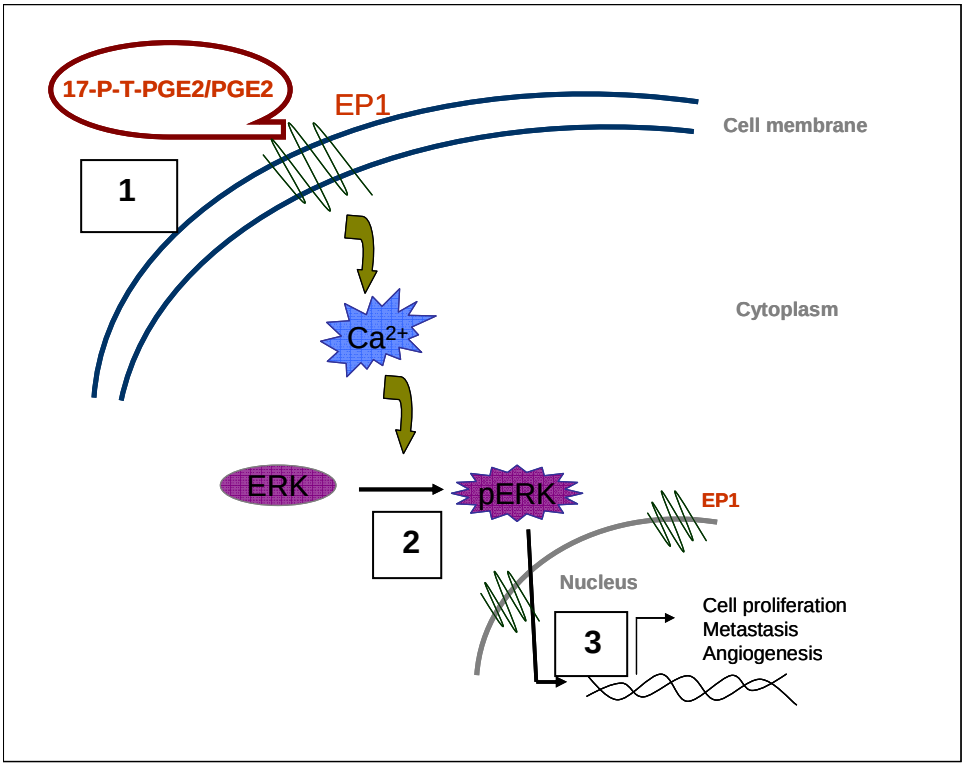


Figure15: Proposed Mechanism of Action

Outlook

We need to further substantiate our results by performing

- gene silencing experiments using siRNA / shRNA to EP1 receptor
- dissection of more downstream effectors to EP1 receptor mediated signaling
- experiments with *in vivo* lung cancer models and
- human tissue samples

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ERKLÄRUNG

Ich erkläre: Ich habe die vorgelegte Dissertation selbständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertaion angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis niedergelegt sind, eingehalten.

Giessen **Pavan Kumar Pamarthi**

9 ACKNOWLEDGEMENTS

My deepest gratitude goes first and foremost to my supervisor, Prof. Dr. Ralph T. Schermuly. His professional guidance, constant encouragement and support kept me all the way on track during my scientific research.

Also I would express heartfelt gratitude to Prof. Dr. Werner Seeger, who motivates me entering pulmonary research and provides all possible conditions for research.

I sincerely thank Dr. Rory Morty, Dr. Soni S. Pullamsetti, Dr. Grazyna Kwapiszewska and Dr. Malgorzata Wygrecka for their excellent tutoring in the MBML (Molecular Biology and Medicine of the Lung) graduate program.

I own great gratitude to Dr. Raj Kumar Savai, not only for his initiation of this project, his intellectual suggestions and helpful comments, but also for his precious care and friendship. It gave me great pleasure to work with him.

I would like to thank all my wonderful colleagues for teaching me techniques, offering experimental tips and more important for the nice working atmosphere they have created specially Joachim Berk for translating a lot of documents including the German version of summary here in the thesis; to Dr. Xia Tian for her instructive suggestions; to Dr. Ewa Kolosionek, Dr.Sevdalina Nikolova, Dr. Sergey B. Udalov, Dr. Piotr Sklepkiewicz, Swati Dabral, Ravi Kumar Komaravolu, Himal Luitel, Gayathri viswanathan, Lal Kurian and Mattias Eschenhagen for all the helps and friendship; to Stephanie Viehmann and Katharina Weidl for their technical assistant; also to other colleagues, too numerous to mention, who helped in their own little way throughout this work.

“Thank you very much”.

My thanks also extend to Prof. Dr. Friedrich Grimminger, Dr. Ardeschir Ghofrani and Dr. Norbert Weissmann for the cooperative support.

Further I highly appreciate my Indian friends for their kind help and moral support during my study.

Last but not least, I am especially indebted to my beloved parents for everything they have done for me all through these years, my wife Mamatha Inturi for her unsummoned patience and sacrifices, my brother and sister for their unconditional love and care. Without the love and encouragement from them, I can never have the chance to finish my study.

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

presence and absence of agonists and antagonists. Downstream molecules to EP receptors playing role in progression and metastasis of lung cancer were measured by western blotting. New directions to the project are under investigation.

Presentations:

- Oral presentations at MBML retreats on the topics of "Role of prostanoid signaling in Pulmonary Arterial Hypertension (PAH)" (2009 & 2010) and "Role of prostanoid signaling in lung cancer (2011)".

- Poster presentation on a topic titled "Role of prostanoid signaling in lung cancer" at *103rd annual meeting of American Association for Cancer Research (AACR)* held during 30th March – 4th of April 2012 in Chicago, USA.

Publications:

Current project is under the process of submission.

1. Therapeutic efficacy of azaindole-1 in experimental pulmonary hypertension.

Eur Respir J. 2010 Oct;36(4):808-18. Epub 2010 Jun 7.
Dahal BK, Kosanovic D, ***Pamarthi PK,*** *Sydykov A, Lai YJ, Kast R, Schirok H, Stasch JP, Ghofrani HA,Weissmann N, Grimminger F, Seeger W, Schermuly RT.*
University of Giessen Lung Center, Giessen, Germany.

Previous Job Experience

Associate Research Scientist (Feb'06 – Sep'08)

Worked as an **Associate Research scientist** at Advinus Therapeutics Pvt.Ltd (A TATA Enterprise), Pune, India.

Job responsibilities:

As an associate scientist, I was involved in planning and executing research protocols. I was responsible to analyze the results and report/document to the groups (assay and molecular biology groups of Drug Discovery wing) involved in Phase I studies.

Job profile:

Cloning and expression of target genes in bacterial and mammalian systems. Tranfection of DNA in to mammalian cells (Eg: HEK; Human Embryonic Kidney and CHO; Chinese hamster Ovary), characterization of clones by calcium and cAMP assays and single cell selection of clones.

My responsibilities involved screening for NCEs (New Chemical Entities) with therapeutic value in metabolic and inflammatory disorders using cell based GPCR assays, by measuring intracellular calcium flux (FLEX STATION II) and cyclic AMP assays. My work also involved screening of therapeutic small molecules against rheumatoid arthritis, a disabling and painful inflammatory condition, which can lead to substantial loss of mobility due to pain and joint destruction. During the course of this work, I have used extremely sensitive cell based assays and cell free assays like radio-ligand binding assay in order to establish a robust screening assay system for high throughput NCE screening.

My job responsibilities also included purification & characterization of targeted proteins from both mammalian and bacterial systems [Characterization - Km & specific activity determination], Studying expression profile of different genes at m-RNA level in different tissues and cell-lines by RT-PCR.

Junior Research Fellow (Oct'04 – Feb'05)

- Worked as a Junior Research Fellow at **TIFR** (Tata Institute of Fundamental Research), Mumbai, India.

Job responsibilities:

- Determination of LhX1 gene expression at mRNA level in different regions of the brain in 11.5 day mouse embryo by Non-radio labeled in-situ hybridization.
- Genotyping of mice colonies by tail DNA PCR for LhX1 +/+ & +/- strains.

MSc Thesis: [January'04 - July '04]

A Seven month training at Tansgenic and Gene Knock-Out Laboratory, Centre for Cellular and Molecular Biology **(CCMB),** Hyderabad on a project titled **"Structural analysis of Regulatory Mechanism in High Level Expression of Casein Genes in Mice"** under the supervision of Prof.Dr. Satish Kumar.

Academic Profile

M.Sc – Micro Biology
University : Barkatullah University
University Teaching Department – Bhopal, India.
Percentage : First class (65%)
Duration : 2002 – 2004.

B.Sc (Microbiology, Bio-chemistry and Chemistry)
University **:** Nagarjuna Univesity
T.J.P.S. College, Guntur, A.P, India.
Percentage : First class (75%)
Duration : 1999 – 2002.

HSSC (Botany, Zoology, Chemistry and Physics)
Board :S.R.K.S.Junior College (Guntur)
Board of Intermediate Education, Hyderabad, A.P, India.
Percentage : First class (73.7%)
Duration : 1996 – 1998.

SSC (Senior Secondary School)
Board : S.M.P.School, Guntur.
Board of secondary education, Hyderabad, A.P, India.
Percentage : First class (80.4%)
Duration : 1996.

Declaration

I here by declare that the above furnished data is true to best of my knowledge and understanding.

(Pavan Kumar Pamarthi)