POTENTIAL ROLE OF ISORHAMNETIN TO SUPPRESS PROLIFERATION, INDUCE APOPTOSIS AND INHIBIT EMT THROUGH THE MODULATION OF PPARγ/BMPR2 SIGNALING PATHWAYS

Ms. LALITHA RAMACHANDRAN
B. Tech., Pharmaceutical Technology
(Department of Biotechnology, Anna University)

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DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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Ms. Lalitha Ramachandran
August 2014

Dedicated to every wonderful child, fighting cancer.
We will find a way.

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SUMMARY

Gastric cancer (GC) is a major malignancy and the second-highest cause of death due to cancer. Even though treatment options such as chemotherapy, radiotherapy and surgery are available, survival rate of patients are low, which in turn creates a burning need to identify novel and efficient chemotherapeutic drugs. Natural compounds have been examined for their anti-cancer properties since time immemorial. In our study, we examine the anticancer effects of one such natural compound, Isorhamnetin, (IH) a 3'-O-methylated metabolite of quercetin, on gastric cancer cells, and its potential impact on the PPARγ activation pathway. The effect of IH was investigated using a combination of *in-silico, in-vitro* and *in-vivo* models. We initially utilized a functional proteomics tumor pathway technology platform to understand the potential activities of isorhamnetin, and later tested the same on gastric cancer cell lines and a xenograft mouse model.

Firstly, using a virtual predictive tumor cell system, we found that IH could modulate various genes involved in apoptosis, proliferation and angiogenesis, including PPARγ, a nuclear receptor involved in controlling cancer cell growth and inducing apoptosis. This led us to hypothesize that IH could act in gastric cancer via the PPARγ pathway. Our results demonstrated that IH exerted significant cytotoxic effect in both drug sensitive and resistant gastric cancer cells. We observed, for the first time, that IH increased PPARγ activity and modulated the expression of the PPARγ regulated genes. Also, the increase in PPARγ activity was partially reversed in the presence of PPARγ specific inhibitor and a PPARγ dominant negative mutant, supporting our hypothesis that IH can act as a ligand of PPARγ. We also observed using molecular docking analysis that IH indeed formed interactions with 7 polar

residues and 6 non-polar residues within the ligand-binding pocket of PPARγ that are reported to be critical for its activity.

Our results demonstrate that IH could inhibit transforming growth factor-β (TGF-β) induced proliferation, migration and invasion of gastric cancer cells. IH modulated the expression of genes such as *N-cadherin*, *E-cadherin*, *Vimentin*, *Snail* and *γ-catenin* that are involved in regulating epithelial mesenchymal transition (EMT), and also down-regulated levels of bone morphogenetic receptor protein-2 (BMPR2) in gastric cancer. Clinical analysis showed that BMPR2 gene expression correlated positively with gastric cancer prognosis and EMT score, thereby indicating its involvement in EMT progression. Supporting the clinical results, our *in-vitro* data showed that knockdown of BMPR2 mitigated the migratory potential of gastric cancer cells and attenuated EMT whereas over-expression of BMPR2 increased the migratory properties of gastric cancer cells.

We further noted that IH could enhance the cytotoxic effects of chemo-therapeutic agents against various gastric cancer cell lines, and significantly potentiate the antitumor effects of capecitabine in a gastric cancer xenograft mouse model, that correlated with suppression of various biomarkers of survival and angiogenesis. Overall, our findings clearly indicate that IH exhibits significant anti-cancer effects both *in-vitro* and *in-vivo* and these actions may be mediated at least in part, through the modulation of the PPARγ activation pathway in gastric cancer.

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LIST OF ABBREVIATIONS

15d-PGJ2	15-Deoxy-$\Delta^{12, 14}$-prostaglandin J2
5-FU	5-fluorouracil
AF-1	Activation function-1
AF-2	Activation function-2
AFB1	Aflatoxin B1
ANOVA	Analysis of variance
ATP	Adenosine tri-phosphate
Bcl-2	B-cell lymphoma protein 2
Bcl-xL	B-cell lymphoma-extra large
BMPR2	Bone morphogenetic receptor 2
BMP	Bone morphogenetic protein
CagA	Cytotoxin-associated gene A
CBP	CREB binding protein
CE	Carboxylesterase
COX-2	Cyclooxygenase-2
CXCL12	C-X-C motif chemokine 12
CXCR4	(C-X-C motif) receptor 4
DAB	3, 3-diaminobenzidine tetrahydrochloride
DBD	DNA binding domain
DCF	Docetaxel/cisplatin/5-fluorouracil (5-FU)
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DR-1	Direct Repeat-1

DupA	Duodenal ulcer promoting A
E-cadherin	Epithelial cadherin
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial to Mesenchymal transition
ERK	Extracellular signal-regulated kinase
EUS	Endoscopic ultrasound
FCM	Flow Cytometry
FDA	Food and Drug Administration
GC	Gastric Cancer
GDF	growth/differentiation factors
GIST	gastrointestinal stromal tumors
GPS2	G-protein pathway suppressor-2
H&E	hematoxylin and eosin
HDAC	Histone deacetylases
HDGC	Hereditary diffuse gastric cancer
HER-2	Human epidermal growth factor receptor 2
IARC	International Agency for Research on Cancer
IKKα/β	IκB kinase
IL-1β	Interleukin-1 β
IL-6	Interleukin-6
iNOS	Inducible nitric-oxide synthase
JNK	c-Jun N-terminal kinases
LBD	Ligand binding Domain

LLC	Lewis Lung Cancer
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MEK	MAP (mitogen-activated protein)/ERK kinase
mTOR	Mammalian target of rapamycin
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
N-cadherin	Nueral cadherin
NCoR	Nuclear receptor corepressors
NF-κB	Nuclear factor kappa B
NOS-2	Nitric oxide synthase
NR	Nuclear Receptor
OipA	Outer membrane protein
PARP	Poly ADP ribose polymerase
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PGC-1	PPAR coactivator
PI3K	Phosphoinositide 3-kinase
PMSF	Phenylmethylsulfonyl fluoride
PP	Peroxisome Proliferator
PPARγ	Peroxisome Proliferator-Activated Receptor gamma
PPRE	Peroxisome proliferator response element
PS	Phosphatidylserine
PTEN	Phosphatase and tensin homolog located on chromosome 10
RCC	Renal Cell Carcinoma
ROS	Reactive Oxygen Species

RPMI 1640	Roswell Park Memorial Institute 1640 Medium
RXR	Retinoid-X-Receptor
SBE	Smad-binding element
SD	Standard deviation
SDF-1α	Stromal cell-derived factor-1α
SDS	Sodium dodecyl sulfate
SDS PAGE	SDS-polyacrylamide gel electrophoresis
SPPARM	Selective modulators of PPARγ
SRC-1	Steroid receptor coactivator-1
TGF-β	Transforming Growth Factor-β
TIROX	S-1 plus irinotecan and oxaliplatin
TNF-α	Tumor necrosis factor-α
TRAIL	TNF-related apoptosis inducing factor
TR-FRET	Time-resolved Fluorescence Resonance Energy Transfer
VacA	Vacuolating cytotoxin
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
ZEB1/2	Zinc finger E-box-binding homeobox protei**n**

LIST OF PUBLICATIONS AND CONFERENCE ABSTRACTS

Publications in Peer Reviewed International Journals

1. **Ramachandran L**, Manu KA, Shanmugam MK, Li F, Siveen KS, Vali S, Kapoor S, Abbasi T, Surana R, Smoot DT, Ashktorab H, Tan P, Ahn KS, Yap CW, Kumar AP, Sethi G (2012). Isorhamnetin inhibits proliferation and invasion and induces apoptosis through the modulation of peroxisome proliferator-activated receptor γ activation pathway in gastric cancer. ***J. Biol. Chem***. 287(45), 38028-38040. doi: 10.1074/jbc.M112.388702 (**IF= 4.651).**

2. Prasannan R, Kalesh KA, Shanmugam MK, Nachiyappan A, **Ramachandran L**, Nguyen AH, Kumar AP, Lakshmanan M, Ahn KS, Sethi G (2012). Key cell signaling pathways modulated by zerumbone: role in the prevention and treatment of cancer. ***Biochem Pharmacol***, 84(10), 1268-1276 **(IF= 4.576).**

3. Manu KA, Shanmugam M, **Ramachandran L**, Li F, Fong CW, Kumar AP, Tan P, Sethi G (2012). First evidence that g-tocotrienol inhibits the growth of human gastric cancer and chemosensitizes it to capecitabine in a xenograft mouse model through the modulation of NF-κB pathway. ***Clinical Cancer Research*** 18(8), p. 2220-2229. doi:10.1158/1078-0432.CCR-11-2470 (**IF= 7.837).**

4. Sethi G, Shanmugam MK, **Ramachandran L**, Kumar AP, Tergaonkar V (2012). Multifaceted link between cancer and inflammation. ***Bioscience Reports*** 32(1), 1-15. doi: 10.1042/bsr20100136 (**IF= 1.876).**

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Book Chapter

1. R Kannaiyan, R Surana, EM Shin, **L Ramachandran**, G Sethi, AP Kumar (2012). Targeted Inhibition of Multiple Proinflammatory Signalling Pathways for the Prevention and Treatment of Multiple Myeloma, Multiple Myeloma – An Overview, Dr. Ajay Gupta (Ed.), ISBN: 978-953-307-768-0, InTech, DOI: 10.5772/30444.

Oral presentations in conferences

1. **L Ramachandran** and G Sethi. Isorhamnetin inhibits the growth of human gastric cancer and chemosensitizes it to capecitabine in a xenograft mouse model through the modulation of NF-κB pathway at the Molecular Medicine Conference, Thailand on 'Alternative strategies against cancer and inflammation', Dec 19-22, 2012

2. **L Ramachandran** and G Sethi. Novel Anti-Cancer Role Of Isorhamnetin In Gastric Cancer, Yong Loo Lin School of Medicine Second Graduate Scientific Congress, Feb 15, 2012

Poster presentations in international conferences

1. **L Ramachandran** and G Sethi. Isorhamnetin inhibits proliferation, invasion, and induces apoptosis through modulation of peroxisome proliferator-activated receptor-gamma activation pathway in gastric cancer. 103rd AACR Annual Meeting, Accelerating Science: Concept to Clinic, March 31- April 4, 2012, McCormick Place West, Chicago, Illinois, USA.

2. **L Ramachandran** and G Sethi, Isorhamnetin inhibits proliferation, invasion, and induces apoptosis through modulation of peroxisome proliferator-activated receptor-gamma activation pathway in gastric cancer. 2nd AACR International Conference on Frontiers in Basic Cancer Research, September 14-18, 2011, InterContinental Hotel, San Francisco, California, USA.

1. INTRODUCTION

1.1. Cancer: the silent killer

Cancers are caused by the abnormal growth of cells that arise from normal tissues in the human body, that have lost their capacity to assemble, and develop, into tissues of normal form and function [1]. In simple words, they can be viewed as a disease of malfunctioning cells. They evolve progressively from being a normal cell, to an invasive and aggressive cell type, that make them responsible for a huge number of deaths worldwide [2]. Research shows that one in three people in developed countries continue to be diagnosed with this disease before the age of 75 years. Estimates suggest that 12.7 million cases of cancer were discovered, and of these, 7.6 million cases led to death worldwide in 2008 [3]. According to the International Agency for Research on Cancer (IARC), the highest number of these deaths were due to cancers of the lung (1.6 million, 19.4% of the total), liver (0.8 million, 9.1%), and stomach (0.7 million, 8.8%). Though the number of cancer related deaths is alarming, survival rates have improved drastically. With researchers constantly striving to bridge the gap between science and medicine, there has been a tremendous increase in early detection methods and treatment regimens. Studies show that there is an improved survival rate over the past decades, with approximately 60% of patients living over 5 years after diagnosis [4]. However, the complexities of cancer environment often make it difficult to settle on an effective mode of treatment.

Even though cancers are strongly regarded as evasive and unpredictable, hallmark characteristics of cancers have been determined, thanks to the continuing research on this disease. These are excessive cell

growth, reprogramming of energy metabolism to support uncontrolled proliferation, immortality, resistance to cell death, induction of angiogenesis, ability to invade and metastasize to distant sites, reprogramming of energy metabolism and evading immune destruction [5, 6]. These six hallmarks have been extensively used by researchers as a foundation for the in-depth understanding of cancer behavior. Even though scientists debate that cancers are a plethora of several complex events, and cannot be regarded as resulting from a few isolated mutations, the identification of common characteristics of different cancers provides us with a unifying theme to ignite our search for the 'perfect target' or a 'magic bullet' [7].

Though researchers have tried to promote marginal benefits of a single agent, to a majority of patients with varied backgrounds, there exists extensive biological complexities within the historical classifications of various tumors, that make it practically impossible to expect similar efficacy of an agent across varied tumors [8]. Solid tumors, that constitute a major percentage of tumors, are especially difficult to treat due to their heterogeneity [9]. Hence, understanding the molecular basis of a cancer type is an important step in determining the perfect therapy.

1.2. Gastric cancer

Gastric carcinoma (GC) is one such type of solid tumor that arises from the inner linings of the stomach. The development of gastric cancer appears to be complex, with genetic predisposition, infection, and diet as important factors. Though other factors such as smoking and alcohol intake have been linked

towards its incidence, the evidence supporting their role is seemingly inconsistent [10].

Currently, five types of gastric cancer are described by the World Health Organization (WHO): papillary, tubular and mucinous adenocarcinoma, poorly cohesive carcinoma (with or without signet ring cells) and mixed carcinoma [11]. They have been classified into two major types (known as Lauren's classification), the diffuse and intestinal types, that stand for two different epidemiological and pathological entities [12]. Generally, intestinal-type appears to be well differentiated with cohesive tumor cells, while the diffuse-type is poorly differentiated with non-cohesive tumor cells [13].

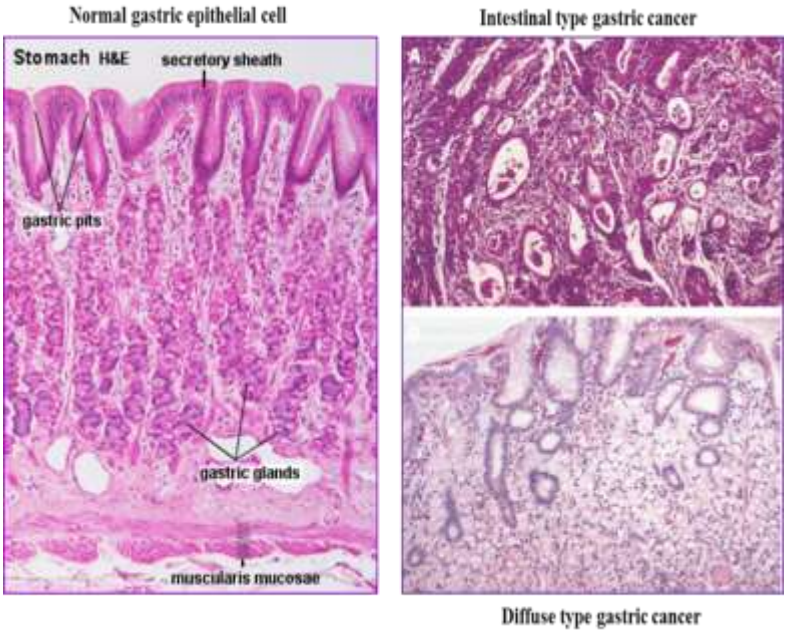


Fig 1. Histological subtypes of gastric cancer

(A) Intestinal type gastric cancer, characterized by infiltrating tubular profiles. (B) Diffuse type carcinoma in which there is diffuse infiltration of the mucosa. In this case, there is no significant sign of metaplasia. Both images were stained with haematoxylin and eosin and pictures obtained at magnification ×200. *Adapted from Gastic cancer. The Lancet, 2003.* ***362****(9380): p. 305-315.*

1.2.1. Risk factors and epidemiology of Gastric Cancer

Gastric cancer has the 2nd highest cancer-related mortality rate worldwide [14]. The risk of developing gastric cancer is said to be 1 in 115, possessing a survival rate of about 20-30% only [15]. The low survival rate has been often attributed to a delay in diagnosing the presence of cancer, usually only at a point when the tumor has already metastasized. However, the seemingly dark situation has begun to brighten, owing to the establishment of screening programmes for early diagnosis and careful surgical resection [10]. According to the International Agency for Research on Cancer, gastric cancer has the sixth highest incidence of cancer in Singapore with equally high mortality rates. The highest risk of developing gastric cancer is thought to occur through the infections with *Helicobacter pylori (H pylori)*, a gram bacillus, discovered by Marshall and Warren in 1983 [16]. A recent study has suggested that *H. pylori* can introduce host genome instabilities either directly, or indirectly through epigenetic modifications, and these seem to be reduced following its eradication [17]. Other risk factors for gastric cancer include diet and genetic abnormalities in the host environment. For example, it was observed in a study in Portugal, that patients with high salt intake were at higher risk of gastric cancer development as compared to those with lesser salt intake [18].

1.2.2. Diagnosis of gastric cancer

Diagnosis of gastric cancer continues to be a difficult feat for physicians. This is attributed to the fact that patients at an early stage of gastric cancer are invariably asymptomatic. However, the symptoms that are commonly observed at a later stage include weight loss, dysphasia, dyspepsia, vomiting,

early satiety, and/or anemia [19]. An experienced pathologist usually diagnoses the disease by a gastroscopic or surgical biopsy, and generates a histology report according to the World Health Organization criteria [IV, C].

More than 85% of gastric cancers are found to be adenocarcinomas, and as mentioned earlier, these are classified according to histology into diffuse and intestinal types. The Clinical Practice Guidelines are not applicable to less commonly observed gastric malignancies such as gastrointestinal stromal tumors (GIST), lymphomas and neuro-endocrine tumors. Although less useful in antral tumors, endoscopy (EUS) has been widely used to find the proximal and distal extent of the tumor, while laparoscopy is utilized in all stages of stomach cancers [20].

1.2.3. Treatment modalities for gastric cancer

1.2.3.1. Surgery

The primary method of treatment of early stage patients is through surgery. Endoscopic resection is also used as an alternative for treatment in well-differentiated early stage tumors [21]. Though there is an ever-growing interest in the use of minimally-invasive techniques for gastric resection, clinicians have to ensure that the outcome is similar to the highly positive results usually achieved through an open surgery [22]. To decide if patients require a combination of surgery and multi-modal therapies, surgeons require improved predictors of nodal disease and prognosis. Studies suggest that majority of patients benefit through use of surgery followed by neo-adjuvant chemotherapy [23] which include drugs such as epirubicin, cisplatin and 5-fluorouracil.

1.2.3.2. Chemotherapy

Chemotherapy has been accepted as the standard mode of treatment in advanced gastric cancer patients and post-operative chemotherapy has especially become a standard option in patients treated for gastric cancer in Asia [24]. Five classes of cytotoxic agents are currently used in GC treatment. They are fluoropyrimidines, platinum compounds, taxanes, topoisomerase inhibitors and anthracyclines. Their mechanism(s) of actions are described below in brief:

Cisplatin: Cisplatin is a metallic (platinum) coordination compound that has been used for treatment of a variety of cancers. It was the first FDA-approved platinum compound for cancer treatment in 1978 [25]. It consists of a doubly charged platinum ion surrounded by four ligands; on the left are the amine ligands that form strong interactions with the platinum ion, and on the right, the chloride ligands or carboxylate compounds that allow the platinum ion to form bonds with DNA bases [26]. Cisplatin is significantly toxic, which often results in nausea, vomiting, nephrotoxicity, and neurotoxicity [27].

Capecitabine and 5-fluorouracil (5-FU): Capecitabine is an oral fluoropyrimidine and prodrug which was initially designed to maintain a continuous supply of 5-FU, the sole aim being to provide prolonged drug exposure and tissue selectivity. It is metabolized in the liver by hepatocyte carboxylesterase (CE) to 5′-deoxy-5-fluorocytidine [28]. The active metabolite competes with deoxyuridine monophosphate and binds to thymidylate synthase thereby inhibiting *de novo* thymidine synthesis, a phenomenon important for DNA replication and cell survival [29]. However, it has side-effects that include the appearance of hand-foot syndrome, leukopenia,

elevated bilirubin and diarrhea [30]. A commonly used combination regimen for advanced gastric cancer is 5-fluorouracil (5-FU) in conjunction with cisplatin, on a bi-weekly or a tri-weekly schedule [31].

Taxanes: Taxanes, which include paclitaxel or docetaxel, act by disrupting the microtubule function and inhibiting the process of cell division. With the potential application of taxanes came the use of novel combination regimens, such as docetaxel/cisplatin/5-fluorouracil (5-FU) (DCF), which is now being utilized as a standard therapy in advanced gastric cancer in a selected cohort of patients [32]. Similarly, a combination of paclitaxel with fluoropyrimidines and/or platinum compounds show considerable improvement in results as compared to mono-therapy [33]. The most common adverse effects observed on administration of taxanes are neutropenia accompanied by sustained fever.

Irinotecan: Irinotecan acts by inhibiting topoisomerase I and thus prevents DNA from unwinding. Trials have shown good tolerance and promising results although they have been closely associated with toxic effects such as diarrhea and neutropenia [34]. A new triplet combination—TIROX, consisting of S-1 (oral dihydropyrimidine dehydrogenase, based on a biochemical modulation of 5-FU) in conjunction with irinotecan and oxaliplatin has shown a marked reduction of tumor burden from baseline in patients [35].

1.2.3.3. Molecular targeted therapies

Gastric cancer is a heterogeneous disease consisting of numerous chromosomal aberrations, genetic and epigenetic changes and alterations in oncogenic pathways. These have led to the discovery of various targeted anticancer agents that are described in brief below.

Cell surface inhibitors: Angiogenesis is an important part of tumor progression and Vascular Endothelial Growth Factor (VEGF) is a critical factor in this phenomenon. It has an essential role in physiologic and pathologic angiogenesis, making it an attractive target for growth inhibition [36]. Bevacizumab is a recombinant humanized IgG1 monoclonal antibody against VEGF which is extensively used in combination with chemotherapy [37]. Sunitinib is an oral, multitargeted tyrosine kinase inhibitor of VEGF receptor, approved for the treatment of advanced renal cell carcinoma (RCC) and imatinib-resistant gastrointestinal stromal tumors (GIST). Even though these drugs are widely used in practice today, there are major drawbacks associated with them. VEGF pathway is critical for the physiological functions and homeostasis of the cell, which are disturbed with the use of its pharmacological inhibitors. The adverse effects associated with the VEGF inhibitors include hypertension, arterial thrombosis, cardiomyopathy and impaired wound healing [38]. Sorafenib is yet another inhibitor of Raf tyrosine kinase that is used in the treatment of RCC and hepatocellular carcinoma [39]. However, patients typically experience hypertension and cardiac ischemia as major side-effects of sorafenib treatment [40].

Epidermal Growth Factor Receptor blockers: EGFR is a transmembrane glycoprotein receptor (EGFR) family of extracellular protein ligands and is overexpressed in several GI malignancies. It is activated and phosphorylated as a result of ligand binding, that results in the activation of Ras/Raf/mitogen activated protein kinase (MAPK) pathway or the Akt/mTORpathway. Cetuximab is an IgG1 type chimeric monoclonal antibody that competitively inhibits the binding of EGF to its receptor [41]. Even though proven to be effective, it has been associated with skin toxicity, including skin rash, dry skin, hair growth disorders, pruritus, and nail changes that can severely affect the well-being of patients [42].

Gefitinib is an orally active EGFR tyrosine kinase inhibitor with promising activity against a range of malignancies in early phase trials [43].The common adverse drug reactions of gefitinib include diarrhea and skin rash, which are generally mild and reversible. A small percentage of people have also reported to experience fatal interstitial pneumonia [44]

Human Epidermal Growth Factor Type 2 Inhibitors: HER-2 is a member of the EGFR family and is active in promoting tumor progression [45]. Trastuzumab, a monoclonal antibody against HER-2, is the only validated receptor tyrosine kinase inhibitor in gastric cancer, while lapatinib is an oral, small molecule, dual tyrosine kinase inhibitor of EGFR and HER-2, found to be effective in trastuzumab-resistant advanced breast cancer [46].

Other molecular targeted agents include the inhibitors of insulin-like growth factor, c-Met tyrosine kinases and fibroblast growth factor tyrosine kinases. Several other drugs have also been developed that target cell-cycle,

Ubiquitin–proteasome pathway, heat shock protein 90 as well as matrix metalloproteinases [11].

Even though several pharmacological inhibitors have been developed to target various oncogenic molecules and their downstream signaling cascades, given the highly complex nature of cancers, targeted therapy has not been as successful in reality as it was expected to be. Hence, it is imperative to develop a multi-targeted approach, possibly by employing a combination of agents with non-overlapping mechanisms(s) of action, but the chances of it becoming a reality is hampered by the limited knowledge on the kind of efficacious agents that can be used in combination, the inability to design or test on multi-sponsor clinical trials, as well as the added toxicities associated with diverse agents [47].

1.2.4. Gastric cancer pathogenesis

Gastric carcinoma is caused by a complicated interaction of the host, environment and bacterial factors. *Helicobacter pylori* is a human gastric pathogen that causes gastric inflammation and is etiologically related to gastric adenocarcinoma [48]. *H. pylori* gastritis possesses various inflammatory infiltrates including neutrophils, lymphocytes, plasma cells, eosinophils, macrophages, and mast cells. The infection typically results in a life-long acute and chronic inflammatory response that leads to progressive mucosal damage. This causes the development of different types of metaplastic and dysplastic epithelia that eventually result in gastric adenocarcinoma [49].

Studies have suggested that *H. pylori*-infected individuals whose diet consist of minimal fresh fruits and vegetables, combined with excessive use of food preservatives and salt have a greater tendency to develop progressive atrophy, which is linked to gastric ulcers and ultimately to gastric cancer. On the contrary, individuals with continuous access to fresh fruits and vegetables, with a healthy diet, have comparatively less mucosal damage and lower incidence of gastric cancer [50]. However, it is interesting to note that even in places of low incidence, gastric cancer has an ability to develop in the presence of polymorphisms in the host coupled with a virulent strain of *H. pylori*. In conclusion, even though *H. pylori* host interactions play an important role in gastric cancer pathogenesis, bacterial virulence seems to be the key factor in predicting the disease outcome.

1.2.4.1. *Helicobacter pylori* virulence factors and their role in gastric cancer pathogenesis

There exist several putative virulence factors such as CagA, vacuolating cytotoxin (VacA), OipA and DupA in *H. pylori* that play a pivotal role in gastric cancer pathogenesis. Out of these factors, CagA, is a highly immunogenic protein that has been incorporated into *H. pylori* by horizontal transfer. The expression of CagA in *H. pylori* varies greatly, from 100% in East Asia to less than 50% in some western countries. Research has shown it can function as an oncogene; transgenic mice containing CagA has been shown to spontaneously develop gastric cancer [51].

VacA is a factor that is present in almost all strains of *H. pylori.* It possesses diverse biological functions, such as membrane channel formation, release of cytochrome c from mitochondria, initiation of a pro-inflammatory response and specific inhibition of T-cell activation and proliferation [52]. Expression of VacA has been consistently associated with the extent and severity of inflammation. OipA is an adhesion, pro-inflammatory response-inducing protein that can induce IL-8 from gastric epithelial cells. It is shown to be involved in the phosphorylation of three different families of mitogen activated protein kinases, namely p38, JNK and ERK [53, 54]. It is also thought to be involved in β-catenin signaling that modulates cell–cell junctions and cell proliferation [55]. *H. pylori* either produces all of these proteins or none of them, and clinical outcome depends on the strain and the virulence factors associated with it. However, the presence of the above indicated factors has been found to directly increase the aggressiveness of the clinical outcome.

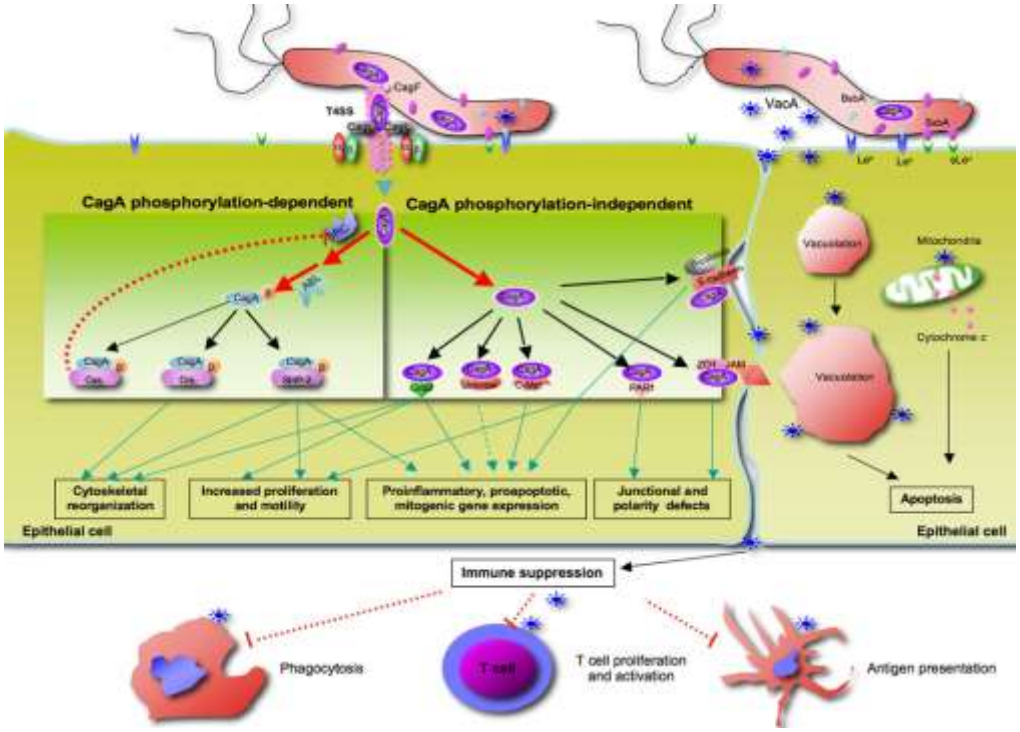


Fig 2. Role of *Helicobacter pylori* in gastric cancer pathogenesis

H. pylori attaches to the gastric epithelial cells, and through its type 4 secretion systems (T4SS), CagA is delivered to them. CagA is in turn tyrosine phosphorylated at EPIYA sites initially by SRC and later by ABL kinases. A combination of the effects of the two proteins, CagA and VacA can contribute to gastric carcinoma development. *Adapted from Cancer Lett. Sep 8, 2009; 282(1): 1–8.*

A number of publications have analyzed the role of diverse pro-inflammatory pathways that are enhanced after bacterial infection, whereas only few studies have focused on characterizing the counter-balancing, anti-inflammatory response generated inside the body. An important study deciphered the role of a transcription factor, peroxisome proliferator-activated receptor-γ (PPARγ) as one of the various anti-inflammatory mediators in gastric cancer [56]. The relationship between PPARγ, its regulated genes and their non-inflammatory actions in gastric cancer will be discussed in the forthcoming chapter.

1.2.4.2. Genetic mutations in gastric cancer

Similar to the profiles of other cancers, gastric cancer is found to be heterogenic, with each patient exhibiting distinct genetic and molecular characteristics [57]. It is now necessary to use advanced molecular methods to identify the optimal method of treatment of gastric cancer, with its complex histological and molecular aberrations [58].

For example, next generation sequencing is a powerful tool for identifying potential therapeutic targets and improving personalized treatment options [59]. Wang and colleagues were the first to publish an exome-sequencing study in gastric cancer that identified 20 genes as top candidate drivers, of which the major finding was the identification of high mutation frequency of ARID1A [60]. Later, Zang *et al*. confirmed the same, and also identified FAT4, a member of the E-cadherin family as a strong candidate driver gene [61]. In 2012, Kim *et al*. showed using RNA-seq study that the central metabolic regulator AMPKα2 (PRKAA2) is a potential functional target in Asian gastric cancer [62]. Only a handful of publications are present that currently describe the potential molecular basis of gastric cancer through NGS. Nevertheless, they provide tremendous insight into the understanding of the genetic alterations in gastric cancer. Further, The International Cancer Genome Consortium (ICGC) has aimed to systematically study 25,000 cancer genomes at the genomic, epigenomic, and transcriptomic levels for at least 50 cancer types, which will serve as valuable resources for identifying novel targets in gastric cancer.

1.3. Peroxisome proliferator-activated receptors

1.3.1. Peroxisomes proliferators and the discovery of PPARs

Peroxisomes, referred to as glyoxysomes or microbodies in plants, are versatile organelles present in eukaryotes that play a critical role in many metabolic pathways [63]. These are related to lipid metabolism, involving β-oxidative degradation of fatty acid, synthesis of cholesterol and other isoprenoids, and synthesis of glycerolipid (plasmalogen). They are dynamic, with the capacity to change their appearance, their association with other organelles, and their enzyme composition [64].

Later studies discovered that peroxisomes respond to a variety of chemical agents, known as peroxisome proliferators (PP), which are a diverse group of chemical entities with various applications. As the name suggests, an increase in rate of proliferation and the number of S-phase cells have frequently been observed when treated with these agents. These compounds include clofibric acid and methylclofenapate [65], and nafenopin [66], some of the first identified peroxisome proliferators. Though these chemicals have been thought to act as hepatocarcinogens in rats, they do not elicit a similar response nor have carcinogenic risk in humans [66].

The peroxisome proliferators were first discovered to activate a form of peroxisome-proliferator activated receptor (PPAR) called PPARα, which is found to be abundant in the liver. PPARs belong to a family of nuclear hormone receptors and the activation of PPARα was found to be responsible for the pleiotropic effects of PPs seen in rodents, such as enzyme induction, hepatocyte DNA synthesis and suppression of hepatocyte apoptosis [67].

1.3.2. Structure of PPARs

Nuclear hormone receptors consist of a large family of ligand-modulated transcription factors including the steroid and thyroid hormone receptors (TRs), the retinoid hormone receptors, and 'orphan' receptors (receptors whose ligands have not been identified yet) [68]. They modulate the expression of various target genes by binding to specific DNA sequences, (called response elements) present in the promoter of these genes [69].

Extensive study of amino acid sequences of various steroid hormone receptors led to the identification of four characteristic domains: the N-terminal A/B domain that comprises of a ligand-independent transactivation function, called activation function 1 (AF-1); the highly conserved C domain containing the DNA-binding domain (DBD); the D domain or so-called "hinge domain", linking the DBD to the ligand-binding domain (LBD); and, the C-terminal E/F domain or LBD, containing a ligand-dependent transactivation function, termed AF-2 [68].

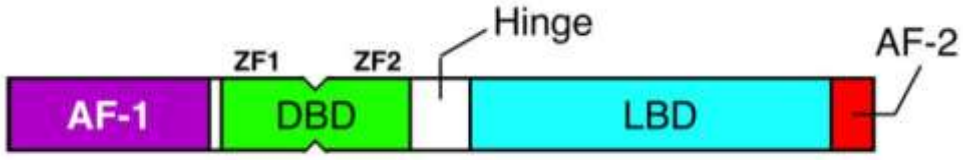


Fig 3. Schematic diagram of the common domain structure of nuclear receptors

Similar to other nuclear receptors, PPAR gamma contains an N-terminal activation function 1 (AF-1), DNA binding domain (DBD) consisting of two zinc fingers (ZF), non-conserved hinge-region (Hinge), ligand binding domain (LBD), and C-terminal AF-2 helix. *Adapted from FEBS Lett. Jan 9, 2008; 582(1): 2–9.*

1.3.3. Types of PPARs

Peroxisome proliferator-activated receptors (PPARs) include 3 members: α, β/δ, and γ, and each of them act as a heterodimer with retinoid-X-receptor (RXR). As described earlier, PPARα, was the first PPAR to be identified, and is the target of the fibrate-class of anti-hyperlipidemic drug or peroxisome proliferators [70]. It is abundantly found in the liver, heart, muscle and kidney where it regulates fatty acid oxidation and apolipoprotein synthesis. PPARα is also thought to play an important anti-inflammatory role, being present in the vascular wall and human macrophage foam cells [71].

Of the three sub-types, PPARβ/δ seems to be the least studied/explored. It is ubiquitously expressed and responds to polyunsaturated fatty acids. PPARδ activation has been shown to induce mitochondrial fatty acid oxidation, energy expenditure and thermogenesis [72]. Deficiency of this receptor promotes obesity and insulin resistance, whereas overexpression protects from diet-induced obesity.

The last subtype, PPARγ has been reported to be the 'master regulator' of adipogenesis and is most abundantly expressed in the adipose tissue. PPARγ is also abundantly expressed in foam cell macrophages in human aortic atherosclerotic lesions, where they decrease atherosclerosis in mice [73]. Overall, it is interesting to observe that all types of PPARs are found to be actively involved in suppressing the inflammatory gene expression in macrophages. This activity of PPARs has also been linked positively to the inhibition of inflammation caused by *H. pylori infection*.

1.3.4. Mechanism (s) of action of PPARs

In the classical model of PPAR activation, PPAR forms a heterodimer with RXR nuclear receptor that further binds to PPRE (PPAR regulatory element) termed DR-1, which consists of direct repeats of AGGTCA separated by a single intervening nucleotide [74]. This activation could be inhibited by the presence of co-repressor proteins [75], such as nuclear receptor co-repressors (NCoR), histone deacetylases (HDAC), and G-protein pathway suppressor 2 (GPS2). Ligand binding causes the dissociation of the co-repressor proteins followed by the recruitment of co-activators such as PPAR co-activator (PGC-1), the histone acetyltransferase p300, CREB binding protein (CBP), and steroid receptor coactivator (SRC)-1 [76]. The kind of heterodimer formed influences promoter recognition on the target gene sequences and determines the effect on different metabolic processes [77].

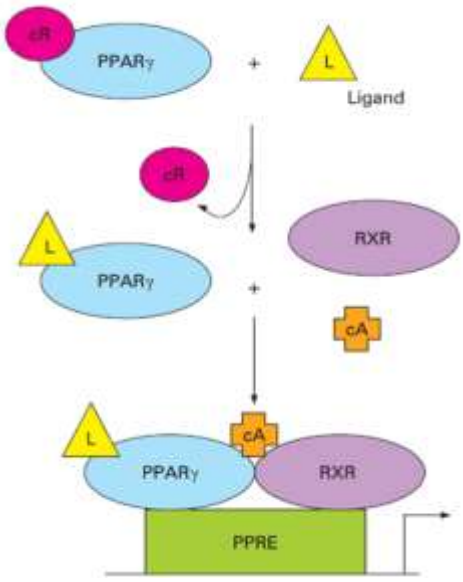


Fig 4. PPARγ binds to RXR receptor to initiate gene transcription

Ligand binding to PPARγ results in the formation of a heterodimer with RXR. This heterodimer in the presence of co-activators or co-repressors binds to the PPRE region to initiate transcription of relevant genes.

1.4. The role of PPARγ activation in *H. pylori* infection

As discussed in the previous section, several studies have demonstrated that PPARγ has an anti-inflammatory role under physiological conditions. The logical mechanism of its anti-inflammatory role is possibly via inhibition of diverse pro-inflammatory pathways thereby modulating the expression of pro-inflammatory genes. An important study reported that activation of PPARγ suppresses *H. pylori*-induced apoptosis in gastric epithelial cells and attributed this effect to the direct inhibition of *H. pylori*-induced NF-κB activation [78]. Yet another group reported that use of a potent PPARγ agonist, ciglitazone can inhibit gastric mucosal inflammation, as evidenced by reduced apoptosis, reduced expression of COX-2, and a drop in the levels of inducible nitric oxide synthase (NOS-2) [79].

A potent virulence factor which is necessary for inflammatory changes in the host is *H. pylori* lipopolysaccharide (LPS). It functions by inducing gastric epithelial cell apoptosis and increasing pro inflammatory cytokine production [80]. This induction of proliferation is strongly linked to transactivation of epidermal growth factor (EGFR). Interestingly, ciglitazone has been shown to suppress this aberrant EGFR activation caused by infection of *H. pylori* [81].

Various research groups have also reported that PPARγ is expressed and functionally active in gastric epithelial cell lines sensitive to *H.pylori*-induced apoptosis, and ligand activation of the receptor could effectively attenuate the apoptotic action of *H.pylori* on gastric epithelial cells [82]. Taken together, these studies emphasize the positive role of PPARγ activation in the inhibition of *H.pylori* induced inflammation.

1.4.1. Role of PPARγ in gastric cancer

The scientific evidence related to the involvement of PPARγ in cancer remains controversial. It is interesting to note that several groups have reported on its pro-tumorigenic role, as opposed to many others who stand by its role as a tumor-suppressor. Several *in-vitro* studies show that PPARγ activation might effectively inhibit the proliferation, prevent metastasis and induce apoptosis in gastric cancer cells [83, 84]. Takahashi *et al.* first demonstrated that activation of PPARγ in a human gastric cancer cell line, MKN45, that has high expression of PPARγ mRNA and protein, inhibited cell growth and induced apoptosis in gastric cancer cells [85]. Yet another research group reported that PPARγ is also expressed in surgically resected specimens, obtained from well, moderately, and poorly differentiated gastric adenocarcinomas, as well as in non-cancerous gastric mucosa with intestinal metaplasia [86]. This inhibitory effect of PPARγ on gastric cancer may be due to diverse molecular mechanisms.

Ligand-induced activation of PPARγ was found to inhibit c-MET [87] and the expression of cyclin D1 and COX-2 [88]. It could also up-regulate the expression of various proteins such as p27 [89], p21, and p53 [90] and could suppress the expression of gastrin. Thus, the pleiotropic ability of PPARγ to inhibit proliferation and metastasis, and induce apoptosis, renders it an attractive target for cancer therapy.

1.4.2. PPARγ ligands

Ligands for PPARγ can be either natural [91] or synthetic [92] in nature. Synthetic ligands have been developed by researchers through a combination of screening, molecular docking with the protein, *in-vitro* and *in-vivo* validation [93, 94]. Though several synthetic ligands have been routinely screened for PPARγ, the only approved use for PPARγ ligands so far is the application of thiazolidinediones (full PPARγ agonists) in type 2 diabetes. These drugs were first used to treat type 2 diabetes in 1990 by decreasing insulin resistance [95].

Several drugs fall into the category of thiazolidinediones, of which troglitazone, was the first approved drug which became available in 1997 and was later withdrawn in 2000 because it induced severe to fatal hepatotoxicity that outweighed its so-called 'benefits' to diabetic patients [96, 97]. Other drugs in the family, rosiglitazone and pioglitazone, are still in clinical use in many countries for glycemic control in the treatment of type 2 diabetes. However, there still exists an uncertainty about the cardiovascular side-effects of rosiglitazone [98], since its treatment has shown an increase in the myocardial infarction and heart failure [99]. Compared to rosiglitazone, pioglitazone has beneficial effects [100], with lower risk of heart failure, but is still limited by occurrence of several adverse effects such as body-weight gain and fluid retention [101]. 15-Deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) is an endogenous ligand for PPARγ produced from a sole precursor, PGD2, and is known to affect the expression of various PPARγ regulated genes regulating relevant transcription factors [102].

1.4.3. Natural sources of PPARγ ligands

The drastic failure of thiazolidinediones in the market was unexpected by researchers, and henceforth, various studies have tried to elucidate the reason for such severe adverse effects. It has been suggested that this could be due to a result of full PPARγ activation in contrast to the weak agonistic effect of natural ligands [103]. Thus, there began a search for the perfect PPARγ agonist with enhanced activation but reduced side-effects, commonly referred to as selective PPARγ modulators or SPPARMs [104]. Weak activators of PPARγ, or partial agonists, elicit the same activation pattern as full agonists but with lower maximal activity [93].

Natural compounds from plants have been used for the treatment of various diseases since ancient times. Till today, they are an important source for discovery and development of new drugs owing to their rich structural diversity [105]. Therefore, there has been a significant interest in assessing natural compounds as alternatives to full agonists that seem to elicit severe side effects. Several active compounds identified in diet, such as amorfrutins [106] and norbixin [107] are weak agonists *per se*, but it is suggested that their metabolites may have a higher efficiency in inducing PPARγ activation. An example is the red clove extract, whose metabolites have been shown to have up to 100-fold higher PPARγ binding affinity than their precursors [108].

Quercetin is one such flavonol compound that has been studied to partially activate PPARγ and exert beneficial effects on hyperglycemia of diabetic animals [109]. Isorhamnetin is a 3'-O-methylated metabolite of quercetin, and we aimed to investigate its effect on the PPARγ signaling cascade in our study.

1.4.4. Flavonoids as PPARγ agonists

Flavonoids are naturally occurring plant polyphenols that can perform a wide variety of biochemical and pharmacological functions [110]. Several agents have been identified as PPARγ agonists, of which a few are discussed below. Luteolin was found to display weak PPARγ agonist behaviour when compared to rosiglitazone, and strong anti-inflammatory activity in the corneal epithelial and endothelial layers [111]. Another flavonoid known as baicalin, isolated from *Scutellaria baicalensis*, induced PPARγ activity and suppressed NF-κB-induced inflammatory response in aging rats [112]. Another study described the isolation of 12 compounds and one PPARγ agonist from *Chromolaena odorata*, an invasive weed used in traditional Vietnamese medicine [113].

1.4.5. Application of flavonoids in gastric cancer therapy

There are numerous studies on the effect of flavonols in cancer and the interest in them continues to expand with every passing decade. Flavonoids, and natural products in general, have been gaining attention in the treatment of cancer not only because they serve as a source of validated structures for further discoveries but also due to their relatively low cost and fewer adverse effects [114]. Flavonoids are divided into seven different groups; flavones, flavonols, flavanones, isoflavones, catechins, anthocyanins, and chalcones. In the following section, we will briefly discuss the reported anticancer effects of a few selected flavonoids against gastric cancer.

Apigenin, one of the most common flavonoids found in abundance in celery, passion flower, and other vegetables and fruits has been shown to inhibit *H. pylori*-induced atrophic gastritis and gastric cancer progression as

well as possessing potent anti-gastric cancer activity [115]. Soybeans contain a major isoflavone, genistein that has been found to exhibit anti-carcinogenic properties; it can induce G2/M cell cycle arrest in gastric cancer cells through the decreased Ser473 and Thr308 phosphorylation of Akt and upregulation of PTEN [116]. Anthocyanins, a group of polyphenols present in many fruit and flowers, are proven antioxidants and chemopreventive agents that were shown to have protective effects against *H. pylori*-induced inflammation [117].

Casticin, a polymethoxyflavone derived from *Fructus viticis* has been shown to induce DR5 expression in gastric cancer cells, and thus potentiate TRAIL-induced apoptotic cell death in gastric cancer cells [118]. Nobiletin is yet another typical polymethoxyl flavone from citrus fruits, which can enhance the action of 5-fluorouracil in p53 mutant tumors [119]. Eupatelin, derived from *Artemisia asiatica* has been found to reduce pro-inflammatory cytokine mediated MMP expression in gastric cancer [120]. Alpinetin is a natural flavonoid widely distributed in *Zingiberaceae* that can alter mitochondrial membrane potential leading to release of cytochrome c from mitochondria, activation of caspase family members and ultimately leading to the apoptosis of human gastric cancer cells [121]. Taken together, these studies emphasize the potential of flavonoids in inhibiting proliferation and inducing apoptosis in gastric cancer cells.

1.5. Isorhamnetin: A novel natural agent

Isorhamnetin (IH), an immediate metabolite of quercetin, also called 3′-O-methylquercetin, has gained significant attention of late, for its anti-inflammatory and anti-proliferative properties in a wide variety of cancers, including colorectal, skin and lung cancers [122-124]. Prior studies so far have focused on quercetin as an anti-inflammatory agent, but recent research has shown that isorhamnetin can induce greater cytotoxicity in tumor cells as compared to quercetin [122]. For example, it was found that aflatoxin B1 (AFB1)-mediated reactive oxygen species (ROS) generation was abrogated significantly by isorhamnetin when compared to quercetin in hepatocellular carcinoma cells [125].

A

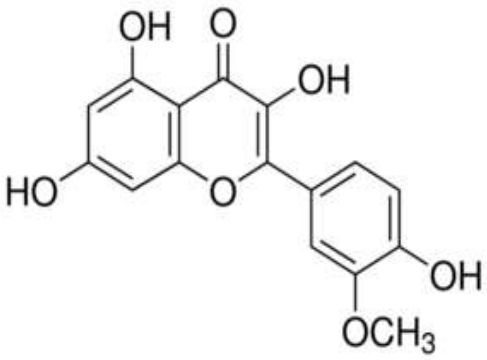


Fig 5. (A) The chemical structure of isorhamnetin (MW: 316.26 g/mol)

B

Fig 5. (B) *Hippophae rhamnoides*: Plant source of isorhamnetin

1.5.1. Role of isorhamnetin in inflammation

Of the various beneficial roles of isorhamnetin, its anti-inflammatory role has been studied in detail by researchers across the globe. A study reported that isorhamnetin could induce heme oxygenase-1 by that lead to reduction in ROS production which in turn resulted in the inhibition of COX-2 expression in response to inflammatory stimuli [126]. Another study also described that isorhamnetin could reduce inducible nitric-oxide synthase (iNOS) expression, and suggested that this might be mediated by inhibition of NF-κB activation [127]. The results were supported by another research group, which reported that this 3′-O-methylated flavonoid could inhibit JNK and AKT/IKKα/β phosphorylation and in turn inhibit the effect of NF-κB regulated genes such as TNF-α, IL-1β and IL-6 [128].

Similar to our hypothesis of involvement of isorhamnetin in inducing PPARγ activation, a group reported that the treatment with *Eruca sativa* extract, which contained isorhamnetin, could significantly increase the transactivation activity of PPARα and thereby suppress the expression of inflammatory cytokines and antimicrobial peptides [129]. A recent review suggested that isorhamnetin might play a fundamental role in inhibiting inflammation, possibly through its targeted effects on a combination of pro-inflammatory pathways [130]. Based on existing evidence, it is suggested that isorhamnetin reduces inflammation primarily by inhibiting COX-2 expression, an effect that is quite common among flavonoids [131]. It has been reported that the O-methylated group in isorhamnetin, could effectively increase the plasma bioavailability of this flavonoid, by preventing the rapid metabolism as it undergoes glucuronidation and sulfation [132]. In fact, the chemopreventive

role of methoxy-flavones have been discussed in detail previously by Dr. Thomas Walle who concluded that the oral bioavailability would be much greater for methoxyflavones as compared to the non-methylated flavones [133]. These desirable characteristics of isorhamnetin make it an attractive agent to be explored further for its potential anticancer effects.

1.5.2. Role of isorhamnetin in cancer

Prior reports on isorhamnetin clearly indicate that it can exhibit significant anticancer effects through the modulation of various oncogenic molecular targets in different cancers [134, 135]. For example, a study showed that isorhamnetin can suppress skin cancer by binding to and inhibiting MAP (mitogen-activated protein)/ERK kinase (MEK) 1 and PI3K. In the same study, researchers found that among the four flavonols commonly consumed by humans, namely, isorhamnetin, quercetin, rutin, and myricetin, isorhamnetin could reduce inflammation, cell proliferation, tumor burden and mortality in a mouse model for colorectal cancer [134]. This observation in colorectal cancer cells is supported by yet another study by Jaramillo *et al*. that also showed that isorhamnetin could induce significant cytotoxic effects in colorectal cancer cells [136].

In an identical study, it was reported that this flavonol compound could effectively inhibit proliferation and induce apoptosis in human esophageal squamous carcinoma cells, though the exact mechanism was not discussed [137]. Yet another study reported that this flavonol could induce substantial apoptosis in Lewis lung cancer cells through mitochondria-dependent caspase activation [138]. An animal model used by the group showed a significant

decrease in tumor size and weight upon treatment with isorhamnetin in tumors excised from LLC bearing C57BL/6 mice.

This plant flavonol can act by modulating multiple molecular targets, as demonstrated by another study which reported that isorhamnetin exhibited its anticancer effects primarily acting via MEK and PI3-K in non-melanoma skin cancer [139]. In the same study, isorhamnetin was found to suppress MEK1 kinase activity through direct binding, and because ERKs are substrates of MEK1, the inhibition of MEK1 by isorhamnetin led to the inhibition of EGF-induced phosphorylation of ERKs. Moreover, in human hepatocellular carcinoma cells, this metabolite of quercetin was reported to exhibit significant cytotoxic effects [140] and was found to permeate the cell membrane into the cell. An important observation of the study was that isorhamnetin was not converted to any active metabolites inside the cell.

Overall, isorhamnetin has been found to have pleiotropic anti-cancer effects in various cancers, but its mechanism(s) of action has not been clearly elucidated till date. Hence, the major objective of our study was to examine the potential anti-cancer effects of isorhamnetin, and to investigate in detail its underlying molecular mechanism(s) of action using diverse gastric cancer cell lines and a xenograft mouse model.

1.6. EMT in cancer

1.6.1. Importance of targeting EMT in cancer

Benign tumors can usually be removed via surgery and treated with the use of chemo-therapeutic agents. On the other hand, metastases in tumors are largely incurable and therefore account for the majority of deaths due to cancer. Various processes are involved in metastasis, of which epithelial–mesenchymal transition is an important reversible process by which polarized epithelial cells convert into motile mesenchymal ones. This process depends on microenvironment signals that interact with various regulators to control the expression of proteins that are involved in cell polarity, cell-cell contact and suppression of epithelial characteristics [141].

It is well established that there are three important stages of tumor progression; invasion, dissemination and metastasis [142]. The entire program is orchestrated by a set of important transcription factors, including Slug, Snail, Twist, ZEB1, and ZEB2, which aid in conversion to a mesenchymal state by repressing the expression of epithelial markers and inducing expression of other markers associated with the mesenchymal state [143]. The signaling events that lead to EMT are not clear even today, but it is suggested that genetic and epigenetic alterations acquired by a cancer cell during its lifetime transform it responsive to EMT-inducing signals [144]. It has largely been regarded that role of EMT in tumor progression is mainly through its effect on the processes of cancer invasion and metastasis [145]. However, studies are now deciphering the active role of EMT in initiation of primary tumors as well.

Apart from simply initiating metastasis, acquisition of mesenchymal characteristics is associated with resistance to drug therapy in certain cancers [146]. Considering the clinically important role of EMT in cancer progression, it is imperative to identify novel pharmacological agents that can modulate this process.

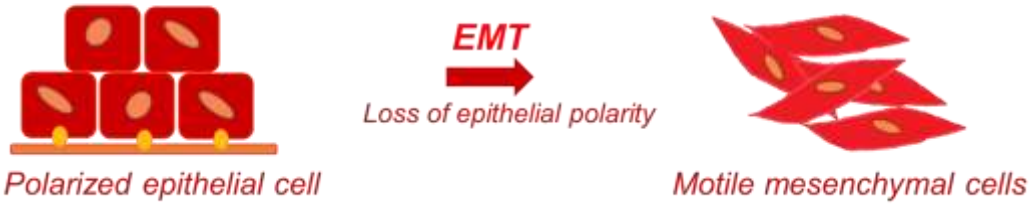


Fig 6. Functional role of EMT in cancer

EMT is a process of transition of polarized epithelial cells into mobile mesenchymal cells. On the molecular level, EMT is defined by the loss of cell–cell adhesion molecules (e.g., E-cadherin), downregulation of epithelial differentiation markers, and induction of mesenchymal markers such as vimentin and N-cadherin. During EMT, cells acquire an invasive capacity to breach basement membrane, initiate the multistep process of metastasis, and spread throughout the host.

1.6.2. Role of Transforming Growth Factor-β (TGFβ) in cancer

Transforming Growth Factor-β (TGFβ) is a multi-functional cytokine that has been extensively studied to understand the diverse effects exerted by it on both epithelial cells and carcinoma cell populations *in-vitro* and *in-vivo* [147]. TGF-β is overexpressed in various human cancers and is extensively linked to their poor prognosis [148]. It seems to perform a dual role in EMT, adapting a tumor-suppressor role in early tumor stage and converting to a tumor promoter in advanced stages [149]. It appears that signaling of TGFβ is at a contextual level, depending on its environment.

The superfamily of TGF-β cytokines comprises of over 40 proteins, including: TGF-beta (β), activins, inhibins, bone morphogenetic proteins (BMPs), and growth/differentiation factors (GDFs) [150]. Specific ligands activate the receptors via different molecular mechanism(s). They transmit their signals through hetero-tetrameric complexes comprising two types of serine-threonine kinase receptor, the type I and type II [151]. After activation of TGFβ Receptor I, the signal activates Smad2 and Smad3 proteins (R-Smad subclass; receptor regulated Smad) bound to the receptors, by phosphorylation of their C-terminal (SXS motif) residues, this complex now targets various genes involved in transcription and regulation of EMT [152]. They can also function in a Smad-independent manner (non-canonical pathways), by activating other pathways, such as the extracellular signal-regulated kinase 1/2 (ERK1/2) and the p38 MAP kinase (p38 MAPK) [153].

1.6.3. Role of BMPR2 in EMT

Bone morphogenetic proteins are members of the TGFβ family that have been well studied in bone formation and embryogenesis [154]. However, recent research has suggested they might possess a pro-tumorigenic role [155]. They transmit their signals by binding to the BMP type II receptors in combination with distinct type I receptors such as Alks 2, 3 and 6 which in turn phosphorylates the Receptor-Smads, Smad1, 5 and 8 [156].

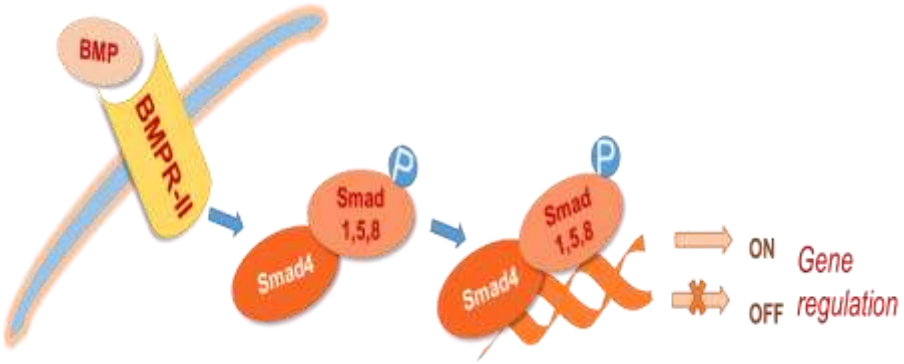


Fig 7. Possible mechanism(s) of action of BMP in EMT

Bone morphogenetic protein (BMP), a member of the TGF-β superfamily, is involved in development, morphogenesis, cell proliferation and apoptosis. Dysregulation of BMP signaling has been suggested in tumorigenesis. BMPs are hypothesized to bind to two types of transmembrane receptors, BMP type I (BMPR-I) and BMP type II (BMPR-II). Both these receptors have intrinsic kinase activity. Upon BMP binding, the heteromeric complex between type I and II receptors initiates intracellular signaling through phosphorylating Smad1, Smad5 and Smad8. Subsequently, these phosphorylated Smads associate with Smad4 and translocate to the nucleus to modulate the transcription of target genes. Three BMP type II receptors, BMPR-II, activin type II receptor (ActRII) and ActR-IIB, have been isolated, the binding affinities of ActR-II and-IIB for BMPs are lower than those for activins

While BMP7 has been shown to block the effect of TGFβ induced cholangiocarcinoma, BMP4 has been shown to induce EMT by upregulation of *Snail* and other EMT regulatory genes [157], showing that they might play a paradoxical role in cancer progression based on the cancer environment. BMPR2 was also found to be over expressed in two prostate cancer cell lines that were observed to have the ability to form osteoblastic lesions *in vivo* [158]. Yet another study showed that BMPRII induced de-epithelialization, in response to either cadherin-6B or BMP. Overall, these observations make BMPs and their receptors as interesting targets for EMT inhibition.

1.7. HYPOTHESIS AND AIMS

The anti-inflammatory and pro-apoptotic properties of isorhamnetin have been studied in detail in various cancer type(s). However, a systematic investigation of its anti-tumor effects in gastric cancer has not been performed so far. Thus, having understood the potential of isorhamnetin as an anti-cancer agent through an extensive review of literature, we hypothesized that isorhamnetin may exhibit its pharmacological effects through the modulation of multiple oncogenic molecular targets.

Our preliminary *in-silico* data showed that isorhamnetin has the capacity to down-regulate various genes involved in proliferation, anti-apoptosis and metastasis in gastric cancer. Therefore, we aimed to analyze if isorhamnetin could inhibit the proliferation of both drug sensitive as well as resistant gastric cancer cells. We also analyzed the pro-apoptotic properties of isorhamnetin in gastric cancer, by examining its effect on various genes involved in inducing apoptosis.

PPARγ has been shown to be a major regulator of inflammation, proliferation and metastasis in gastric cancer. We hypothesized that the anti-cancer activities of isorhamnetin could be mediated at least partially through the modulation of PPARγ signaling pathway. Considering the pivotal role of EMT in cancer progression, we further aimed to determine if isorhamnetin could inhibit the migratory and invasive properties of gastric cancer cells and whether these anti-metastatic effects could be mediated through the inhibition of BMPR2, a BMP receptor observed to be involved in bone metastasis in aggressive tumors.

Lastly, we employed a gastric cancer xenograft model to examine the potential anticancer effects of isorhamnetin *in-vivo*. The mouse model was used to understand whether isorhamnetin can also reduce tumor growth and enhance the effects of chemotherapy. Overall, our aim was to investigate the anti-cancer potential of isorhamnetin, and decipher its mechanism(s) of action through diverse *in silico*, *in vitro* as well as *in vivo* approaches.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Cell culture maintenance and transfection reagents

▪ DMEM (Dulbecco's Modified Eagle's Medium)	Hyclone (Logan, Utah, USA)
▪ RPMI 1640 (Roswell Park Memorial Institute 1640 Medium) ▪ 0.4% trypan blue vital stain ▪ Antibiotic-antimycotic mixture ▪ Lipofectamine® 2000 reagent ▪ Lipofectamine® RNAiMAX Reagent	Life Technologies (Carlsbad, CA, USA)
▪ FBS (fetal bovine serum)	BioWest (Miami, FL, USA)
▪ Trypsin EDTA	Sigma-Aldrich (St. Louis, MO, USA)

2.1.2. Cell lines

Human gastric cancer cells AGS, MKN28, YCC1, oxaliplatin-resistant NUGC3 and cisplatin-resistant AZ521 were kindly provided by Prof. Patrick Tan (DUKE-NUS Graduate Medical School, Singapore). Human gastric cancer SNU5 cells, human head and neck carcinoma cell line CAL27, human hepatocellular carcinoma cell line HepG2 and human breast cancer cell line MDA-MB-231 were obtained from American Type Culture Collection (Manassas, VA). MKN45 gastric cancer cells were obtained from JCRB

(Japanese Collection of Research Bioresources), Japan. HFE-145 normal gastric epithelial cells were kindly provided by Dr. Hassan Ashktorab (Howard University Cancer Center, Washington, DC). Human androgen-independent DU145 prostate cancer cell lines were kindly provided by Prof. Shazib Pervaiz (Department of Physiology, YLLSOM, NUS). AGS, HepG2 and CAL27 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 1X antibiotic-antimycotic mixture (100 U/mL penicillin, and 100 μg/mL streptomycin). AZ521, NUGC3, SNU5, MKN28, MKN45, DU145, MDA-MB-231 cells and HFE-145 cell lines were cultured in Roswell Park Memorial Institute 1640 media, supplemented with 10% FBS and 1X antibiotic-antimycotic mixture. YCC1 cells were cultured in DMEM media supplemented with 10% FBS, 1X antibiotic-antimycotic mixture and 5mM sodium pyruvate. All the cells were maintained at 37°C in an atmosphere of 5% CO_2 and 95% air.

2.1.3 (A) Test compounds

We used the following test compounds in different experiments in our study.

▪ Isorhamnetin (IH) (> 90% purity) ▪ 5-fluorouracil ▪ Cisplatin ▪ Troglitazone	Sigma-Aldrich (St. Louis, MO, USA)
▪ GSK0660 ▪ GW0742	Tocris Bioscience (Ellisville, MO, USA)
▪ 15d-PGJ2 ▪ GW9662	Cayman Chemicals (Michigan, USA)
▪ Capecitabine	Duheng International Trading Company Ltd., Shanghai, China.

2.1.3 (B) Functions of test compounds used in the study

Test compounds	Recognized functions
5-fluorouracil	Irreversible inhibitor of thymidylate synthase
Cisplatin	Platinum-containing anti-cancer drug
Troglitazone	Synthetic PPARγ agonist
GSK0660	Inhibitor of PPARβ
GW0742	PPARβ agonist
15d-PGJ2	15-Deoxy-Delta12,14-Prostaglandin J2, an endogenous PPARγ ligand
GW9662	Irreversible PPARγ antagonist
Capecitabine	5-fluorouracil precursor, thymidylate synthase inhibitor

2.1.4. Reagents and Chemicals

▪ Propidium iodide (PI) ▪ Crystal violet	Sigma-Aldrich (St. Louis, MO, USA)
▪ Annexin V-FITC assay kit ▪ DAPI (4',6-Diamidino-2-Phenylindole, dihydrochloride)	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
▪ MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reagent ▪ SDS (sodium dodecyl sulfate) ▪ Dimethylformamide ▪ The Live and Dead Viability/Cytotoxicity	Life Technologies (Carlsbad, CA, USA)

Assay Kit ▪ Trizol reagent ▪ Hoechst stain	
▪ CXCL12 ▪ TGFβ	Pro-Spec-Tany TechnoGene Ltd. (Rehovot, Israel)
▪ RNAase	Roche (USA)
▪ Calcein-AM	Becton Dickinson (Bedford, MA, USA)
▪ Chemiluminescence ECL	GE Healthcare, (Little Chalfont, Buckinghamshire, UK)
▪ Immunohistochemistry kit	DAKO LSAB kit, Dako Corporation, (Carpinteria, California, USA)
▪ BD BioCoat Matrigel Invasion Chamber	BD Biosciences (Bedford, MA)
▪ Luciferase Assay System with Reporter Lysis Buffer	Promega Pte Ltd.

2.1.5. Antibodies

Antibodies used in the study are listed as follows:

Antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA) are:

Bcl-2 (sc-509)

Bcl-xL (sc-8392)

Cyclin-D1 (sc-753)

PARP (sc-7150)

PPARγ (sc-7196)

VEGF (sc-057496)

E-cadherin (sc-8426)

N-cadherin (sc-7939)

Snail	(sc-10432)
Vimentin	(sc-6260)
PARP	(sc-7150)
MMP-9	(sc-10737)
COX-2	(sc19999)
BMP4	(sc12721)
β-actin	(A5316) was purchased from Sigma-Aldrich
γ catenin	(ab12083) was purchased from Abcam while
Ki-67	(# 9027)
CD31	(# 3528)
BMPR2	(#6979)
p-Smad 3	(#9520)
Smad 3	(#9513)

were purchased from Cell Signaling Technology, Inc, Danvers, USA

2.1.6. Plasmids, siRNAs and transfection methods

GAL4-mPPARγ-LBD, GAL4-PPARδ-LBD chimeric constructs and GAL4-luc reporter plasmid were kindly provided by Dr. Javier F. Piedrafita belonging to the Torrey Pines Institute for Molecular Studies, California, USA. PPRE X3-tk-luc (three DR1 sites upstream of a luciferase reporter) reporter construct and pTA-luc empty vector encoding for mouse PPARγ were kindly provided by Dr. Ronald M. Evans (The Salk Institute for Biological Studies, San Diego, CA, USA). The complete circular plasmid map of PPRE X3-tk-luc as obtained from Addgene (non-profit organization that shares plasmids with researchers) is provided below:

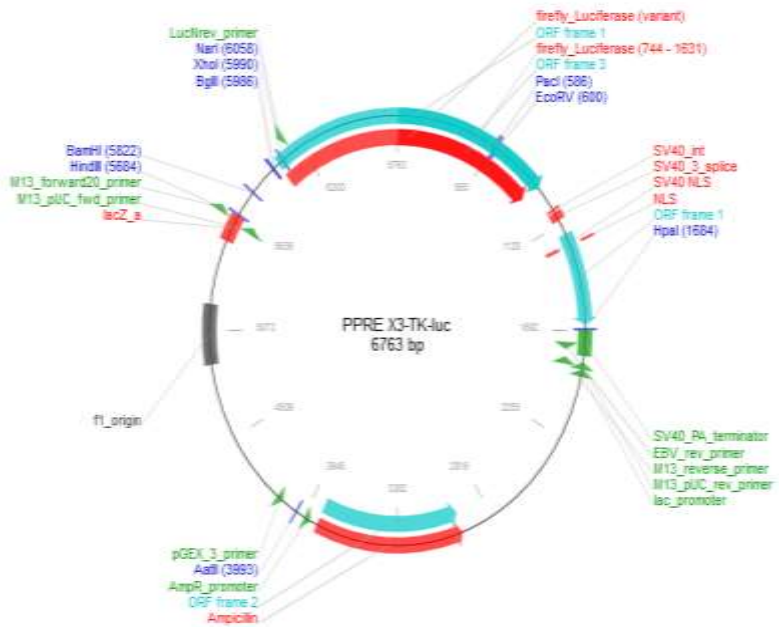


Fig. 2.1.6 (A) Circular map of PPRE X3-tk-luc

Dominant negative mPPARγ mutant (pCMX-mPPARγC126A/E127A) was generously given by Dr. Christopher K. Glass (University of California, San Diego, CA, USA).

BMPR2 Human cDNA ORF Clone (RG208673) was obtained from OriGene Technologies, Inc., Rockville, MD and the plasmid map provided by the company is shown below:

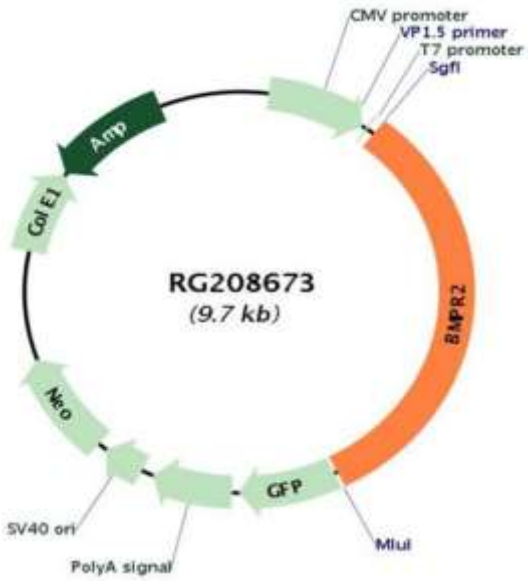


Fig. 2.1.6 (B) Circular map of BMPR2 Human cDNA ORF Clone

BMPR2 (ID: 659) Trilencer-27 Human siRNA was also obtained from OriGene Technologies, Inc., Rockville, MD. It consists of 3 unique 27mer siRNA duplexes of 2 nmol each, which were dissolved in SR30005, RNAse free siRNA duplex resuspension buffer, provided by OriGene.

Transfection

Cells to be transfected were allowed to grow till 70% confluency and left to attach to the plate surface for at least 24 hours before transfection. DMEM serum free media (DMEM media in the absence of FBS and antibiotics) was used to dissolve the plasmids/siRNA and lipofectamine reagents, at required concentrations, according to manufacturer's instructions. On 6 well titer plates, 9 µg of plasmid DNA was used, along with 9-12 µL of

lipofectamine2000. In 96-well plates, 2.5 µg of DNA was used alongside 1.5-2 µL of lipofectamine2000. For BMPR2 siRNA transfections, 40 nM of siRNA was used along with 9 µL of RNAiMAX. The mixture was then added drop-wise to all wells, and allowed to remain for 4-6 hours. Following transfection, serum-free media was replaced by fresh, serum containing media, and allowed to incubate for 48-72 hours. During the incubation, cells were periodically assessed for cell death visually. Thereafter, cells were treated with drugs, or harvested immediately for further analysis.

2.2. Methods

2.2.1. Drug treatment

Isorhamnetin, troglitazone, capecitabine, 5-fluorouracil, GSK0660, GW0742 and GW9662 were dissolved in 100% DMSO and stored at -20°C where they were stable for at least three months. 15d-PGJ2 was dissolved in pure ethanol solvent while cisplatin was prepared in pure distilled water. Further dilutions were freshly prepared in DMEM or RPMI media as and when necessary. Cancer cells required in the study were seeded on to the plates and allowed to adhere for a minimum of 24 hours, after which they were treated at various doses and time-points, as mentioned in the respective figure legends. Thereafter, they were harvested and used for various experiments.

2.2.2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The cytotoxic effect of isorhamnetin against various cancer cells was determined by the MTT assay. $5x10^3$ cells per well were seeded in triplicate in

a 96-well plate and allowed to attach overnight in a final volume of 200 µL media at 37°C. The following day, cells were treated with indicated concentrations of isorhamnetin. They were then allowed to incubate for the required time points. At the end of each time point, 20 mL MTT solution (5 mg/mL MTT in PBS) was added to each well. After 4 hours of incubation in the dark at 37°C, 100 µL lysis buffer (20% SDS, 50% dimethylformamide) was added and incubated for 2 hours at 37°C. Thereafter, cell viability was measured by a Tecan plate reader (Durham, NC, USA) at an optical density of 570nm.

2.2.3. Molecular docking analysis

Please refer to Appendix-I

2.2.4. PPARγ competitive binding assay

A binding assay was performed in a white 384-well polypropylene assay plate to test if isorhamnetin could competitively bind to PPARγ using LanthaScreen™ TR-FRET PPARγ Competitive Binding Assay kit (Life Technologies (Carlsbad, CA, USA). A terbium-labeled anti-GST antibody was used to indirectly label a nuclear receptor (NR) by binding to its GST tag. When a fluorescent ligand (tracer) binds to the receptor, energy transfer from the antibody to the tracer occurs, and a high TR-FRET ratio is observed. Competitive ligand binding to the NR is detected by a test compound's ability to displace the tracer from the NR, which results in a loss of FRET signal between the antibody and the tracer. The assay was performed with various concentrations of isorhamnetin as described in figure legends. Isorhamnetin was dissolved in DMSO and incubated for one hour with human PPAR-LBD

tagged with GST, terbium-tagged anti-GST antibody and fluorescently labeled pan-PPARγ ligand (Fluormone^{TM} Pan-PPAR Green). Radiometric emissions at 520 nm were normalized against terbium emissions at 495 nm and subsequently plotted against the indicated concentrations of isorhamnetin to assess its PPARy binding ability The positive control, GW1929 was a kind gift from Prof. Shazib Pervaiz, and was analyzed using the same method as described above. The curve was plotted using a sigmoidal dose-response equation with varying slope using Prism® software from GraphPad™ Software, Inc.

2.2.5. Flow cytometric analysis

To determine the effect of isorhamnetin on the cell cycle, gastric cancer cells were first seeded at a density of $2x10^5$ cells per well in a 6-well titer plate and incubated at 37°C overnight. They were then treated with isorhamnetin for the various time intervals as described in figure legends. Thereafter, cells were washed, fixed with 70% cold ethanol, and incubated for 30 minutes in ice. Cells were then washed again, resuspended, and stained in PBS containing 25μg/ml propidium iodide (PI) and RNase and kept in the dark for 30 minutes at room temperature. Cell cycle distribution was examined using a CyAn ADP flow cytometer (Dako Cytomation) as described previously [159].

2.2.6. Annexin V assay

Similar to the above described procedures, gastric cancer cells were first seeded at a density of $2x10^5$ cells per well in a 6-well titer plate and allowed to attach at 37°C overnight. After treatment with isorhamnetin for the indicated

time intervals, cells were trypsinized, washed with binding buffer, and resuspended in annexin V-FITC and PI containing binding buffer for 15 minutes at room temperature under dark conditions. Cells were analyzed with a flow cytometer (BD FACS Calibur, BD Biosciences, US) and the data recorded were analyzed using WINMDI software as described previously [160].

2.2.7. Wound healing assay

The migration of cells was investigated using a 'wound-healing' assay. Gastric cancer cells were seeded in a 6-well microtiter plate until about 80% confluent. To observe the effects of over-expression or knock down of BMPR2, cells were first transfected as described previously (2.1.6) before beginning the assay. Using a pipette tip, a 'wound' was created on the uniform layer of seeded cells, the location of the wound marked on the micro titer plate, and each well lightly washed with PBS to remove detached cells. Images of the wounds observed under the microscope before treatments were recorded. The cells were then treated with specific drugs, or simply allowed to migrate for the indicated time-points. At the end of the time-point, the microscopic observation of the cells was again recorded to compare the gap difference before and after treatment.

2.2.8. Invasion assay

The BD BioCoat Tumor Invasion system contains a BD FluoroBlok PET membrane (8.0 μm pore size) uniformly coated with BD Matrigel™ Matrix (BD biosciences). $5x10^4$ gastric cancer cells were suspended in serum-free

media and seeded into the Matrigel transwell chambers and allowed to incubate overnight. The cells were treated with the required reagents and allowed to invade at the time-points indicated in the figure legends. Media containing 10% FBS was used in the bottom chamber to act as a chemo-attractant. In experiments using CXCL12, it was added at this step in the lower chamber to facilitate further invasion of cells. Following incubation, the upper surfaces of the transwell chambers were wiped with cotton swabs and the invading cells were fixed and stained with crystal violet solution. The invading cells were then observed under the microscope, and counted in 5 randomly selected areas as described previously [160].

2.2.9. Luciferase assay

The activity of PPAR-γ was investigated using luciferase assay. Firstly, $5x10^3$ gastric cancer cells per well were seeded in a 96-well micro titer plate and allowed to adhere to the plate overnight. The cells were incubated in serum free DMEM medium for at least 1 hour followed by transfection with pPPRE-tk-Luc as described in section 2.1.6. In the experiment to study the activation of PPARs by isorhamnetin, the cells were first transfected with either GAL4-PPAR-β LBD or GAL4-PPAR-γ LBD plasmids, together with GAL4-Luc. For experiments requiring a mutant PPARγ, the cells were transfected with PPARγ dominant negative plasmid or pCMX-PPARγ plasmid together with pPPRE-tk-Luc. Cells were lysed in reporter lysis buffer (Promega, USA) and luciferase activity was immediately measured with a Tecan (Durham, NC, USA) plate reader and normalized against Renilla activity. The Relative Luciferase Unit per µg of each treatment group was then plotted against the

control group. The data was analyzed using Prism® software from GraphPad™ Software, Inc.

2.2.10. Western blot analysis

For detection of various proteins, gastric cancer cells were first seeded at a density of $3x10^5$ cells per well on a 6 well micro-titer plate and treated with isorhamnetin for different time intervals. The cells were then washed with 1X PBS and incubated on ice for 30 minutes in 0.05 ml lysis buffer (2.0 mM Tris (pH 7.4), 2.50 mM NaCl, 2 mM EDTA (pH 8.0), 0.1% Triton X-100, 0.01 mg/mL aprotinin, 0.005 mg/mL leupeptin, 0.4 mM PMSF, and 4 mM Na_3VO_4). The lysate was then centrifuged at 12,000 rpm for 3 minutes to remove the cell debris and the supernatant was collected. Whole-cell extract protein (ranging from 30-100 μg) was resolved on 12%, 10%, 15% SDS-PAGE depending on the size of protein of interest, electro-transferred onto a nitrocellulose membrane, blocked (Blocking One, Nacalai USA, inc.) for 60 min and blotted with antibodies against relevant proteins. Antibodies were prepared in either 1:100, 1:500 or 1:1000 dilutions in Bovine Serum Albumin (A9418 obtained from Sigma-Aldrich). The proteins were then detected by chemiluminescence (ECL; GE Healthcare**,** Little Chalfont, Buckinghamshire, UK) using a Gel Doc system. The densitometry analysis of the scanned blots was done using Image J software and the results are expressed as fold change relative to the control.

2.2.11. RNA extraction and real-time PCR analysis

Total RNA was extracted using the Trizol reagent (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. Reverse transcription was then carried out as described previously [161]. Briefly, for a 50 μL reaction, 10 μL of RT product was mixed with 1x Taq-Man® Universal PCR Master mix, 2.5 μL of 20x TaqMan probes for *Bcl-2, Bcl-XL* and *CyclinD1* respectively, 2.5 μL of 20x 18S RNA TaqMan probe as the endogenous control for each targeting gene, and topped up to 50 μL with sterile water. A negative control for RT, in which sterile water replaced the RNA template, was included. Another control, where RT mix was replaced with sterile water, was included to check for DNA contamination. Real-time PCR was done using 7500 Fast Real-Time PCR System (ABI PRISM 7500, Applied Biosystems, Foster City, CA, USA) with the following protocol; 50˚C for 2 minutes, 95˚C for 10 minutes, followed by 40 cycles of denaturing at 95˚C for 15 seconds and extension at 60˚C for 1 minute. Results were analyzed using Sequence Detection Software version 1.3 provided by Applied Biosystems. Relative gene expression was obtained after normalization with endogenous human 18S RNA and determination of the difference in threshold cycle (Ct) between treated and untreated cells using 2-ΔΔCt method. Primers and probes for human *Bcl-2, Bcl-XL,* and *CyclinD1* were purchased as kits from Applied Biosystems (Assays-on-Demand) as described previously [161].

2.2.12. Immunocytochemistry for E-cadherin localization

AGS cells were seeded in Nunc Lab-Tek Chamber Slide (Thermo Fisher Scientific) in serum containing media and were left to attach overnight. On the

following day, the cells were washed with PBS. The cells were then fixed with cold acetone for 15 minutes. Upon fixing, the slide was placed for an hour in 5% normal goat serum for blocking. The cells were then incubated with mouse monoclonal anti-human E-cadherin antibody (dilution, 1/100). After overnight incubation, the slides were washed which was followed by incubation with goat anti-mouse immunoglobulin G (IgG)-Alexa Fluor 594 (dilution, 1/100) for 1 hour and counterstained for nuclei with 4′,6-diamidino-2-phenylindole (DAPI; 0.5 μg/mL) for 15 minutes. At the end of the procedure, the slides were mounted with mounting medium (Sigma-Aldrich) and analyzed under a fluorescence microscope (Olympus DP 70).

2.2.13 *In-Silico* analysis

Please refer to Appendix-II

2.2.14 Xenograft tumor model

All procedures involving animals were approved by NUS Institutional Animal Care and Use Committee. Six week-old athymic nu/nu female mice (Animal Resource Centre, Australia) were used to develop a xenograft model. In the right flank of the mice, subcutaneous implantation was performed with SNU-5 cells (3x 10^6 cells/100 μL saline). When tumor size reached 0.25 cm in diameter, the mice were randomized into the following four different treatment groups (n = 5/group) (a) untreated control (corn oil, 100 μL daily); (b) isorhamnetin alone (1 mg/kg bodyweight, suspended in corn oil, intraperitoneal injection thrice/week); (c) capecitabine alone (60 mg/kg bodyweight, suspended in corn oil, twice weekly by gavage); and (d)

combination: isorhamnetin (1 mg/kg bodyweight, intraperitoneal injection, thrice/week) and capecitabine (60 mg/kg bodyweight, twice weekly by gavage). Therapy was continued for 4 weeks, and the animals were euthanized 1 week later and the final tumor volume was measured as $V=4/3\pi r3$, where r is the mean radius of the three dimensions (length, width, and depth). Tumor tissues obtained were fixed in formalin and embedded in paraffin for immunohistochemistry analysis.

2.2.15. Immunohistochemical analysis of tumor tissues

Solid tumors from control and isorhamnetin treated groups were fixed with 10% phosphate buffered formalin, processed and embedded in paraffin. Sections were cut and treated with xylene, dehydrated in graded alcohol and finally hydrated in water. Antigen retrieval was performed by boiling the slide in 10 mM sodium citrate (pH 6.0) for 30 minutes. Immunohistochemistry was performed following manufacturer instructions (DAKO LSAB kit). Briefly, endogenous peroxidases were quenched with 3% hydrogen peroxide. Non-specific binding was blocked by incubation in the blocking reagent in the LSAB kit according to the manufacturer's instructions. Sections were incubated overnight with primary antibodies as follows: anti-PPARγ, anti-Bcl-2, anti-CD31, anti-BMPR2, anti-COX-2 and anti-MMP-9 (each at 1:100 dilutions). Slides were subsequently washed several times in Tris buffered saline with 0.1% Tween 20 and were incubated with biotinylated linker for 30 min, followed by incubation with streptavidin conjugate provided in LSAB kit according to the manufacturer's instructions. Immunoreactive species were detected using 3, 3-diaminobenzidine tetrahydrochloride (DAB) as a substrate.

Sections were counterstained with Gill's hematoxylin and mounted under glass cover slips. Images were taken using an Olympus BX51 microscope (magnification, 20X). Positive cells (brown) were quantitated using the Image-Pro plus 6.0 software package (Media Cybernetics, Inc.).

2.2.16. Clinical Analysis

Please refer to Appendix III

2.2.17. Statistical analysis

Statistical analysis was performed by student's unpaired t test. One way ANOVA test was used when multiple groups had to be compared and Bonferroni method was used for post-test comparisons among the groups. In each case, *p values less than 0.05, **$p<0.01$, and ***$p<0.001$ were considered statistically significant. Bar graphs were plotted and statistical analysis was performed using Prism® software from GraphPad™ Software, Inc.

3. RESULTS

3.1. *In silico* analysis of anti-cancer effects of isorhamnetin

Please refer to Appendix-II

3.2. Anti-gastric cancer effects of isorhamnetin *in vitro*

3.2.1. Isorhamnetin significantly suppresses the viability of diverse gastric cancer cells

The most important characteristic of a cancer cell remains its ability to sustain proliferation. The cellular pathways that control proliferation in normal cells are perturbed in most cancers [162]. Tumor cells can proliferate using alternate strategies: autocrine signaling through which they might produce growth factors themselves and respond to it with their own cognate receptors or by manipulating normal cells in providing them with growth factors [163]. Thus, we first analyzed the effect of isorhamnetin on the viability of gastric cancer cells using a MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) assay, in which the reduction of MTT dye by active mitochondrial enzymes to formazan crystals is proportional to the amount of viable cells. Different gastric cancer cell lines including AGS, MKN45 and SNU-5 were employed for these experiments. The cells were exposed to 0, 5, 10, 25 and 50 μM concentrations of isorhamnetin for 0, 1, 2 and 3 days and then subjected to MTT assay. The data obtained indicated that isorhamnetin can significantly inhibit the viability of all gastric cancer cells examined in a dose- and time-dependent manner.

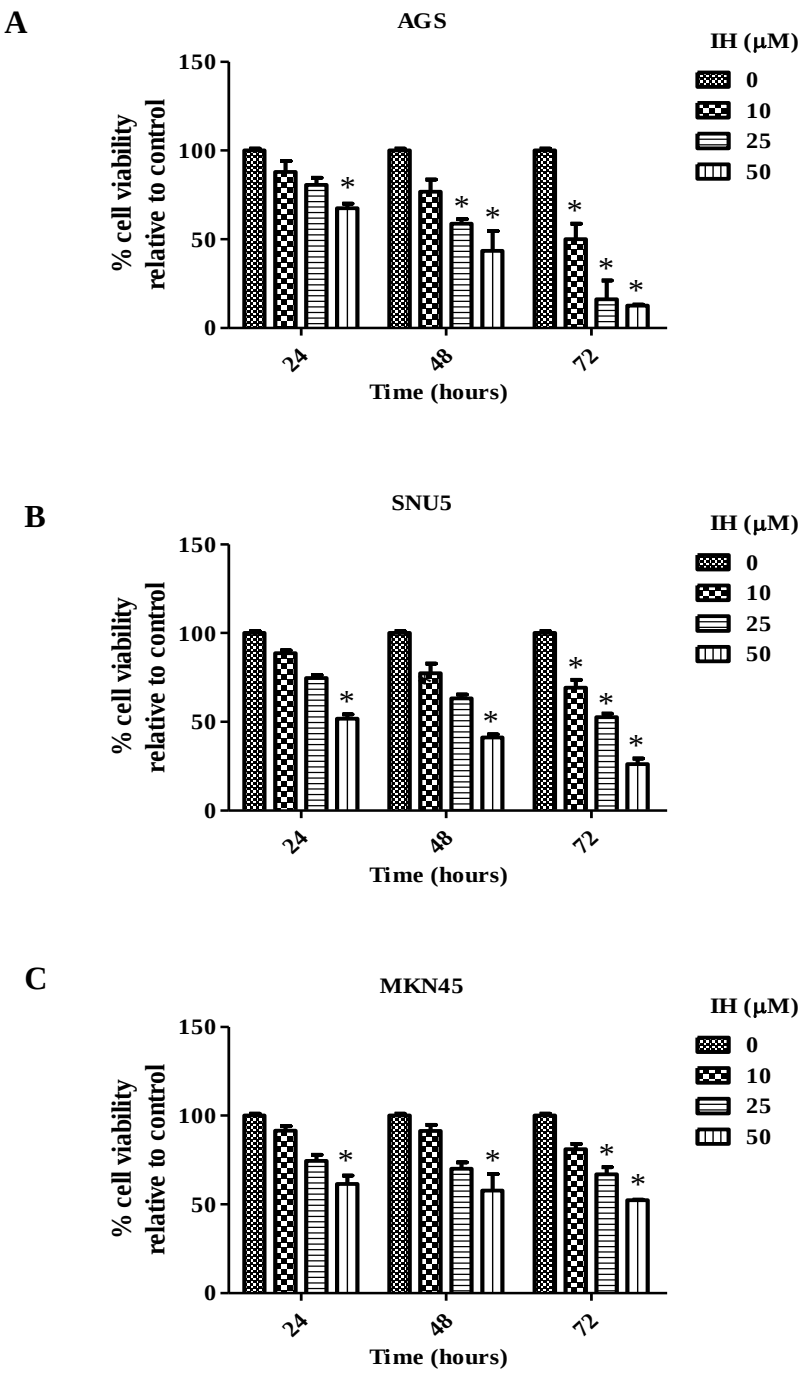


Fig 3.2.1: Isorhamnetin inhibits the viability of gastric cancer cells.

(A) AGS, (B) SNU-5 and (C) MKN45 cells were treated with 0, 10, 25 and 50 μM of IH for 0, 1, 2 and 3 days. At the end of each time point, 20 μL of MTT solution was added and cells were incubated in the dark at 37°C for 4 hours. Following this, cells were lysed using freshly prepared lysis buffer as mentioned in "Materials and Methods". Cell viability was measured at 570nm at the end of treatment using a Tecan plate reader (Durham, NC, USA). As seen in figure, viability of cells treated with isorhamnetin reduced significantly when compared to the control group (*$p < 0.05$) after treatment for indicated time points. Data expressed as mean ± standard deviation (SD) of two independent experiments.

3.2.1.1. Isorhamnetin suppresses the viability of drug-resistant gastric cancer cells

Cancer cells respond well to chemotherapeutic drugs in the initial stages of treatment. However, with prolonged treatment, they begin to develop resistance to the first-line drugs [164]. Since the mechanisms of chemo-resistance are unclear, combination therapy has been gaining attention recently as a means of chemo-sensitizing the cells to therapy [165]. Thus, we investigated the anti-proliferative effects of isorhamnetin in drug resistant gastric cancer cells. Two drug-resistant gastric cancer cells, namely, oxaliplatin-resistant NUGC3 and cisplatin-resistant AZ521 cells were treated with 0, 10, 25 and 50 μM of isorhamnetin for 0, 1, 2 and 3 days and then subjected to MTT assay. The data obtained indicated that isorhamnetin inhibits the viability of drug-resistant gastric cancer cells in a dose- and time-dependent manner, as shown in Fig.3.2.1.1, thus providing evidence that isorhamnetin could sensitize drug resistant gastric cancer cells and could potentially be used in combination with chemo-therapeutic drugs for cancer therapy.

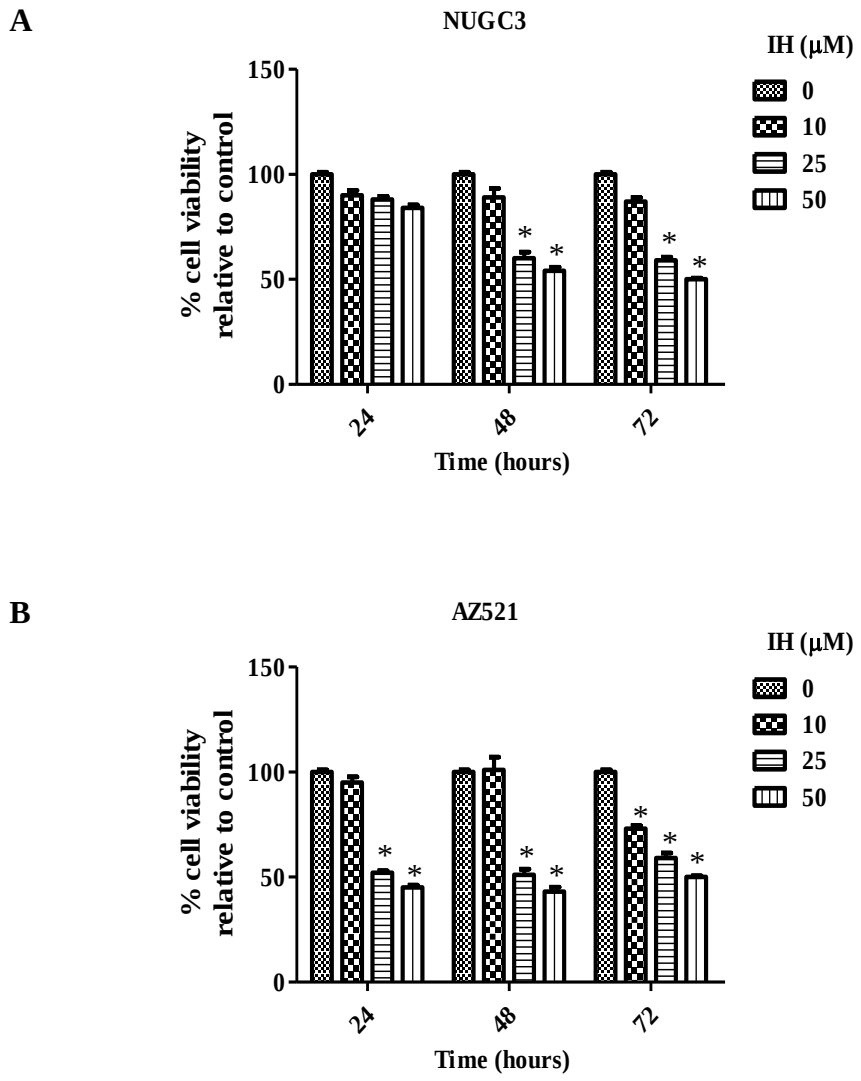


Fig 3.2.1.1: Isorhamnetin inhibits the viability of drug-resistant gastric cancer cells.

(A) Oxaliplatin resistant NUGC3 and (B) cisplatin resistant AZ521 cells were treated with 0, 10, 25 and 50 μM of IH for 0, 1, 2 and 3 days. At the end of each time point, 20 μL of MTT solution was added and cells were incubated in the dark at 37°C for 4 hours. Following this, cells were lysed using freshly prepared lysis buffer as mentioned in "Materials and Methods". Cell viability was measured at 570nm at the end of treatment using a Tecan plate reader (Durham, NC, USA). As seen in figure, viability of cells treated with isorhamnetin reduced significantly when compared to the control group (*$p < 0.05$) after treatment for indicated time points. Data expressed as mean ± standard deviation (SD) of two independent experiments.

3.2.1.2. Isorhamnetin can suppress the viability of other tumor cell types

To analyze the anti-proliferative effects of isorhamnetin on other tumor cell type(s) apart from gastric cancer, MTT assay was used to study the effect of isorhamnetin on the viability of breast, liver, prostate and head and neck cancer cells. Breast cancer (MDA-MB-231) cells, liver cancer (HepG2) cells, prostate cancer (DU145) cells and head and neck cancer (CAL27) cells were treated with 0, 10, 25 and 50 μM isorhamnetin for 0, 24, 48 and 72 hours and MTT assay was performed. The data clearly indicates that isorhamnetin can significantly reduce the viability of various tumor cell types, thereby indicating that its cytotoxic effects are not limited to just one tumor type.

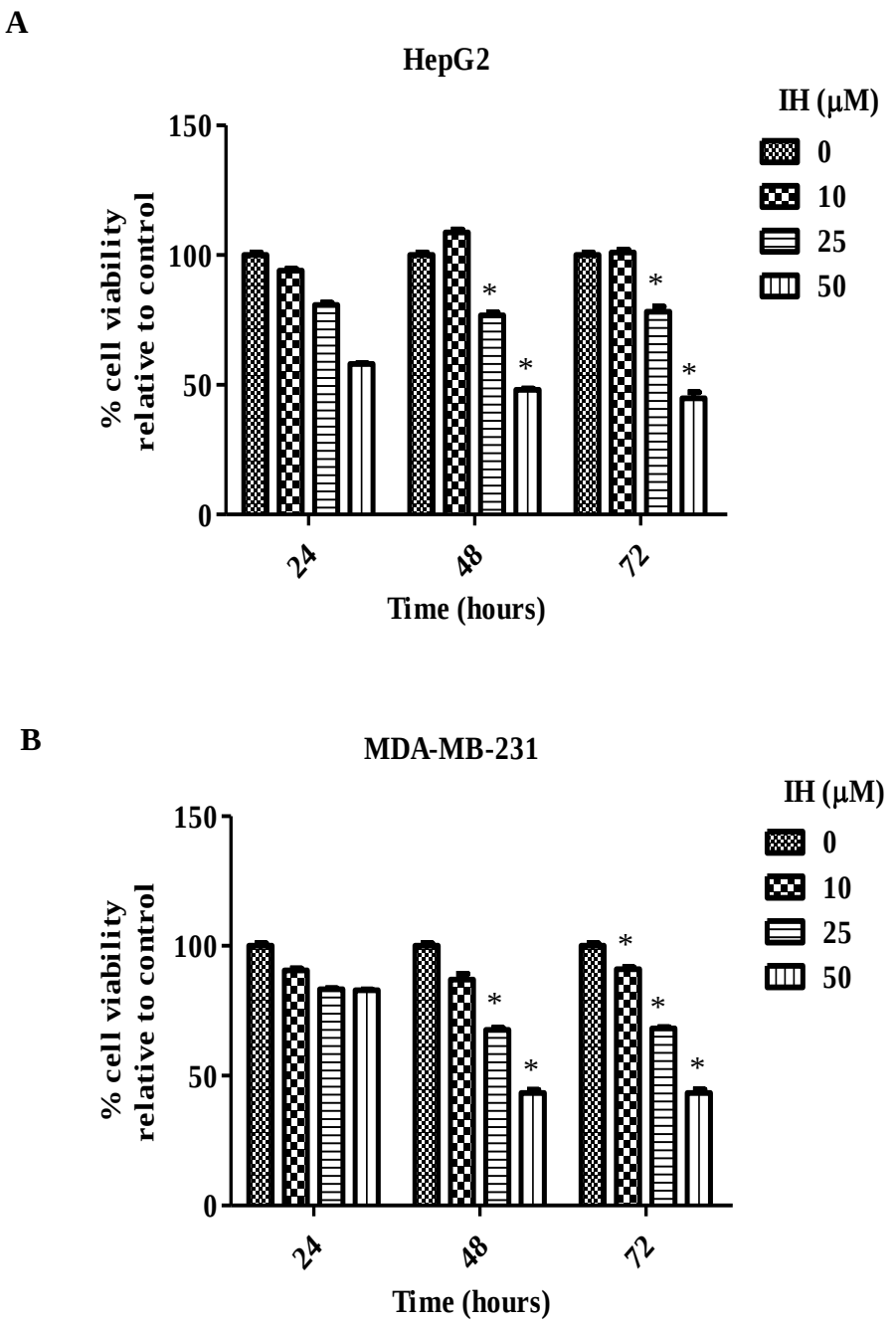


Fig 3.2.1.2: Isorhamnetin inhibits the viability of other tumor cell type(s).

(A) HepG2 and (B) MDA-MB-231 cells were treated with 0, 10, 25 and 50 μM of isorhamnetin for 0, 1, 2 and 3 days. At the end of each time point, 20 μL of MTT solution was added and cells were incubated in the dark at 37°C for 4 hours. Following this, cells were lysed using freshly prepared lysis buffer as mentioned in "Materials and Methods". Cell viability was measured at 570nm at the end of treatment using a Tecan plate reader (Durham, NC, USA). As seen in figure, viability of cells treated with isorhamnetin reduced significantly when compared to the control group (*$p < 0.05$) after treatment for indicated time points. Data expressed as mean ± standard deviation (SD) of two independent experiments

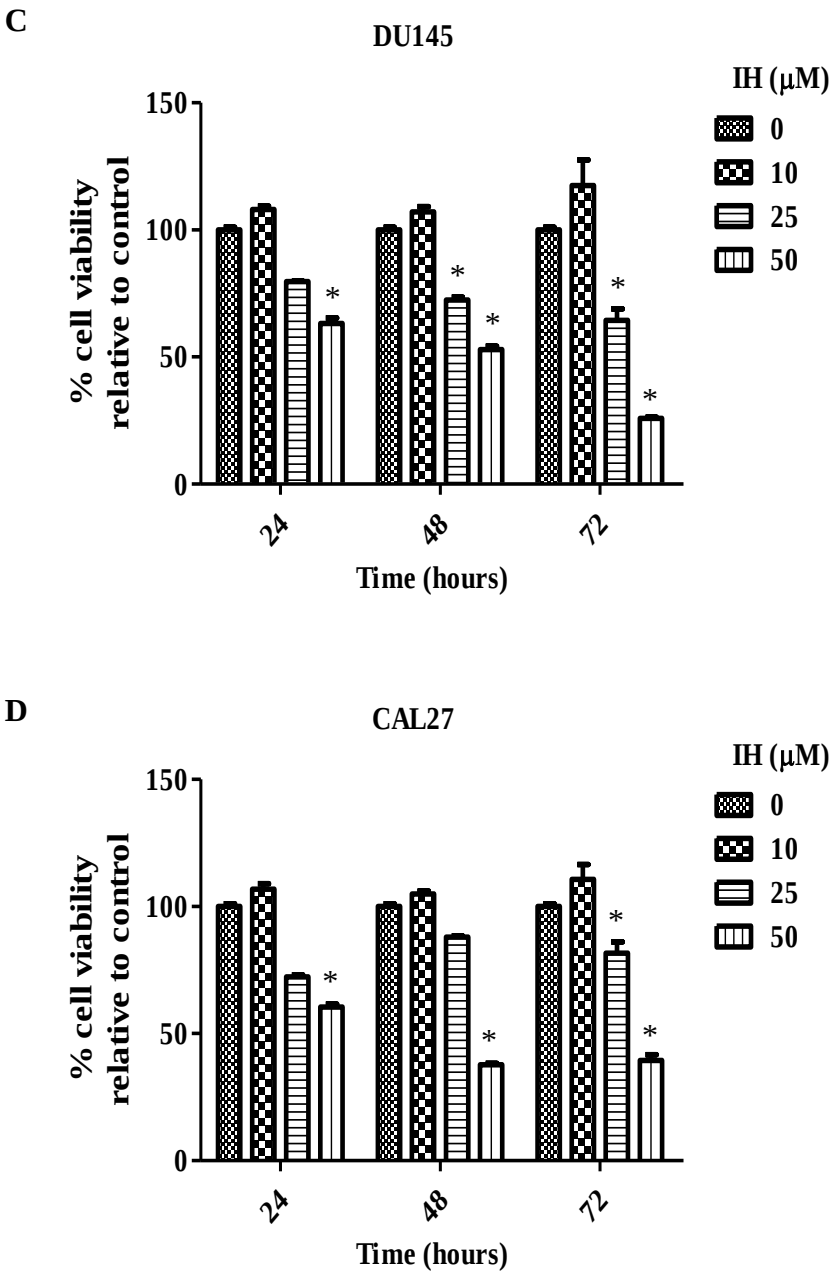


Fig 3.2.1.2: Isorhamnetin inhibits the viability of other tumor cells (contd.).

(C) DU145 and (D) CAL27 cells were treated with 0, 10, 25 and 50 µM of isorhamnetin for 0, 1, 2 and 3 days. At the end of each time point, 20 µL of MTT solution was added and cells were incubated in the dark at 37°C for 4 hours. Following this, cells were lysed using freshly prepared lysis buffer as mentioned in "Materials and Methods". Cell viability was measured at 570nm at the end of treatment using a Tecan plate reader (Durham, NC, USA). As seen in figure, viability of cells treated with isorhamnetin reduced significantly when compared to the control group (*$p < 0.05$) after treatment for indicated time points. Data expressed as mean ± standard deviation (SD) of two independent experiments.

3.2.1.3. Isorhamnetin does not significantly inhibit the viability of normal gastric epithelial cells

It is well established that conventional chemotherapeutic agents deliver the drug to both normal and cancerous tissues, thus leading to undesirable adverse effects [166]. Several chemotherapeutic drugs currently used in treatment such as tamoxifen and capecitabine have been observed to induce undesired side-effects on normal cells, such as atrophy and cardiomyopathy respectively [167, 168]. Thus, whether isorhamnetin could inhibit the viability of normal gastric epithelial cells was analyzed using MTT assay. HFE145 gastric epithelial cells were treated with 0, 20, 40 and 60 µM of isorhamnetin for 0, 1, 2 and 3 days and then subjected to MTT assay. The data showed that isorhamnetin does not significantly inhibit the viability of normal gastric epithelial cells, thereby indicating its specificity towards tumor cells.

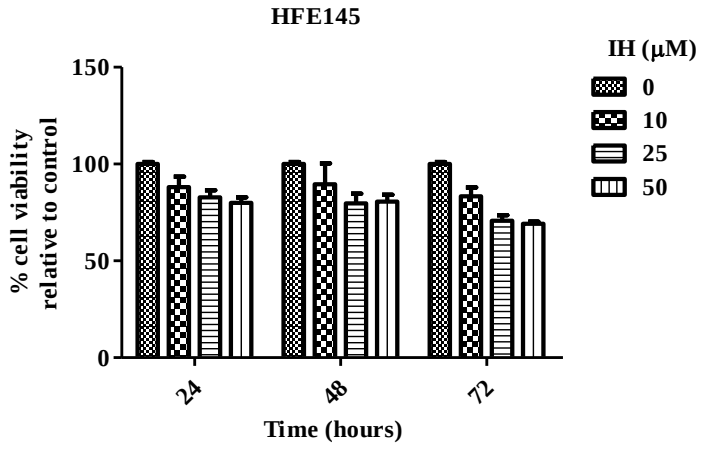


Fig 3.2.1.3: Isorhamnetin does not affect the viability of normal gastric epithelial cells.

HFE145 were treated with 0, 10, 25 and 50 µM of isorhamnetin for 0, 1, 2 and 3 days and subjected to MTT assay. As observed, cell viability was not affected in the presence of various doses of isorhamnetin. Data expressed as mean ± standard deviation (SD) of two independent experiments.

3.2.2. Isorhamnetin induces apoptosis in gastric cancer cells

3.2.2.1. Isorhamnetin induces early apoptosis in gastric cancer cells

During early apoptosis, the membrane phospholipid phosphatidylserine swiftly accumulates and moves from the cytoplasmic region to the extracellular surface [169]. This precedes other apoptotic processes such as loss of plasma membrane integrity, DNA fragmentation, and chromatin condensation. [159]. The loss of membrane symmetry can be detected by utilizing the binding properties of AnnexinV which is a 35-36 kDa, calcium dependent, phospholipid-binding protein with a high affinity for phospholipid phosphatidylserine (PS). It acts as an extrinsic membrane and is therefore an excellent tool to detect cell surface exposed to PS *in vitro* and *in vivo* and is considered to be a sensitive technique to detect early apoptosis [170]. The Annexin V staining assay was performed as described in "Materials and Methods". The results showed that treatment of cells with isorhamnetin was able to increase the number of annexin V-positive cells substantially in a time-dependent manner, thus indicating induction of early apoptosis (Fig. 3.2.2.1).

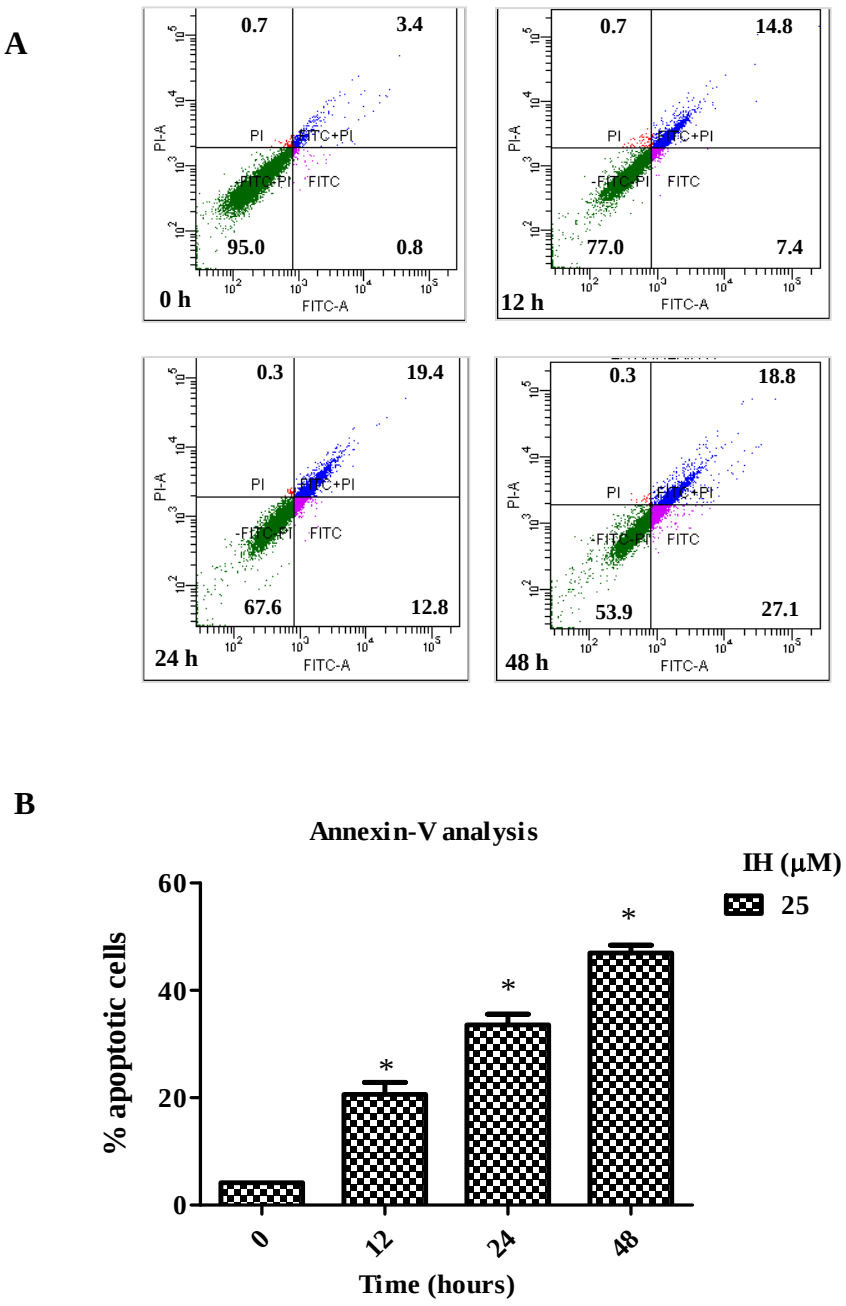


Fig 3.2.2.1: Isorhamnetin induces early apoptosis in gastric cancer cells.

(A) AGS cells were treated with 25 µM of isorhamnetin for 0, 12, 24 and 48 hours. Cells were then washed with PBS, stained with Annexin V and PI and analyzed using flow cytometry. Representative image from each treatment group is shown in the figure. (B) Percentage of apoptotic cells from two independent experiments was calculated and data obtained was plotted in bar graphs as mean ± standard deviation (SD). * indicates p value < 0.05 as compared to control.

3.2.2.2. Isorhamnetin causes increased accumulation of gastric cancer cells in sub G1 phase

Flow Cytometry (FCM) is an important technique for the accurate quantification of apoptosis which differentiates apoptotic cells from non-apoptotic cells by DNA staining. Apoptosis is characterized by altered cell morphology in which plasma membrane excludes uptake of DNA-specific fluorochromes like propidium iodide (PI) [170]. The apoptotic cells with degraded DNA appear as cells with hypo diploid DNA content, seen as "sub-G1" peaks on DNA histograms [171]. Initially, the cells were treated with 25 µM isorhamnetin for 0, 12, 24, and 48 hours followed by fixing and staining as indicated in “Materials and Methods”. As evident from Fig. 3.2.2.2, our results showed that isorhamnetin can cause substantial increased accumulation of the cell population in the sub-G1 phase of the cell cycle in a time-dependent manner, thereby inducing apoptosis in gastric cancer cells with increasing time points.

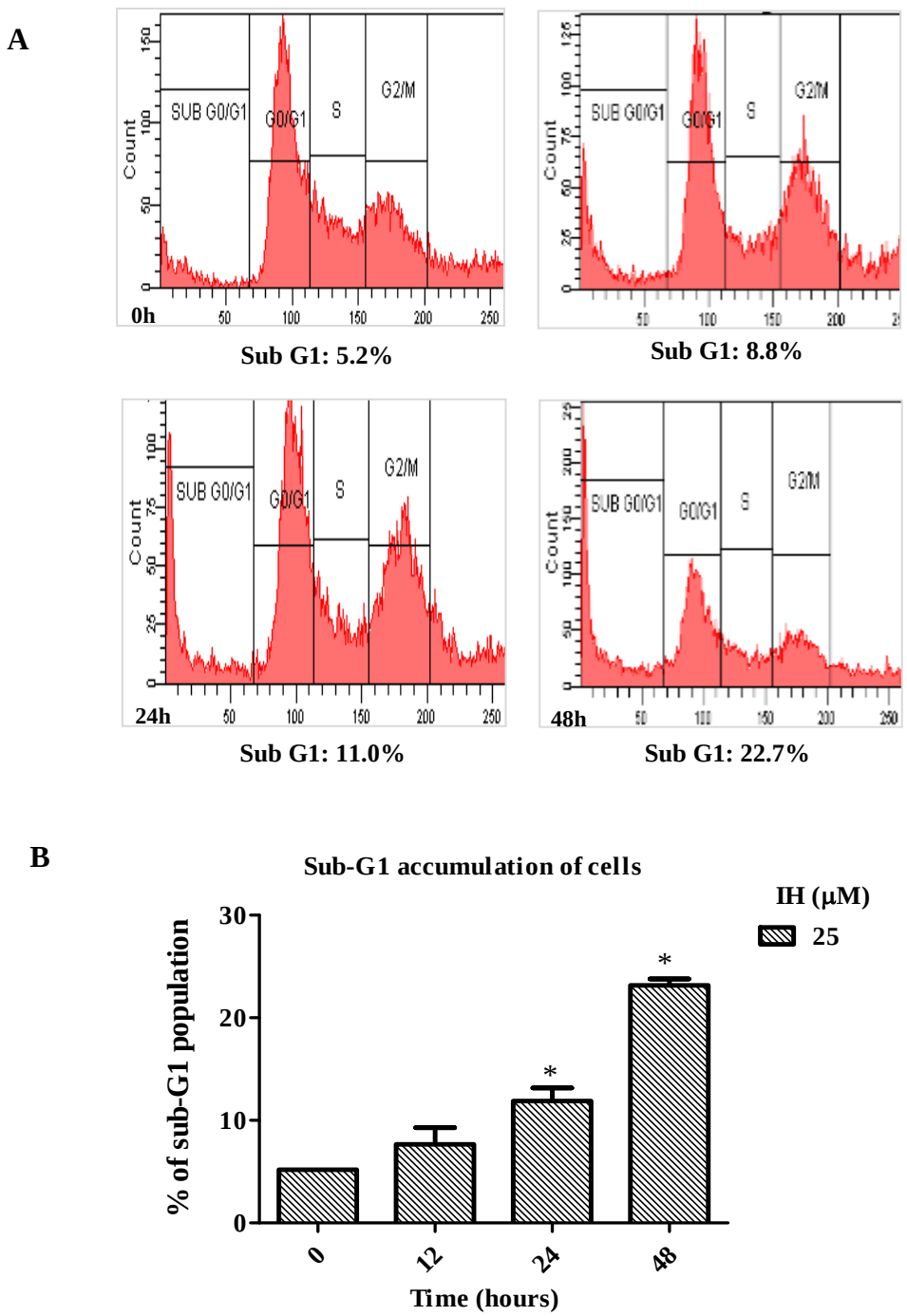


Fig 3.2.2.2: Isorhamnetin induces sub-G1 accumulation in gastric cancer cells.

(A) AGS cells were treated with 25 μM of isorhamnetin for 0, 12, 24 and 48 hours. Cells were then washed with PBS, fixed with ethanol, stained with PI and further analyzed using flow cytometry. Representative image from each treatment group is shown in the figure. (B) Percentage of apoptotic cells from two independent experiments was calculated and data obtained was plotted in bar graphs as mean ± standard deviation (SD). * indicates p value < 0.05 as compared to control.

3.2.2.3. Isorhamnetin induces PARP cleavage in gastric cancer cells

Apoptosis is marked by changes in cellular morphology, as well as by cleavage of poly(ADP-ribose) polymerase (PARP) [172]. PARP cleavage aims at preventing the activation of PARP and hence targets at preserving cellular energy for certain ATP dependent steps of apoptosis [173]. It is subsequently cleaved into 89- and 24-kDa fragments that contain the active site and the DNA-binding domain of the enzyme, respectively, during drug-induced apoptosis. We next analyzed if isorhamnetin could induce PARP cleavage in two different gastric cancer cells, namely AGS and SNU-16. Our results shows that there was a gradual decrease in the level of full length PARP and time-dependent increase in cleaved PARP in both the cell lines thereby indicating that isorhamnetin at 25 µM induces apoptosis in a time-dependent manner in gastric cancer cells.

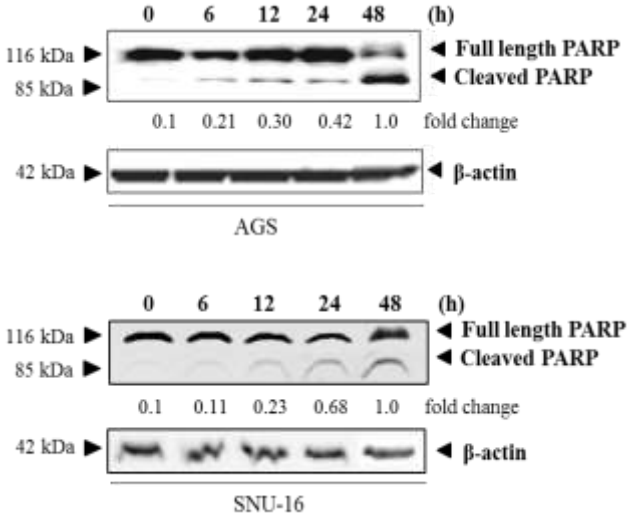


Fig 3.2.2.3: Isorhamnetin increases PARP cleavage in gastric cancer cells.

AGS cells were treated with 25 µM of isorhamnetin for 0, 6, 12, 24 and 48 hours. Whole cell extract was prepared, separated on SDS-PAGE and subjected to western blot analysis to detect protein expression using PARP specific antibody. β-actin was used as a loading control. Densitometry analysis was performed to determine differences in fold change in protein bands between treated and control groups.

3.2.2.4. Isorhamnetin causes downregulation of expression of various oncogenic proteins in gastric cancer cells

The BCL-2 protein family determines the commitment of cells to apoptosis and recent research has focused on the development of novel therapeutics that target these proteins [174]. Cyclin D1 is one of the most important proteins to regulate cell cycle, and related with the development of many cancers. Regulation of Cyclin D1 can induce G1 arrest and inhibit cell growth [175]. To analyze if isorhamnetin could also abrogate the expression of Bcl-2, Bcl-xL and in gastric cancer cells, western blot analysis was performed. AGS and SNU-16 cells were treated with 25 μM for 0, 6, 12, 24 and 48 hours. Cells were then harvested and western blot analysis was done using specific antibodies to detect the various anti-apoptotic proteins. It was found that isorhamnetin can downregulate the expression of the above indicated proteins in a time-dependent manner in both the gastric cancer cell lines (Fig.3.2.2.4).

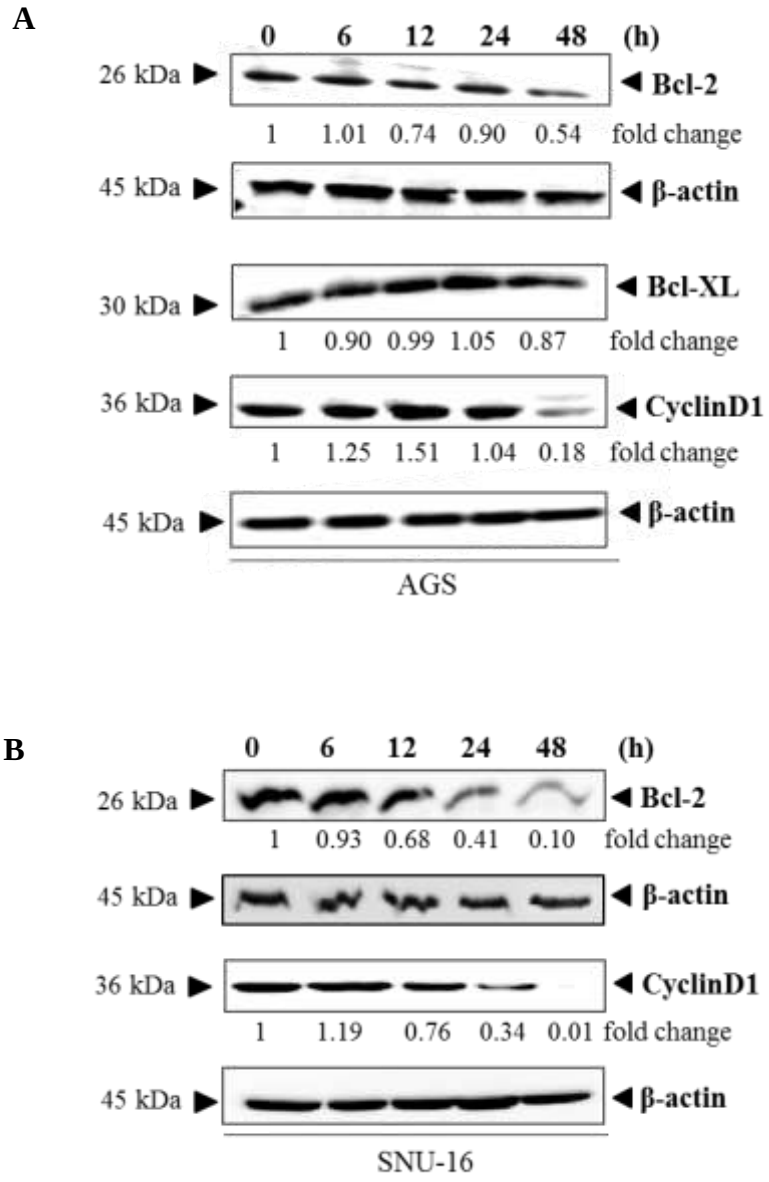


Fig 3.2.2.4: Isorhamnetin inhibits the expression of Bcl-2, Bcl-XL and Cyclin D1 in gastric cancer cells.

AGS and SNU-16 cells were treated with 25 µM of isorhamnetin for 0, 6, 12, 24 and 48 hours. Whole cell extract was prepared, separated on SDS-PAGE and subjected to western blot analysis to detect protein expression using Bcl-2, Bcl-xL and Cyclin D1 specific antibodies. β-actin was used as a loading control. All experiments were done twice, and the best representative blot has been shown. Densitometry analysis was performed to determine differences in fold change in protein bands between treated and control groups.

3.2.2.5. Isorhamnetin causes downregulation of expression of various oncogenic genes involved in the proliferation and survival of gastric cancer cells

Our western blot analysis data showed that isorhamnetin could downregulate the expression of various anti-apoptotic/proliferative proteins and induce apoptosis in gastric cancer cells (Fig.3.2.2.5). Real time PCR was performed because it is currently the most sensitive method to determine the amount of a specific DNA in a complex biological sample [176]. To analyze if isorhamnetin could modulate the expression of proteins at the transcriptional level, gastric cancer cells were treated with 25 μM for 0, 2 and 4 hours and real time PCR analysis was done as described in "Materials and Methods". The results clearly reveal that isorhamnetin also downregulated the expression of various oncogenic proteins at the transcriptional level in a time-dependent manner.

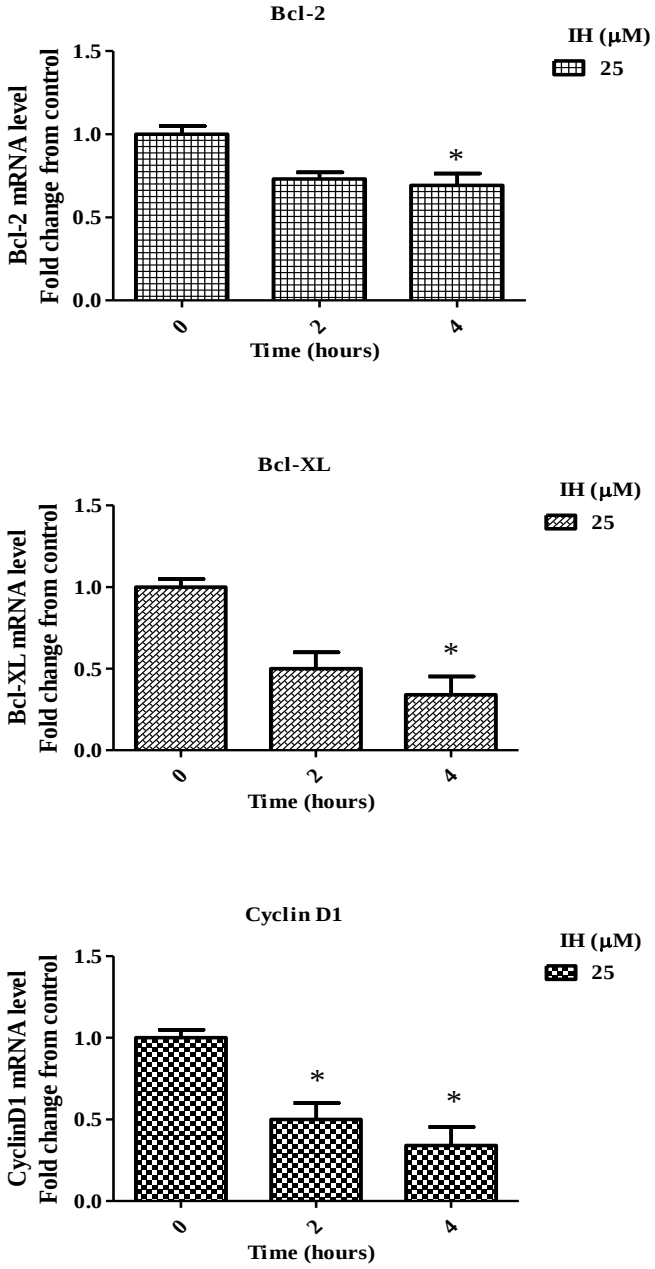


Fig 3.2.2.5: Isorhamnetin inhibits the transcription of anti-apoptotic and proliferative genes in gastric cancer cells.

AGS cells were treated with 25 μM of isorhamnetin for 0, 2 and 4 hours. RNA samples were extracted. 1 μg portions of the respective RNA extracts were subjected to reverse transcription to generate corresponding cDNA. Real time PCR was performed to measure the relative quantities of mRNA. Each RT product was targeted against Bcl-2, Bcl-xL, and cyclin D1 TaqMan probes, with 18S RNA as endogenous control for measurement of equal loading of RNA samples. The results were analyzed using Sequence Detection Software version 1.3 provided by Applied Biosystems, * indicates p value < 0.05 as compared to control.

3.3. PPARγ as a possible molecular target of isorhamnetin

3.3.1. Molecular docking of isorhamnetin with PPARγ

Please refer to appendix 2

3.3.2. Competitive binding of isorhamnetin with PPARγ

An *in vitro* binding assay was performed to determine whether isorhamnetin could competitively bind to PPARγ and to determine its half maximum inhibitory concentration (IC50) [177]. Serial dilutions of isorhamnetin were prepared in a 384-well polypropylene assay plate. Fluormone^{TM} Pan-PPAR Green, PPAR-γ-LBD, and Tb anti-GST Ab were then added to each sample well as described in the protocol. The results demonstrate that isorhamnetin could indeed bind competitively to PPARγ in a dose-dependent manner.

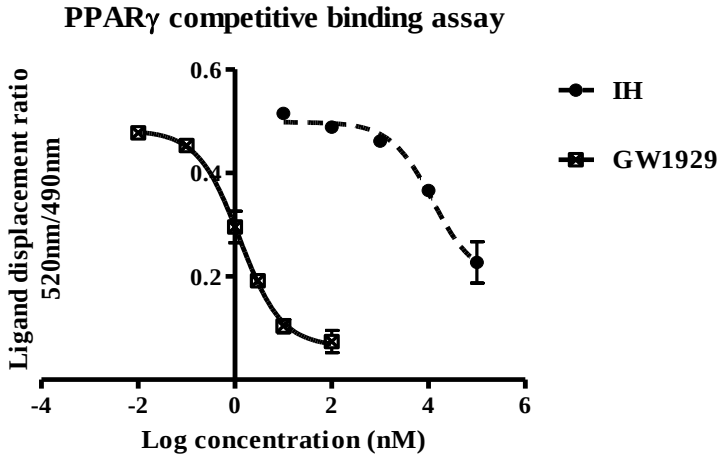


Fig 3.3.2: Isorhamnetin effectively binds to PPARγ in a dose dependent manner.

Serial dilutions of isorhamnetin and positive control GW1929 (both in 1% final DMSO concentration) were prepared in a white 384-well polypropylene assay plate. FluormoneTM Pan-PPAR Green, PPARγ-LBD, and Tb-anti-GST Ab were then added to each sample well as described in the protocol (LanthaScreen™ TR-FRET PPARγ Competitive Binding Assay kit). The assay mixture was incubated for 1 hour at room temperature prior to measuring the 520-nm/490-nm emission ratio of each well using a Tecan (Durham, NC, USA) plate reader. The error bars represent the S.D. of duplicate wells (n=2) and is the result of two independent experiments.

3.3.3. Effect of isorhamnetin on the PPARγ signaling cascade in gastric cancer cells

3.3.3.1. Isorhamnetin activates PPARs in gastric cancer cells

Based on the results of our previous experiments, we found that isorhamnetin could competitively bind to PPARγ. Therefore, we next analyzed if isorhamnetin could activate PPARγ in gastric cancer cells. AGS cells were co-transfected with a chimeric receptor composed of PPARγ Ligand Binding Domain (LBD) fused to the GAL4 DNA Binding Domain (GAL4-*m*PPARγ LBD), along with a GAL4-responsive luciferase reporter construct, and renilla plasmid as internal control. In parallel experiments, cells were transfected with GAL4-*m*PPARδ LBD. Transcriptional activation of the respective PPAR isoforms by isorhamnetin was then assessed by luciferase assay as described in "Materials and Methods". As shown in Fig.3.3.3.1, isorhamnetin was able to significantly increase the activity of two different PPAR isoforms namely PPAR-γ and PPAR-β/δ in AGS cells.

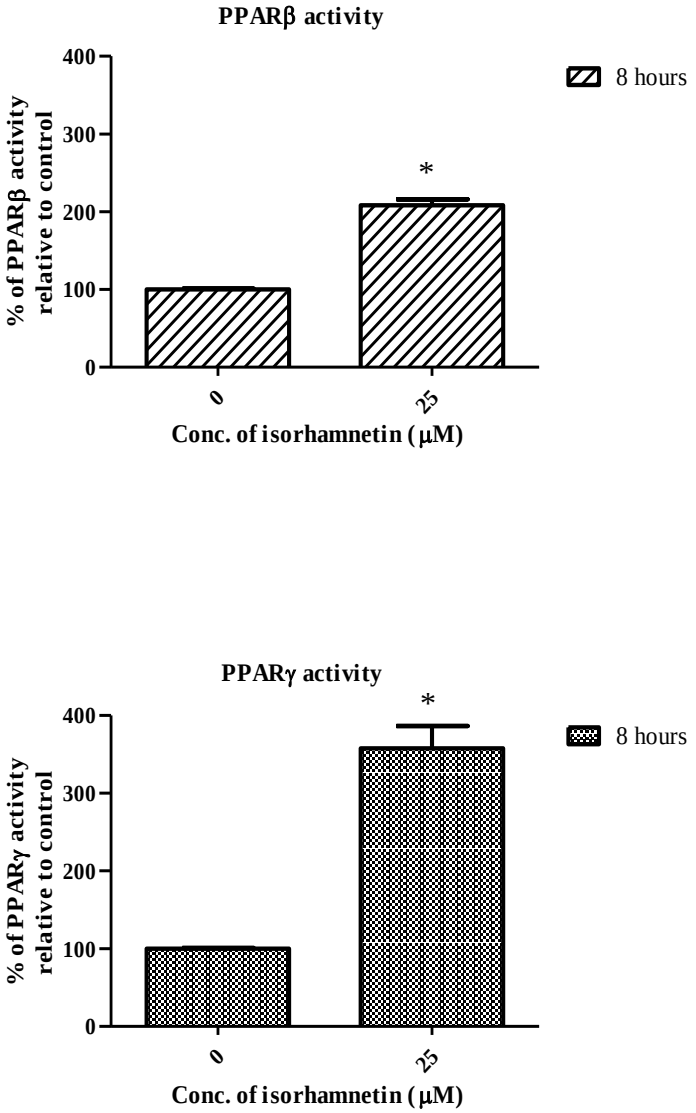


Fig 3.3.3.1: Effect of isorhamnetin on PPAR activity in gastric cancer cells

AGS cells were transfected with either GAL4-PPARβ-LBD or GAL4-PPARγ-LBD plasmids, along with GAL4-Luc and Renilla plasmids. The transfection was allowed for 4 hours following which cells were treated with 25 μM of isorhamnetin for 8 hours. After treatment, cells were lysed in reporter lysis buffer (Promega, USA) and the luciferase activity generated was immediately measured in the dark with a Tecan plate reader (Durham, NC, USA) and normalized against Renilla activity. The bars denote the percentage of PPAR activity compared to control. The plotted values represent the means ± SD of two independent experiments. * indicates p value < 0.05 as compared to control.

3.3.3.2. Isorhamnetin induces significant transcriptional activation of endogenous PPARγ in gastric cancer cells

Given that data from the initial screening reflected LBD transactivation of exogenous transfectants, we employed an additional luciferase system to study effect of isorhamnetin on endogenous PPARγ transcriptional activation. For this, AGS cells were transiently transfected with a luciferase-linked PPARγ reporter construct containing 3 X PPARγ response element (PPRE), or pTA-luciferase (pTA-luc) empty vector before exposure to various doses of isorhamnetin. As shown in Fig. 3.3.3.2, isorhamnetin elicited robust dose-dependent transcriptional activation of endogenous PPARγ receptor in AGS cells as demonstrated by 3xPPRE-tk-luc transfected cells, with negligible luciferase activity elicited in pTA-luc empty vector transfected control cells. Further kinetic studies revealed that isorhamnetin-induced activation of PPARγ was evident at the shortest time point tested (2 hours after treatment), and increased substantially in a time- and dose-dependent manner, reaching a peak at 8 hours after treatment. Similarly, the protein expression of PPAR-γ was significantly increased after isorhamnetin treatment in AGS cells.

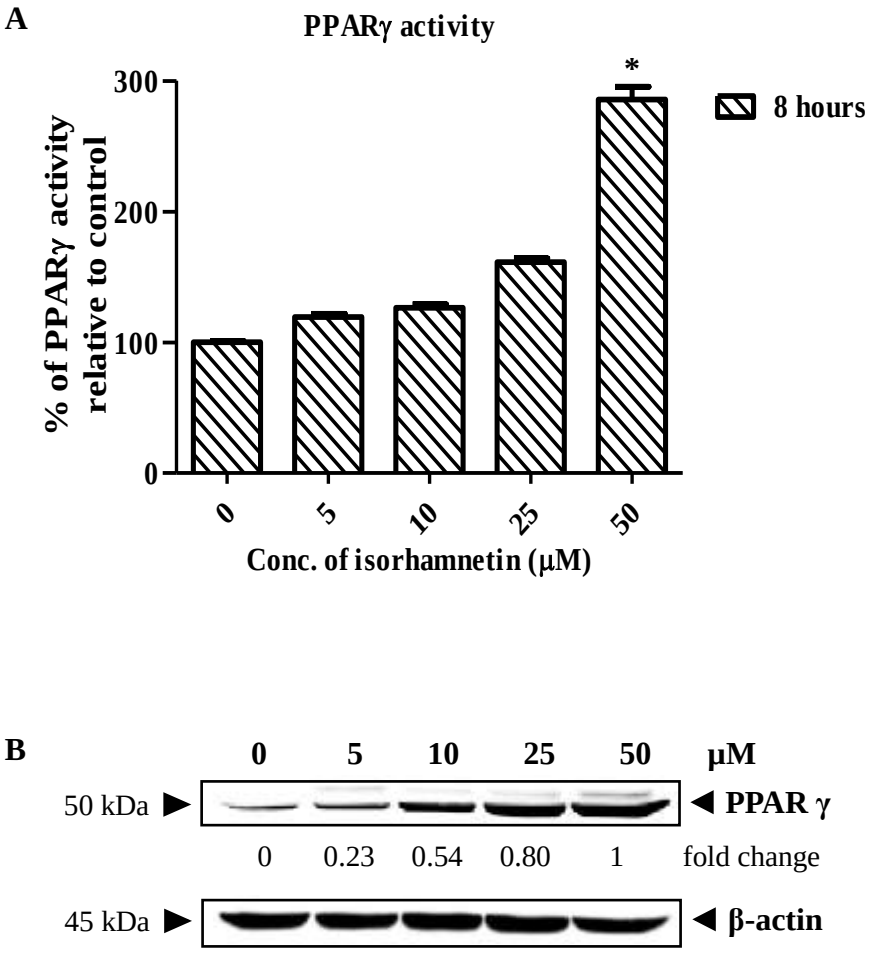


Fig 3.3.3.2: Isorhamnetin increases PPARγ activity and expression in a dose- dependent manner in gastric cancer cells.

(A) AGS cells were transfected with pPPRE-tk-Luc and Renilla plasmid for 4 hours. The cells were then exposed to increasing doses of isorhamnetin. Following treatment, cells were lysed in reporter lysis buffer (Promega, USA) and luciferase activity was immediately measured with a Tecan plate reader (Durham, NC, USA) and normalized against Renilla activity. The data obtained as mean ± standard deviation (SD) of two independent experiments are expressed as percentages of the PPARγ activity relative to the control. * indicates p value < 0.05 as compared to control. (B) Gastric cancer cells were treated with increasing doses of isorhamnetin; 0, 5, 10, 25 and 50 μM. Whole cell extract was obtained, subjected to SDS PAGE analysis, transferred to a nitrocellulose membrane and analyzed using PPARγ specific antibody. Two independent experiments were performed and representative blot is shown. Densitometry analysis was performed to determine differences in fold change in protein bands between treated and control groups.

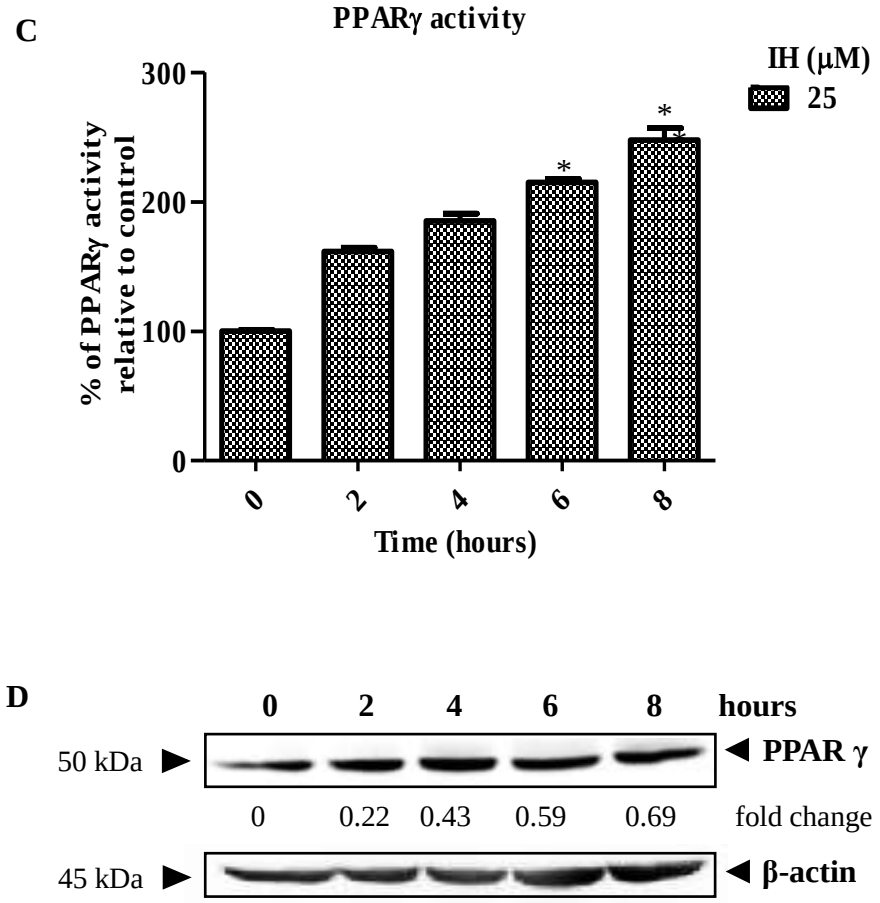


Fig 3.3.3.2: Isorhamnetin increases PPARγ activity and expression in a time- dependent manner in gastric cancer cells

(C) Similar to the previous figure, AGS cells were seeded in a 24-well plate and allowed to adhere overnight. They were then transfected with pPPRE-tk-Luc and Renilla plasmid for 4 hours followed by exposure to 25 μM of isorhamnetin for 0, 2, 4, 6 and 8 hours. Following treatment, cells were lysed in reporter lysis buffer (Promega, USA) and luciferase activity was immediately measured with a Tecan plate reader (Durham, NC, USA) and normalized against Renilla activity. The data obtained are mean ± standard deviation (SD) of two independent experiments. The plotted bars denote the percentages of the PPARγ activity relative to the control. * indicates p value < 0.05 as compared to control. (D) Gastric cancer cells were treated with 25 μM of isorhamnetin for 0, 2, 4, 6 and 8 hours. Whole cell extract was obtained, subjected to SDS PAGE analysis, transferred to a nitrocellulose membrane and analyzed using PPARγ specific antibody. Two independent experiments were performed and representative blot is shown. Densitometry analysis was performed to determine differences in fold change in protein bands between treated and control groups.

3.3.3.3. Pharmacological blocker (GW9662) reverses isorhamnetin-induced PPARγ activity in gastric cancer cells

We next determined if isorhamnetin induced PPARγ transcriptional activation requires ligand binding to the receptor. To study this, we pre-incubated AGS cells with GW9662, a specific and irreversible antagonist of PPARγ, which acts by covalently modifying a cysteine residue in PPARγ ligand binding domain. Notably, it is known that this specific antagonist of PPARγ has negligible effect on the activity of PPARα and δ [178]. As illustrated in Fig.3.3.3.3, we observed that GW9662 could significantly attenuate ligand-dependent PPARγ-activation induced by both isorhamnetin and an endogenous ligand, 15d-PGJ2 that can activate PPAR response elements [179].

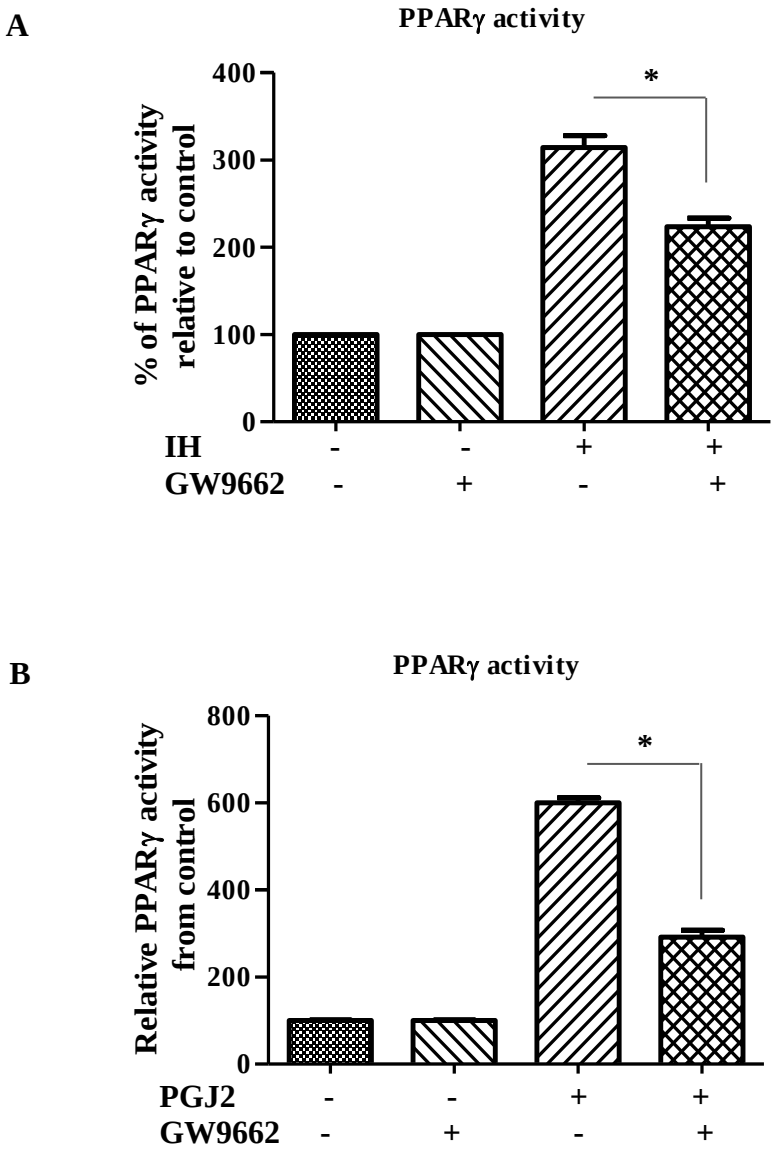


Fig 3.3.3.3: Isorhamnetin induced PPARγ activity could be partially reversed by GW9662, a pharmacological inhibitor of PPARγ.

AGS gastric cancer cells were seeded onto a 24-well plate and allowed to adhere overnight. The cells were then transfected with GAL4-PPARγ-LBD plasmids along with GAL4-Luc and Renilla plasmid for 4 hours. Following transfection, the cells were exposed to 10 μM or 20 μM GW9662 for 2 hours. Once exposed to the PPARγ antagonist, cells were now treated with 25 μM isorhamnetin (A) or 20 μM 15d-PGJ2 (B), a PPARγ agonist, both for 18 hours. After the treatment, cells were lysed in reporter lysis buffer (Promega, USA) and luciferase activity was immediately measured with a Tecan plate reader (Durham, NC, USA) and normalized against Renilla activity. The data obtained are mean ± standard deviation (SD) of two independent experiments. The plotted bars denote the percentages of the PPARγ activity relative to the control. * indicates p value < 0.05 as compared to control.

3.3.3.4. DBD mutation of PPARγ partially reverses isorhamnetin mediated activation of PPARγ in gastric cancer cells

Consequently, to investigate whether DNA-binding function of PPARγ was required for isorhamnetin induced PPARγ activation, we transfected AGS cells with either an empty vector or a DNA-binding defective form of PPARγ. This dominant negative form of PPARγ (PPARγ $^{C126A/E127A}$) contains a double amino acid substitution in the DNA-binding domain (DBD), which annuls its ability to bind PPRE without affecting its potential for ligand binding [180]. This finding is consistent with previous studies which demonstrated that point mutations of these two critical amino acids in the first zinc finger of the DBD renders the receptor completely dysfunctional for transactivation [181].

Our data indicate that transfection with a dominant negative mutant PPARγ construct could partially reverse isorhamnetin induced activation of PPARγ (Fig.3.3.3.4 (A)). Also, we found that the dominant negative mutant could partially block isorhamnetin mediated apoptosis, as seen in Fig. 3.3.3.4(B), by rescuing the expression of Bcl-2 and decreasing PARP cleavage. Taken together, our results show that isorhamnetin mediated anti-apoptotic effects might be at least partially due to DNA binding function of PPARγ.

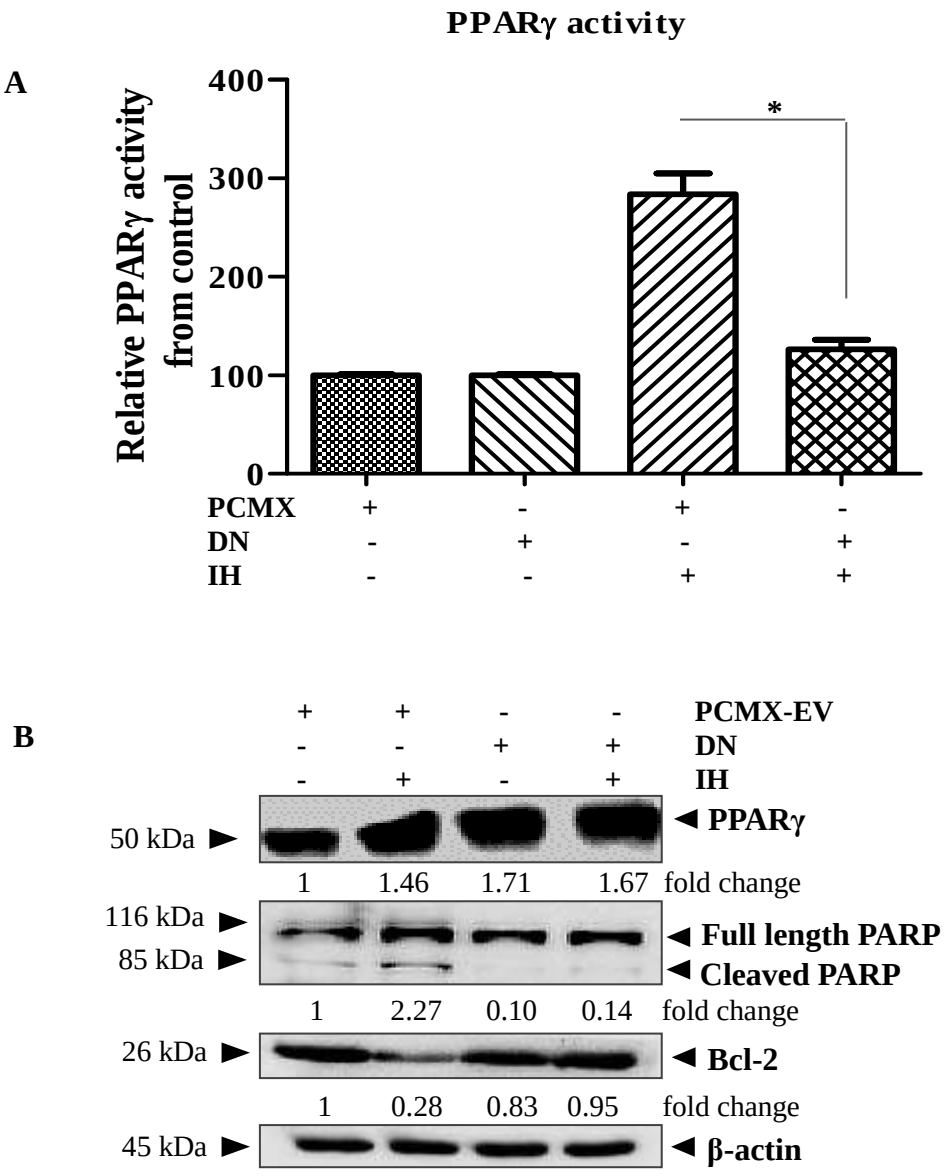


Fig 3.3.3.4: Transfection with dominant negative PPARγ partially reverses isorhamnetin-induced PPARγ activity.

(A) AGS gastric cancer cells were first seeded on to a 24-well plate and allowed to adhere overnight. Upon checking the attachment of cells to the plate visually, they were transfected with PPARγ DN, along with pPPRE-tk-Luc and Renilla plasmids for 4 hours. Upon transfection, cells were allowed to grow for 48 hours and then treated with 25 μM of isorhamnetin for 18 hours. The data obtained are mean ± standard deviation (SD) of two independent experiments. The plotted bars denote the percentages of the PPARγ activity relative to the control. * indicates p value < 0.05 as compared to control. (B) The cells were transfected with PPARγ DN plasmids and treated with 25 μM of isorhamnetin for 24 hours. Whole cell extract was obtained, subjected to SDS PAGE analysis and probed with PARP and Bcl-2 specific antibodies. Two independent experiments were performed and representative blot is shown. Densitometry analysis was performed to determine differences in fold change in protein bands between treated and control groups.

3.3.3.5. Selective role of isorhamnetin in activating PPARγ

We have previously observed that isorhamnetin could also induce the activation of PPARβ in gastric cancer cells. Considering the similarity of different isoforms of PPAR, it was important to analyze whether this activation was a specific or non-specific phenomena. Therefore, we employed an established agonist of PPARβ, GW0472 and an antagonist, GSK0660, that can compete with an agonist at the cellular level [182], to examine the selective role of isorhamnetin in activating PPARγ. Our data, as shown in Fig. 3.3.3.5 (A), showed that GSK0660, was unable to reverse isorhamnetin-induced PPARβ activity, whereas similar concentrations of GSK0660 could reverse the effect of GW0472 (Fig. 3.3.3.5 (B)), suggesting that the effect of IH on PPARβ activation was non-specific. Overall, through our experiments so far, we demonstrate for the first time that isorhamnetin exhibits anti-proliferative and pro-apoptotic effects in gastric cancer cells and this effect is mediated at least in part, via the modulation of PPARγ pathway.

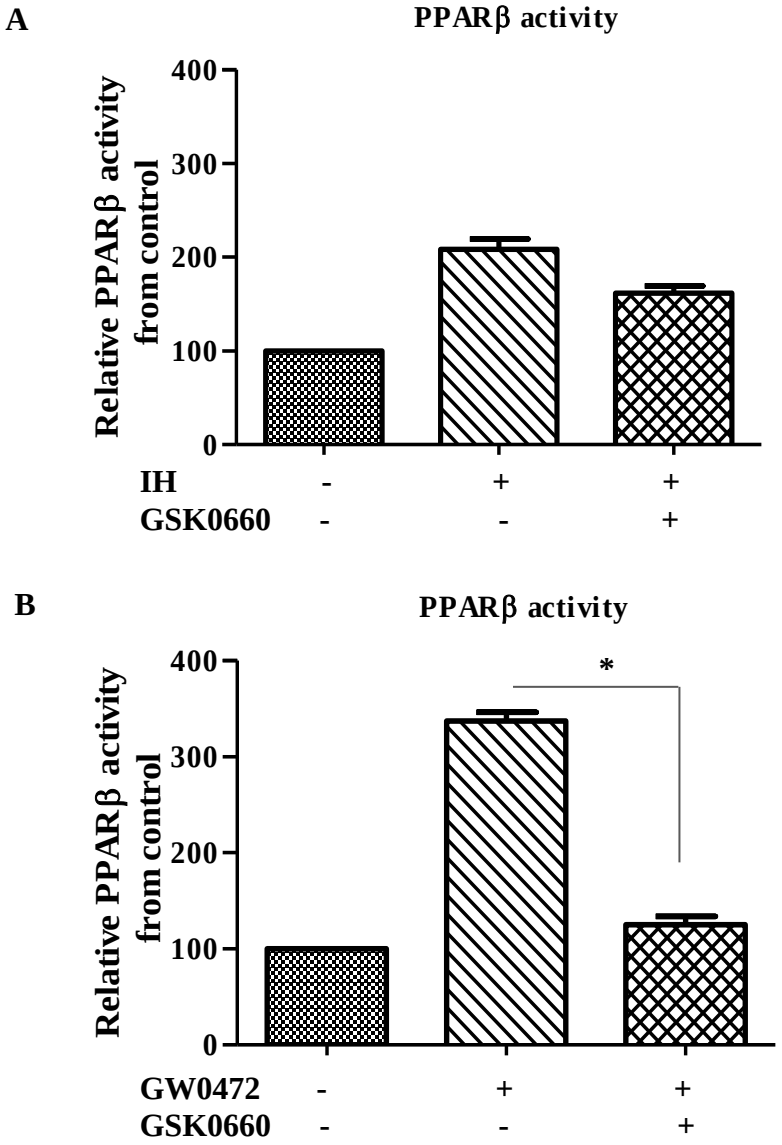


Fig 3.3.3.5. Pharmacological inhibitor of PPARβ, GSK0660, could not block isorhamnetin-induced PPARγ activity.

AGS gastric cancer cells were seeded on a 24-well plate and allowed to adhere overnight. They were later transfected with GAL4-PPARβ-LBD plasmids in combination with GAL4-Luc and Renilla plasmid for 4 hours. Following transfection, cells were allowed to grow for 48 hours after which they were pretreated with 50 µM GSK0660 for 4 hours followed by exposure to 25 µM isorhamnetin (A) or 10 µM GW0742 (B), a PPARβ agonist, both for 18 hours. Following treatment, cells were lysed in reporter lysis buffer (Promega, USA) and luciferase activity was immediately measured with a Tecan plate reader (Durham, NC, USA) and normalized against Renilla activity. The data obtained as mean ± standard deviation (SD) of two independent experiments are expressed as percentages of the PPARγ activity relative to the control.
* indicates p value < 0.05 as compared to control.

3.4. Role of isorhamnetin in overcoming epithelial to mesenchymal transition

3.4.1. Isorhamnetin inhibits CXCL12-induced migration of gastric cancer cells

Clinical studies suggest that mortality rates of patients with advanced gastric cancer is high due to the complications caused by metastases of existing tumors [183]. Therefore, preventing metastasis is an effective approach for the successful treatment of gastric cancers. In recent years, numerous bioactive compounds obtained from natural sources have gained recognition as a source of development of novel agents that can considerably halt the progression of metastasis [184]. Cell migration is a complex cellular behavior that serves as an important step in the progression of metastasis in gastric cancer cells [185]. CXCL12 is a well characterized chemokine that can induce migration in a variety of cancer cells [186]. Therefore, we investigated the effect of isorhamnetin on the migratory potential of gastric cancer cells in the presence and absence of CXCL12 using the wound healing assay as shown in Fig. 3.4.1(A).

Our results showed that isorhamnetin could significantly inhibit the migration of gastric cancer cells. We also found that cancer cells migrated faster under the influence of CXCL12 and this effect was also reduced significantly upon treatment with isorhamnetin.

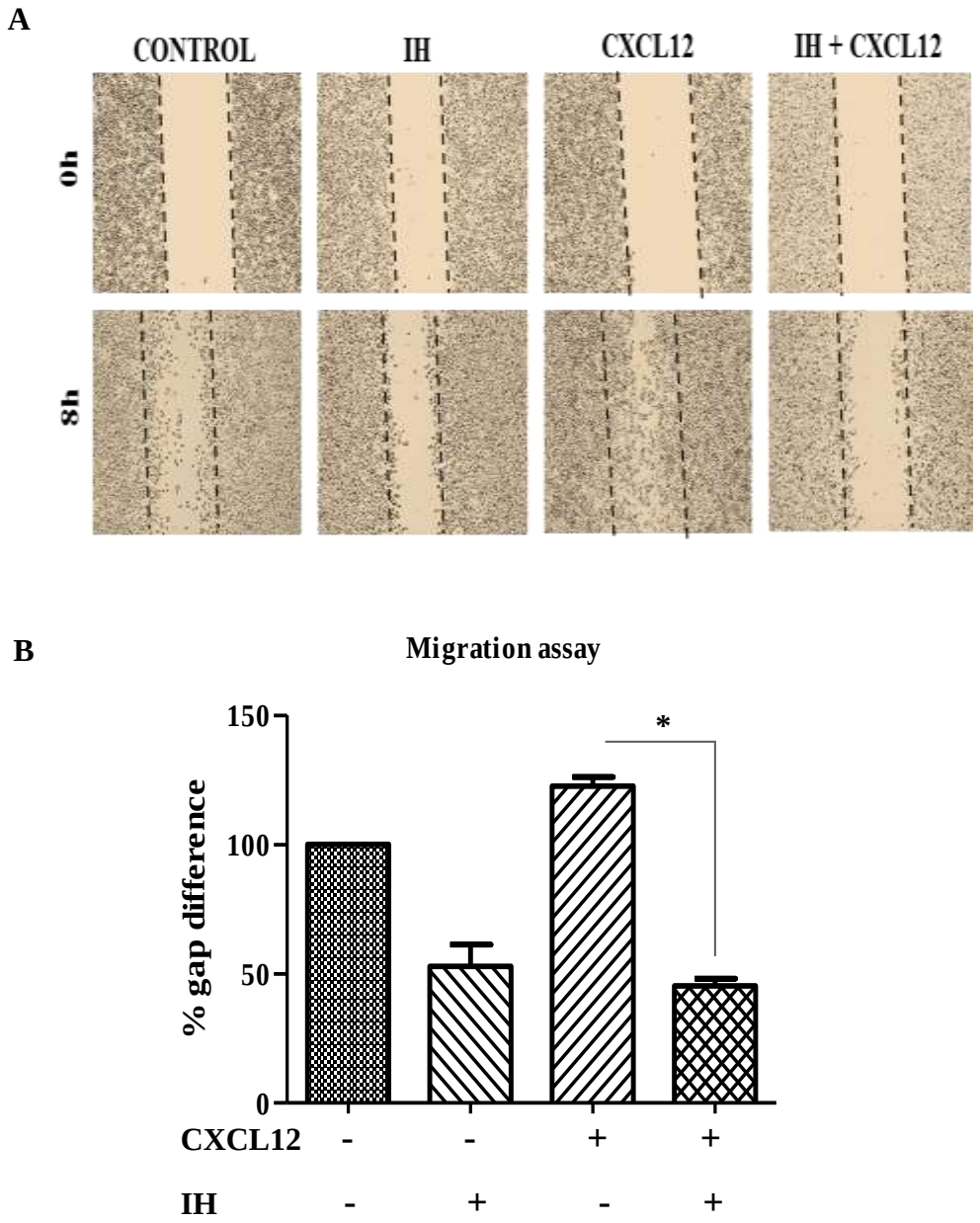


Fig 3.4.1: Isorhamnetin inhibits CXCL12 induced migration in gastric cancer cells.

(A) AGS cells were seeded on a 6-well plate and allowed to grow until a uniform mono-layer was obtained. Following this, a wound was swiftly created on the plate using a pipette tip. The images of the wound were recorded under the microscope. Then, the cells were either treated with 25µM isorhamnetin and/or CXCL12 (100 ng/ml) for 8 hours. Gap difference was measured at the end of treatment and microscopic images were recorded. Representative images from each treatment group are shown. (B) The percentage of the gap difference between the treated and untreated groups was normalized and plotted in the form of bar charts. The values plotted are the means ± S.D. of two independent experiments. * indicates p value < 0.05 as compared to control.

3.4.2. Isorhamnetin inhibits CXCL12-induced invasion of gastric cancer cells

Tumor invasion consists of discrete biological processes in which tumor cells move from the primary neoplasm to the underlying stroma; this process involves the loss of adherence to other cells as well as cell adhesion to the extracellular matrix (ECM) [187]. Chemokines also contribute to invasion by inducing the infiltration of tumors by releasing proteases and other inflammatory molecules [188]. Recently, it has been reported that chemokine stromal cell-derived factor-1α (SDF-1α, also known as CXC-chemokine ligand 12, CXCL-12) and its receptor, CXC-chemokine receptor 4 (CXCR4), are involved in gastric cancer invasion and metastasis [189]. To analyze if isorhamnetin could inhibit the invasive capacity of gastric cancer cells, we used a BD biocoat tumor invasion system that consists of matrigel transwell chambers containing a light-tight polyethylene terephthalate membrane with 8-μm diameter pores and coated with a reconstituted basement membrane gel. Upon treatment with isorhamnetin, there was a significant reduction in the number of cells that could invade the chamber, indicating that isorhamnetin could indeed inhibit the invasive property of gastric cancer cells. Moreover, isorhamnetin could also significantly reduce the number of cells that were induced to invade in the presence of CXCL12, confirming the inhibitory capacity of this novel compound on gastric cancer cell invasion.

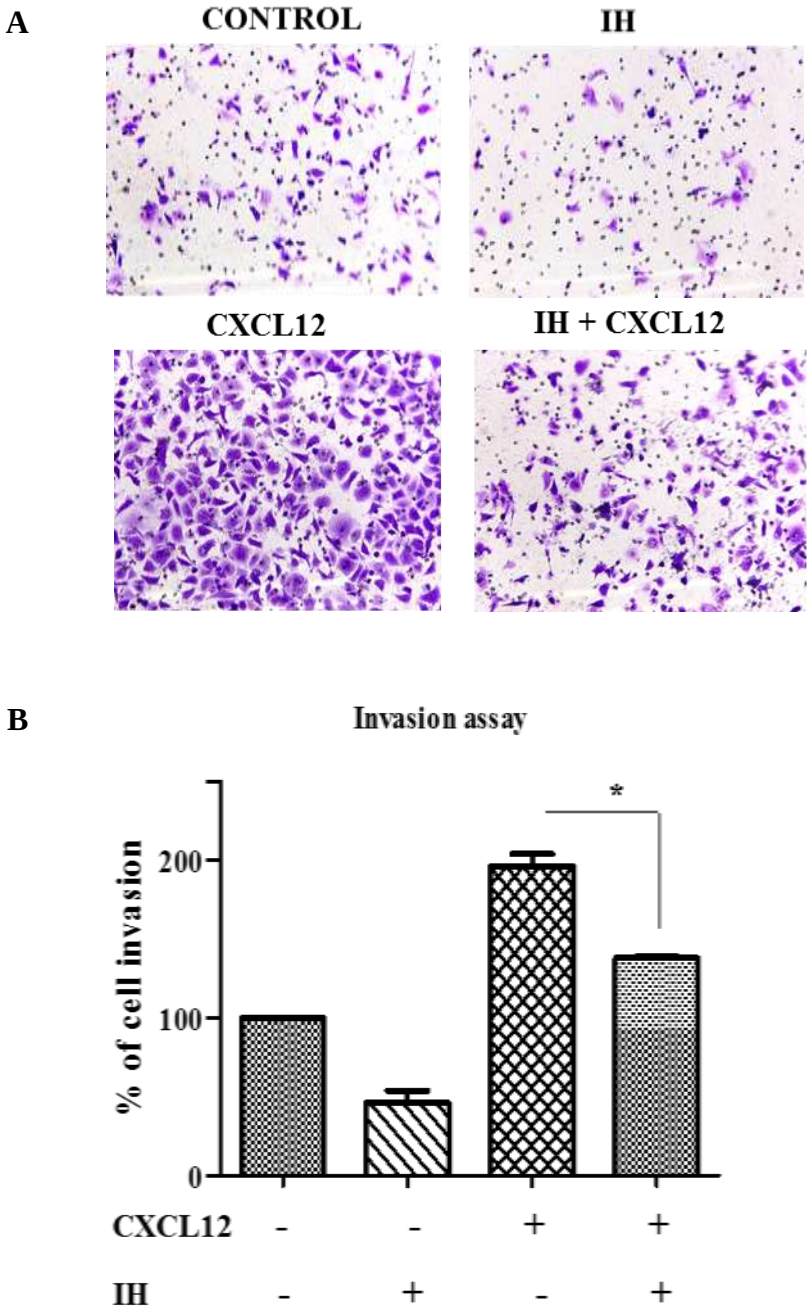


Fig 3.4.2: Isorhamnetin inhibits CXCL12 induced invasion in gastric cancer cells.

(A) A BD biocoat matrigel invasion chamber was used to study the invasion of gastric cancer cells. AGS gastric cancer cells were either treated with 25 µM isorhamnetin and/or CXCL12 (100 ng/ml) for 8 hours. They were then fixed with 4% paraformaldehyde before staining with 0.5% crystal violet and pictures were recorded. Numbers of cells invading were counted at the end of treatment. Representative images from each treatment group are shown. (B) The percentage of the gap difference between the treated and untreated groups was normalized and plotted in the form of bar charts. The values plotted are the means ± S.D. of two independent experiments. * indicates p value < 0.05 as compared to control.*p<0.05

3.4.3. Effect of isorhamnetin on migration and invasion is partially reversed in the presence of pharmacological PPARγ specific inhibitor

Previous studies have shown that ligand induced activation of PPARγ acts to inhibit the migration and invasion of cancer cells [190, 191]. A recent study also showed that both ectopic over-expression of PPARγ or its activation by an agonist, rosiglitazone, could suppress the migration and invasion of the hepatocellular carcinoma cells *in vitro* and also inhibit the distant metastases from liver in an orthotopic hepatocellular carcinoma model *in vivo* [192]. To study if the inhibitory activity of isorhamnetin on migration of gastric cancer cells was via the PPARγ pathway, we employed a pharmacological blocker of the PPARγ, called GW9662 in our experiments, as mentioned previously. It is an irreversible, synthetic antagonist that can be utilized to distinguish PPARγ-dependent and -independent effects of its ligands [193]. The effect of isorhamnetin on the migratory potential of gastric cancer cells was investigated using the wound healing assay. A 'wound' was created on a confluent mono-layer of cells, and the ability of cells to migrate was observed by measuring the gap difference before and after treatment. Cells were initially treated with GW9662 for 2 hours followed by exposure to 25 μM of isorhamnetin. The cells were then allowed to migrate for 8 hours. Our results as seen in Figs. 3.4.3(A) and 3.4.3(B) indicate that isorhamnetin significantly suppressed the migration of gastric cancer cells, and pretreatment with GW9662 reversed the anti-migratory effects of isorhamnetin.

Similarly, the effect of isorhamnetin on the invasive capacity of gastric cancer cells was analyzed using a BD biocoat tumor invasion system. Gastric cancer cells were pre-treated with GW9662 for 2 hours before treatment with isorhamnetin for 8 hours. Our results show that isorhamnetin could inhibit the invasive capacity of gastric cancer cells and this effect was reversed in the presence of GW9662 as shown in Figs 3.4.3(C) and 3.4.3(D). Overall, the above experiments show that the anti-migratory and anti-invasive properties of isorhamnetin is primarily mediated through the PPARγ dependent pathway since the pretreatment with a specific PPARγ antagonist, GW9662, could partially reverse these observed effects of isorhamnetin.

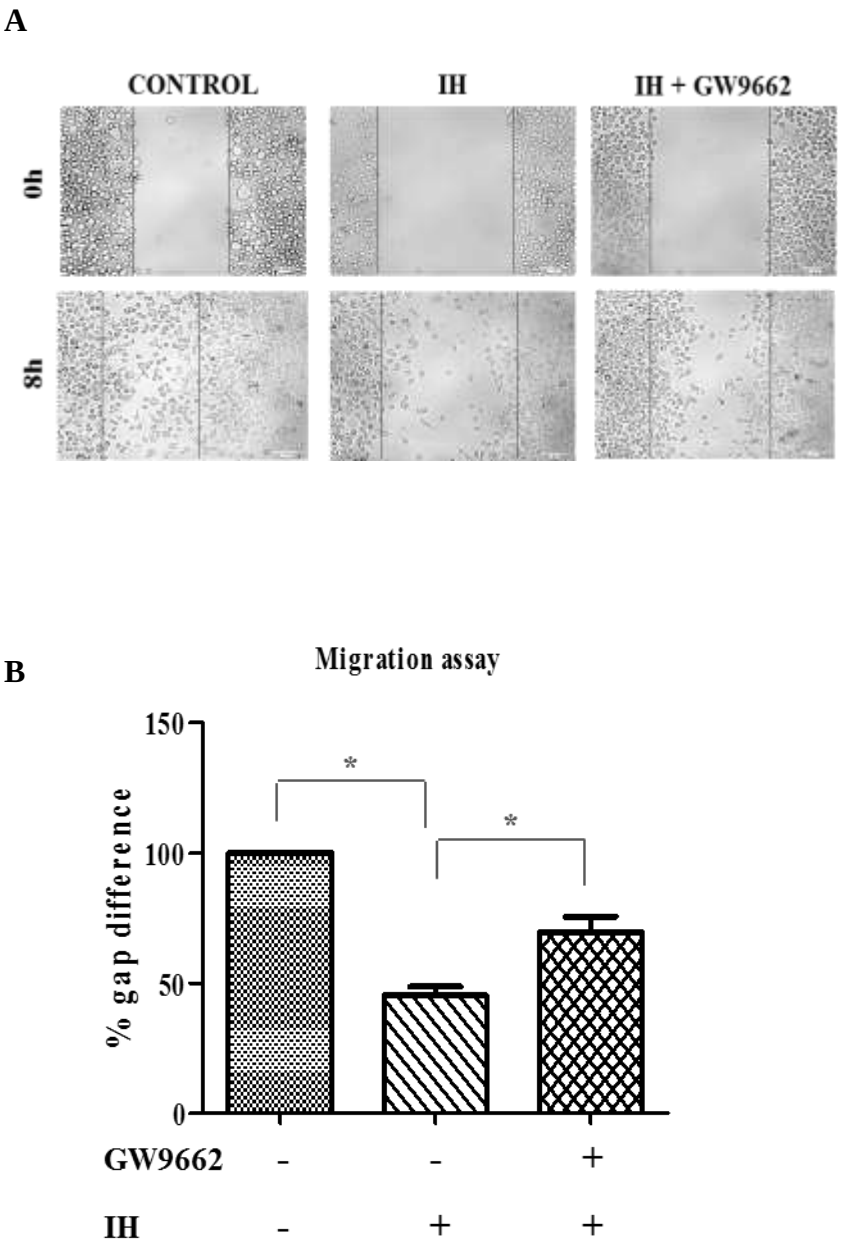
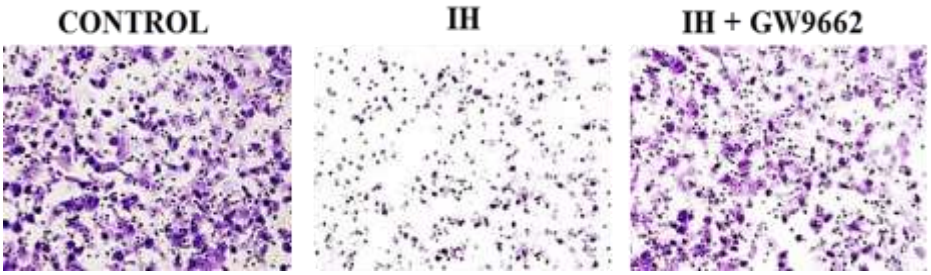


Fig 3.4.3: GW9662 reverses anti-migratory property of isorhamnetin in gastric cancer cells.

(A) A wound was created with a pipette tip on 90% confluent AGS cells. Microscopic observation of the migration of the cells after pretreatment with GW9662 (20 µM for 2 hours), followed by incubation with isorhamnetin (25 µM) for 8 hours was recorded. (B) The percentage of the gap difference between the treated and untreated groups of 3 replicates was normalized and plotted in the form of bar charts. The values plotted are the means ± S.D. of two independent experiments. * indicates p value < 0.05 as compared to control.

C



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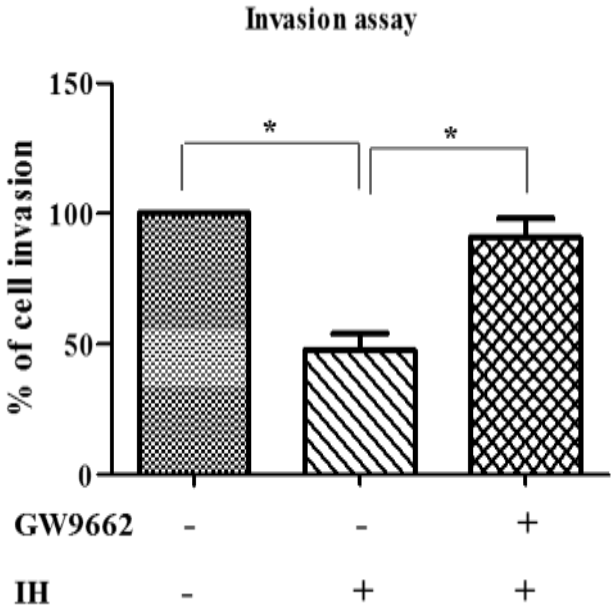


Fig 3.4.3: GW9662 reverses anti- invasive property of isorhamnetin in gastric cancer cells.

(C) The cell invasion assay was performed for evaluating the inhibitory effect of isorhamnetin on gastric cancer cell invasion. AGS cells were pretreated with GW9662 (20 µM for 2 hours) followed by incubation with isorhamnetin for 8 hours. They were then fixed with 4% paraformaldehyde after which they were stained with 0.5% crystal violet and pictures were recorded. (D) The percentage of the invaded cells of the treated group was normalized against the untreated group. The values are the means ± S.E. of two independent experiments. * indicates p value < 0.05 as compared to control.

3.4.4. Transforming growth factor-β (TGF-β) treatment induces epithelial to mesenchymal transition in gastric epithelial and cancer cells

Transforming growth factor-β signaling functions in several biological processes, including cell proliferation, differentiation and migration [194]. There is increasing evidence that TGF-β signaling promotes invasion and metastasis by induction of epithelial to mesenchymal transition (EMT) [143]. EMT is also characterized by breakdown of cell junctions and loss of epithelial phenotypes which leads to depolarization of cells, thus contributing to cancer progress [195]. Our initial aim was to test if TGF-β could induce EMT in two gastric cell lines; non-neoplastic gastric epithelial cell line HFE145 and gastric-epithelial-like cancer cell line, YCC1. Cells were treated with TGFβ (10 ng/ml) for 72 hours and microscopic images were captured after treating with crystal violet stain. As shown in Figs. 3.4.4(A) and 3.4.4(C), TGF-β could induce phenotypic changes consistent with EMT in gastric cancer cells, including abnormal epithelial cell morphology, fibroblast-like properties, and reduced intercellular adhesion [196].

The cells after treatment with TGF-β were also analyzed by western blot assay for various EMT marker proteins. Several EMT markers have been characterized in detail, including E-cadherin, Vimentin and gamma catenin. Reduced levels of E-cadherin have been extensively demonstrated in many cancers, in fact, E-cadherin-inactivating germline mutations have been reported to be a primary cause of hereditary diffuse gastric cancer (HDGC) [197]. Vimentin is considered a marker of mesenchymal differentiation and has been shown to induce invasive behavior in many epithelial carcinoma cell lines [198]. γ-catenin also known as plakoglobin, has been found to be

important in the normal epithelial tissue architecture. Reduced expression of γ-catenin in human cancers has been closely associated with increased tumor progression and adverse clinical outcome [199]. Thus, considering the importance of the above indicated proteins in the EMT process, we analyzed if TGF-β could alter the expression of these proteins in both gastric epithelial and gastric cancer cells. As evident in the Figs. 3.4.4(B) and 3.4.4(D), TGF-β treatment resulted in a decreased expression of the epithelial markers E-cadherin and γ-catenin and increased expression of the mesenchymal marker vimentin in both the cell lines. Considering that our above results indicated that TGF-β could induce EMT in the gastric epithelial and cancer cells, we next analyzed if this phenomena could be reversed upon treatment with isorhamnetin.

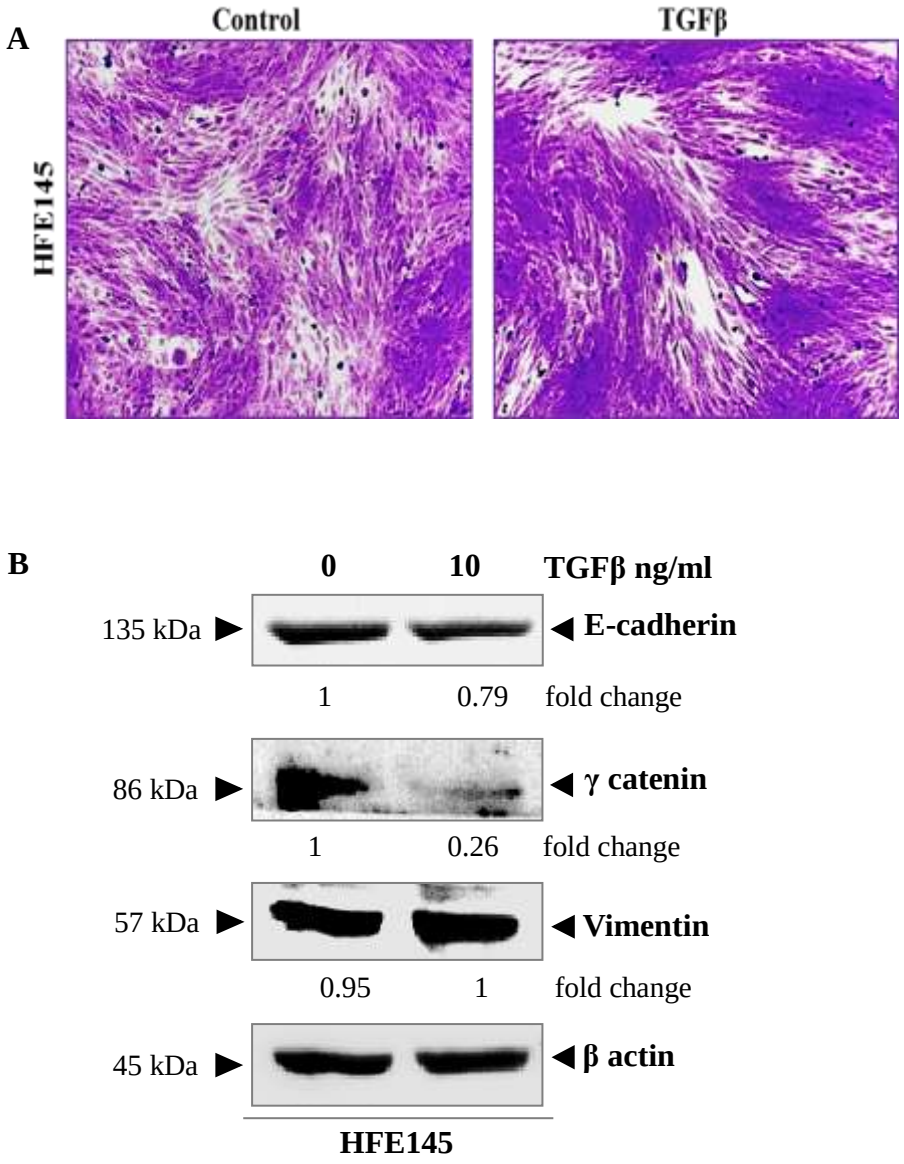


Fig 3.4.4: TGF-β treatment induces epithelial to mesenchymal transition in HFE145 gastric epithelial cells.

(A) HFE145 cells were treated with 10 ng/ml of TGF-β for 72 hours. At the end of treatment, cells were stained with 0.5% crystal violet, washed with PBS and observed for morphological differences under the microscope, and photographs were recorded. (B) HFE145 cells were treated with 10 ng/ml of TGF-β for 72 hours. Whole cell extract was prepared, separated on SDS-PAGE and subjected to western blot analysis to detect protein expression. β-actin was used as a loading control. Data representative of at least two independent experiments have been shown. Densitometry analysis was performed to determine differences in fold change in protein bands between treated and control groups.

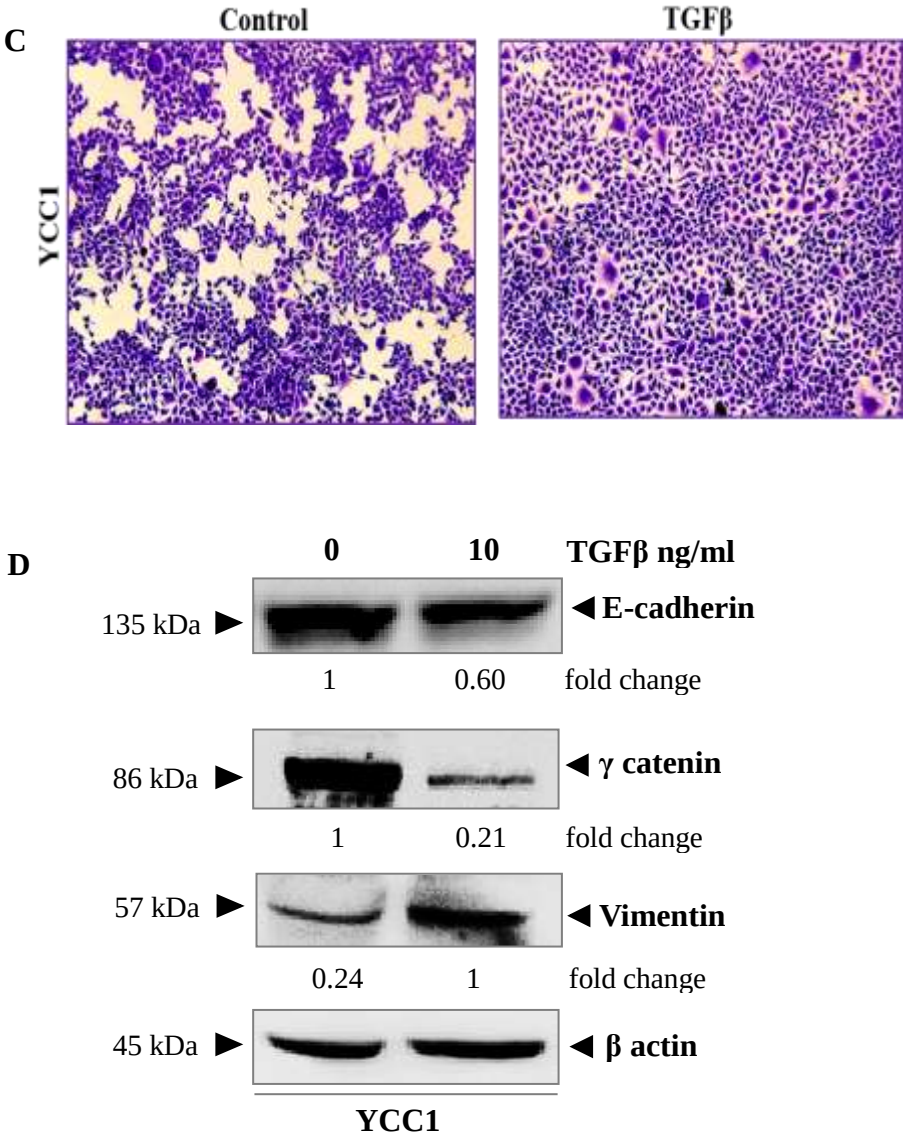


Fig 3.4.4: TGF-β treatment induces epithelial to mesenchymal transition in YCC1 gastric cancer cells.

(C) YCC1 cells were treated with 10 ng/ml of TGF-β for 72 hours. At the end of treatment, cells were stained with 0.5% crystal violet, washed with PBS and observed for morphological differences under the microscope, and photographs were recorded. (D) YCC1 cells were treated with 10ng/ml of TGF-β for 72 hours. Whole cell extract was prepared, separated on SDS-PAGE and subjected to western blot analysis to detect protein expression. β-actin was used as a loading control. Data representative of at least two independent experiments have been shown. Densitometry analysis was performed to determine differences in fold change in protein bands between treated and control groups.

3.4.5. Isorhamnetin reverses transforming growth factor-β (TGF-β) treatment induced epithelial to mesenchymal transition in gastric epithelial and cancer cells

Cells that undergo EMT will switch from a polarized epithelial phenotype to a spindle-shaped, fibroblast-like mesenchymal phenotype [200]. To analyze if isorhamnetin treatment could reverse TGF-β induced EMT in gastric epithelial and cancer cells, we pre-treated the cells with isorhamnetin before exposing them to TGF-β (10 ng/ml) for 72 hours. Images of the cells captured under the microscope showed that isorhamnetin could substantially reverse the spindle-shaped mesenchymal phenotype of cells to a polarized epithelial type.

During EMT, tumor cells lose expression of proteins, such as E-cadherin, that promote cell-to-cell contact and acquire mesenchymal markers such as vimentin, and N-cadherin, which can drive cancer progression, invasion, and metastasis [201]. SNAIL is a transcription factor that represses epithelial genes by binding to E-box DNA sequences through their carboxy-terminal zinc-finger domains, well-illustrated by its activity on the E-cadherin promoter [202]. To analyze if isorhamnetin treatment could reverse TGF-β induced EMT in gastric epithelial and cancer cells, we pre-treated the cells with isorhamnetin before exposing them to TGF-β (10 ng/ml) for 72 hours. The results indicated that increasing doses of isorhamnetin could reverse the TGF-β induced epithelial–mesenchymal transition. Treatment with TGFβ (as seen in the second lanes of Figs. 3.4.5(A) and 3.4.5(C)) induces a mesenchymal phenotype, as characterized by reduced expression of E-cadherin (epithelial marker) and increase in the expression of mesenchymal markers such as N-cadherin, Vimentin and SNAIL.

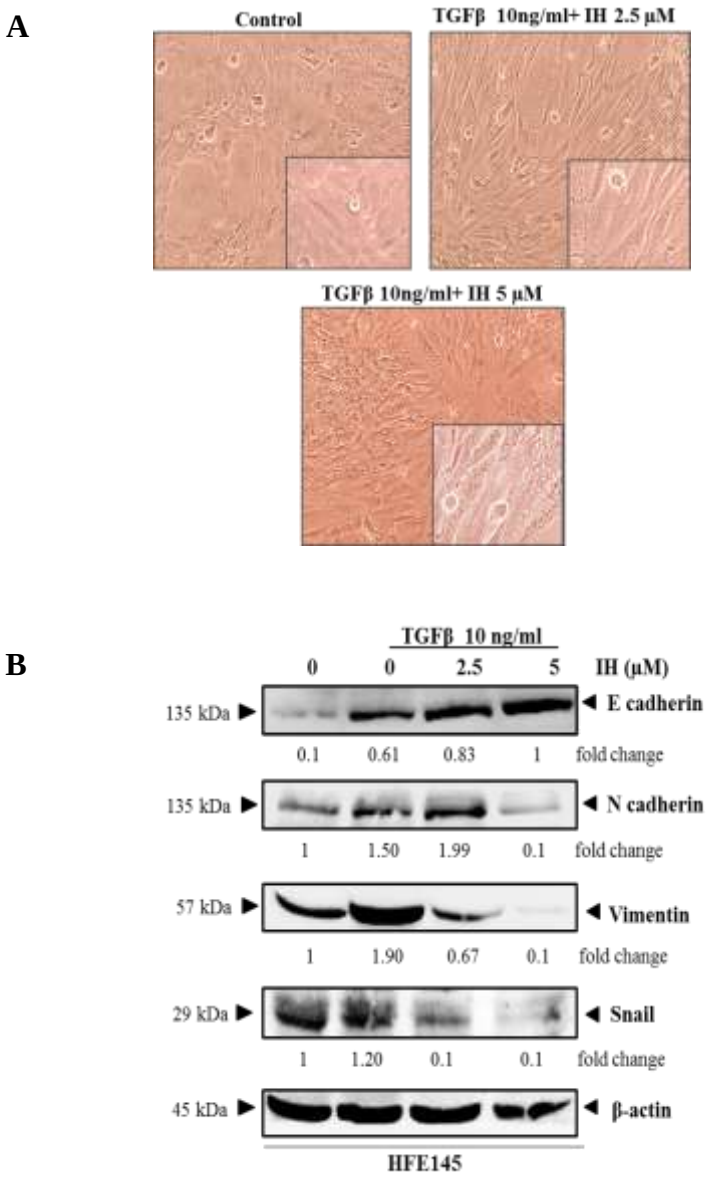


Fig 3.4.5: Isorhamnetin reverses TGF-β treatment-induced epithelial to mesenchymal transition in HFE145 gastric epithelial cells.

(A) HFE145 cells were initially treated with 10 ng/ml of TGF-β before treatment with increasing doses of isorhamnetin for 72 hours. At the end of treatment, cells were exposed to 0.5% crystal violet, rinsed with PBS and observed for morphological differences under the microscope, and photographs were recorded. (B) HFE145 cells were pre-treated with 10ng/ml of TGF-β before treatment with increasing doses of isorhamnetin for 72 hours. Whole cell extract was prepared, separated on SDS-PAGE and subjected to western blot analysis to detect protein expression. β-actin was used as a loading control. Data representative of at least two independent experiments have been shown. Densitometry analysis was performed to determine differences in fold change in protein bands between treated and control groups.

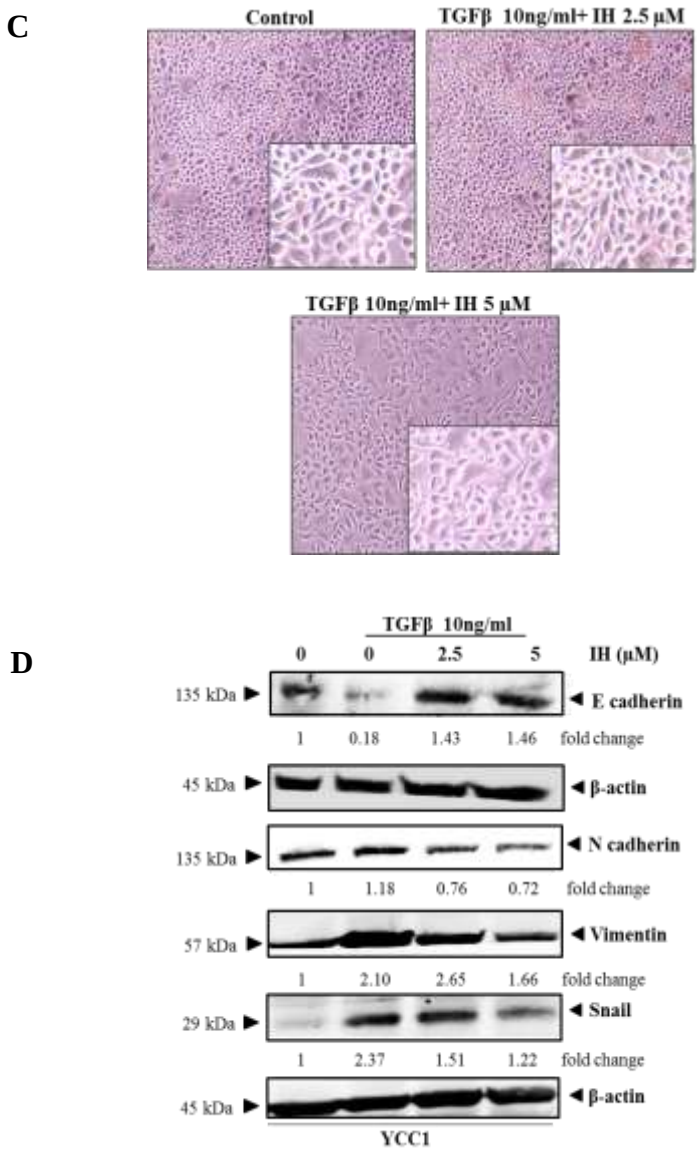


Fig 3.4.5: Isorhamnetin reverses TGF-β treatment-induced epithelial to mesenchymal transition in YCC1 gastric cancer cells.

(C) YCC1 cancer cells were initially treated with 10 ng/ml of TGF-β before treatment with increasing doses of isorhamnetin for 72 hours. At the end of treatment, cells were were exposed to 0.5% crystal violet, rinsed with PBS and observed for morphological differences under the microscope, and photographs were recorded. (D) YCC1 cells were pre-treated with 10 ng/ml of TGF-β before treatment with increasing doses of isorhamnetin for 72 hours. Whole cell extract was prepared, separated on SDS-PAGE and subjected to western blot analysis to detect protein expression. β-actin was used as a loading control. Data representative of at least two independent experiments have been shown. Densitometry analysis was performed to determine differences in fold change in protein bands between treated and control groups.

3.4.6. Isorhamnetin induces relocalization of E-cadherin to the region of cell-cell adherent junction

E-cadherin (Ecad), an important component of adherent junction in the epithelia acts as the master regulator of cell–cell adhesion and is known to function as a tumor suppressor in a majority of cancers due to its role in inhibiting cellular invasion [203]. The central role of Ecad in intercellular adhesion has been well studied and it has been shown that the loss of the protein can enhance gastric cancer progression in both mice and humans [204]. Since expression of Ecad is important in preventing tumor progression, we used an immunofluorescence staining method to analyze its expression in MKN28 gastric cancer cells. Using immunofluorescence, E-cadherin was shown to be expressed at a low level in MKN28 cells and to be localized mainly in the cytoplasm without significant membrane staining. Treatment with isorhamnetin for 24 hours showed a marked increase in E-cadherin. Interestingly, increased membranous staining was noted in the gastric cancer cells following treatment, suggesting relocalization of E-cadherin to the region of cell–cell adherent junctions, in addition to a substantial increase in its protein expression (shown next in Fig. 3.4.7(B)).

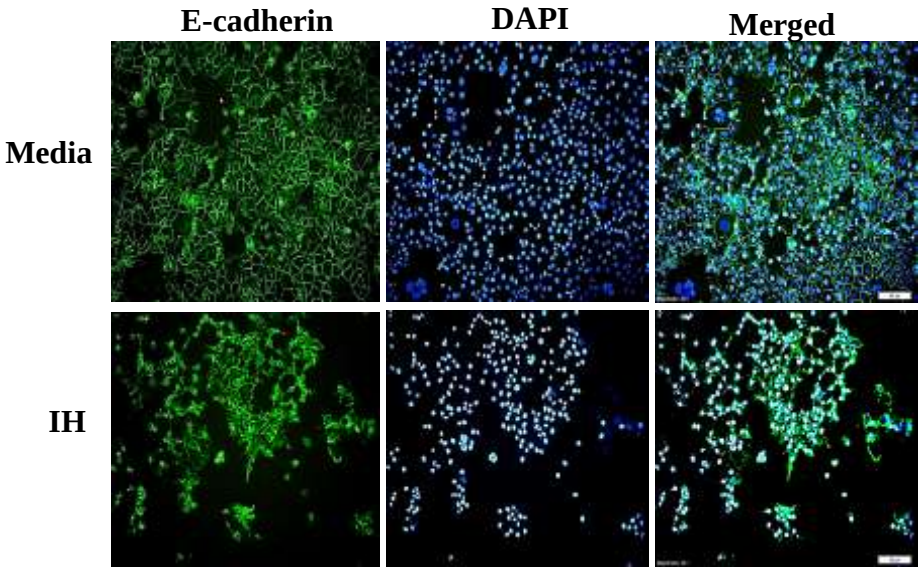


Fig 3.4.6: Isorhamnetin up-regulates E-cadherin expression in gastric cancer cells.

MKN28 cells were plated on a chamber slide and treated with isorhamnetin for 24 hours. The cells were then subjected to immunofluorescence analysis. They were incubated with mouse monoclonal E-cadherin antibody (1:100) followed by incubation with goat anti-mouse IgG-Alexa 594 (1:100) and counterstained for nuclei with Hoechst (50 ng/ml) for 5 min. Stained cells were mounted and analyzed under a fluorescence microscope. Green staining shows E-cadherin levels and blue staining shows the nucleus. The images were later merged using Olympus cell Sens Standard software. Representative image for each treatment group has been shown (n=2).

3.4.7. Isorhamnetin reverses epithelial to mesenchymal transition in mesenchymal gastric cancer cells

Flavonoids have been extensively studied for their role in reversing EMT in various cancers. For example, naringenin, a natural predominant flavanone, significantly inhibited the transcription of TGF-β1-induced Smad3, and reduced the binding probability of TGF-β1 to its specific receptor TβRII, thus suppressing the subsequent downstream signal transduction events [205]. Another study reported that some other flavonoids namely, rhamnetin and cirsiliol also showed increased expression of E-cadherin and decreased expression of vimentin and fibronectin, which consequently alleviated radiation-induced EMT both in *in vitro* and *in vivo* models [206]. Therefore, our next aim was to determine if isorhamnetin could reverse EMT in two different mesenchymal gastric cancer cells that were invasive in nature. Since EMT involves acquisition of mesenchymal markers such as vimentin by epithelial carcinoma cells and loss of epithelial cell adhesion molecules such as E-cadherin [207], we proceeded to investigate whether isorhamnetin could reverse EMT by analyzing the expression levels of these proteins. AGS and MKN28 gastric cancer cells were treated with increasing doses of isorhamnetin and subjected to western blot analysis to study the expression of various epithelial and mesenchymal markers. E-cadherin and N-cadherin protein levels were observed to increase and decrease respectively in both the cell lines. Expression levels of vimentin in MKN28 (Fig. 3.4.7(B)) cells were also found to decrease, thereby indicating that isorhamnetin could modulate the expression of both epithelial and mesenchymal markers in gastric cancer cells.

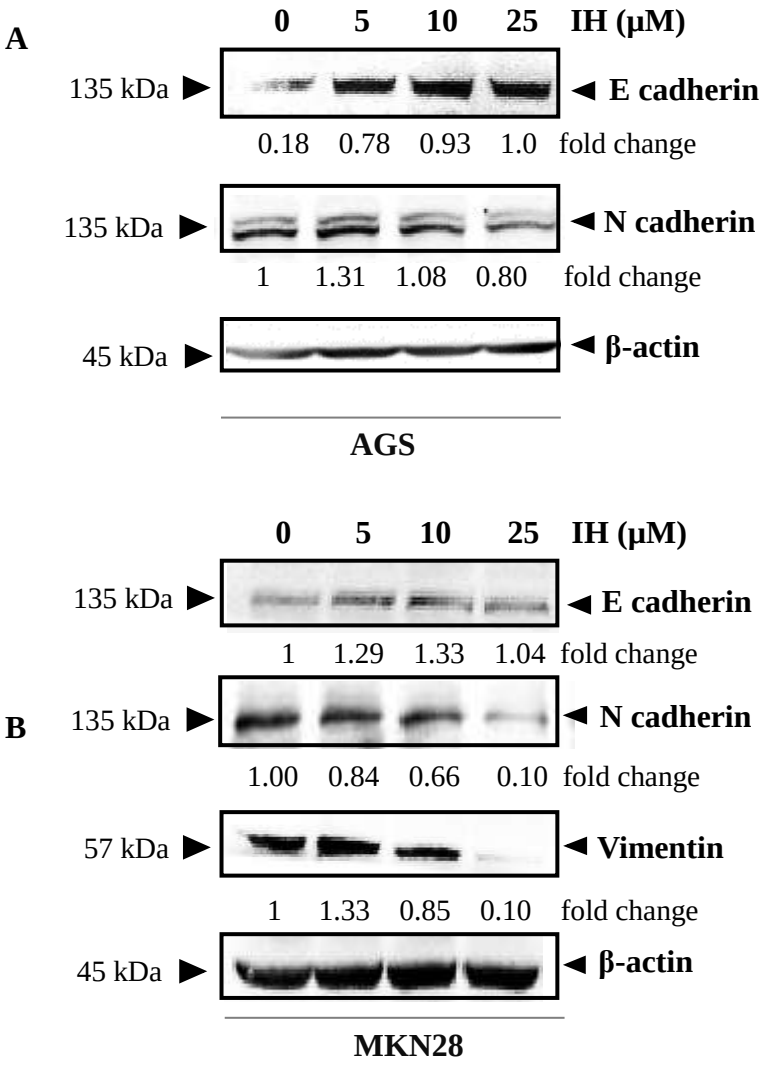


Fig 3.4.7: Isorhamnetin reverses EMT in AGS and MKN28 gastric cancer cells.

(A) AGS cells were treated with 0, 5, 10 and 25 μM of isorhamnetin for 24 hours. Whole cell extracts were prepared, and lysates were subjected to western blot analysis using antibodies against E cadherin and N cadherin. β-actin was used as a loading control. Data representative of two independent experiments are shown. (B) MKN28 cells were treated with 0, 5, 10 and 25 μM of isorhamnetin for 24 hours. Whole cell extracts were prepared, and lysates were subjected to western blot analysis using antibodies against E cadherin, N cadherin and Vimentin proteins. β-actin was used as a loading control. Data representative of two independent experiments are shown. Densitometry analysis was performed to determine differences in fold change in protein bands between treated and control groups.

3.4.8. Isorhamnetin inhibits expression of p-Smad3 in gastric cancer cells.

TGF-β signaling can be Smad-mediated or non-Smad-mediated depending on the kind of cellular mechanism(s) involved [208]. The Smads are a group of intracellular proteins that transmit TGF-β ligand signals to the nucleus [209]. Activated TGF-β receptor phosphorylates a sub-class of Smads called receptor-activated Smads (R-Smads) such as Smad3; phosphorylated Smad3 (p-Smad3) and then proceeds to bind to Smad-binding element (SBE) present in DNA sequences to regulate various TGF-β responsive genes [210]. Smad3 phosphorylation as well as its downstream signaling has been shown to mediate the invasive and proliferative properties of cancer cells that are required for its progression [211].

Considering that p-Smad3 has been associated with a more invasive phenotype, and suggested to be a potential new prognostic marker of gastric carcinoma [212], we hypothesized that pharmacological inhibition of Smad3 phosphorylation may help to repress gastric cancer progression. To analyze this aspect, gastric cancer AGS cells were treated with increasing doses of isorhamnetin for different time points and subjected to western blot analysis. As shown in Fig. 3.4.8(B), levels of p-Smad3 were found to decrease substantially both in a dose- and time- dependent manner upon isorhamnetin exposure thereby indicating that this flavonoid may block EMT through the inhibition of Smad3 mediated TGF-β signaling.

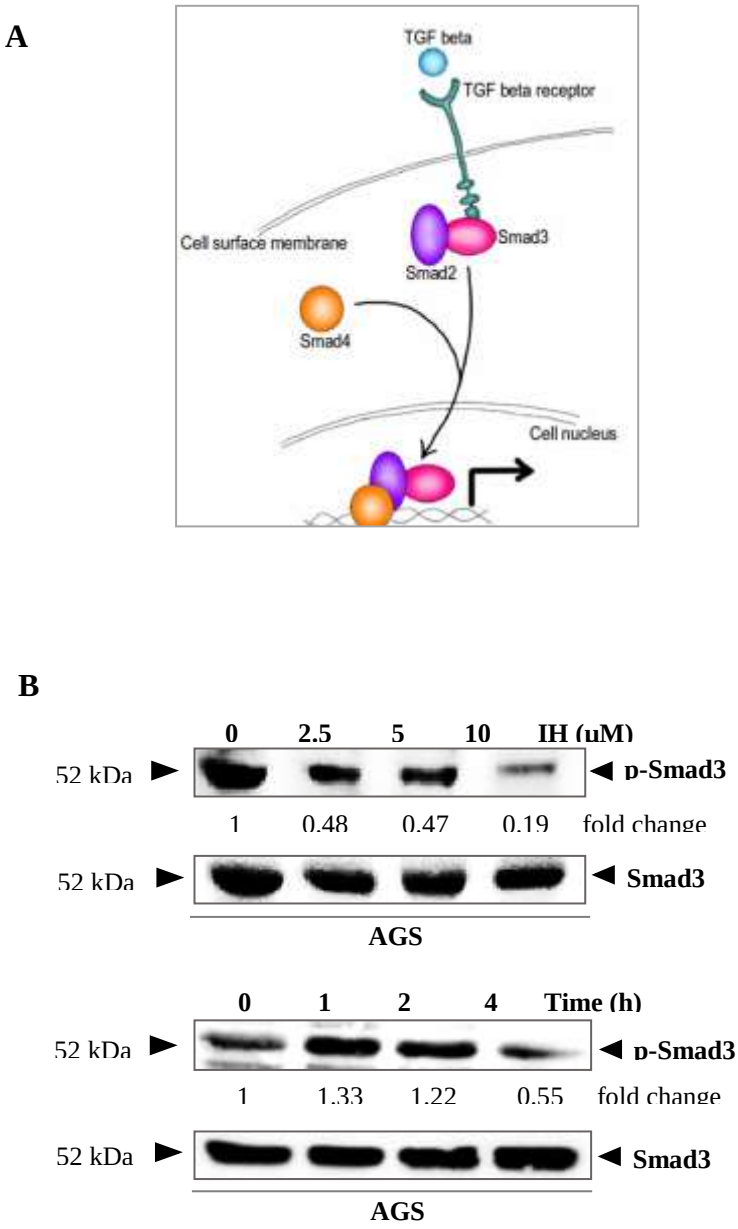


Fig 3.4.8: Isorhamnetin inhibits p-Smad3 expression in gastric cancer cells.

(A) Transforming growth factor-β (TGF-β) superfamily signaling plays a critical role in a wide range of biological systems. Signaling is initiated with ligand-induced oligomerization of serine/threonine receptor kinases and phosphorylation of the cytoplasmic signaling molecules Smad2 and Smad3 for the TGF-β/activin pathway. (B) AGS cells were treated with isorhamnetin in a dose- and time-dependent manner as indicated above. Whole cell extracts were obtained and subjected to western blot analysis using antibody against p-Smad3. The membrane was later stripped and re-probed with Smad3 as a loading control. Data representative of two independent experiments are shown. Densitometry analysis was performed to determine differences in fold change in protein bands between treated and control groups.

3.5. BMPR2 is a possible target of PPARγ

3.5.1. PPRE search database identifies BMPR2 as a target gene of PPARγ

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β family that bind to two types of serine-threonine kinase receptors, known as type I and type II receptors [213]. BMPs have been shown to favor angiogenesis by stimulating the secretion of pro-angiogenic growth factors, such as VEGF [214]. A recent study has also suggested that BMPR2 could serve as a potential therapeutic target for renal cell carcinoma [215]. Upon understanding the angiogenic role of BMPR2, we first proceeded to determine if it was a putative target of the transcription factor, PPARγ.

PPARγ has a highly conserved DNA binding domain that recognizes specific DNA sequences known as Peroxisome Proliferator Response Elements (PPREs) [216]. Upon ligand binding, PPARγ translocates from cytoplasm to nucleus and forms a heterodimer with Retinoic-X-Receptor (RXR). PPAR/RXR complex then binds to PPRE located in the promoter region of PPAR target genes [217]. The PPRE region is composed of a Direct Repeat (DR) spaced by one nucleotide, DR1 or spaced by two nucleotides, DR2 [218]. We used a PPRE search database [219], which uses an *in-silico* approach, and identified that BMPR2 contains a putative PPRE in its promoter region. This led us to hypothesize that BMPR2 possibly contained a possible PPARγ binding site.

Search Criteria:

Sequence mismatchs = 2 Binding efficiency = 1 Directed repeats = 1 Flanking Match = 1

PPRE Name	Binding efficiency	PPRE pattern	Pubmed id	Flanking Sequence & Flanking Match	Gene Sequence	Gene Position	Competitive binders
>gi|189339276|ref|NM_001204.6| Homo sapiens bone morphogenetic protein receptor, type II (serine/threonine kinase) (BMPR2), mRNA							
PPAR gamma(in vitro)	12	AGGTCACAGGTTA	17650321	CAAAGAT6	AGGTCCCAGTTTA	11453	ARF1
PPAR gamma(in vivo)	85	AGGTCCTAGTTCA	1502166	CAAAGAT6	AGGTCCCAGTTTA	11453	ARF1
PPAR alpha(in vivo)	66	AGGTCCTAGTTCA	1502166	CAAAGAT6	AGGTCCCAGTTTA	11453	ARF1

Fig 3.5.1: PPRE search database identifies BMPR2 as a possible target of PPARγ

FASTA sequence of 'Homo sapiens bone morphogenetic protein receptor, type II (serine/threonine kinase) (BMPR2), mRNA' with NCBI Reference Sequence: NM_001204.6 was used as an input sequence in the PPRESearch database. The PPRE region is composed of a Direct Repeat (DR) spaced by one nucleotide, DR1 or spaced by two nucleotides, DR2. The database contains the PPRE repeats from the literature, shown to be experimentally validated through *in vitro* or *in vivo* binding assays. The PPRE isoform specificity, binding efficiency, pubmed id and their experimental validation assay type (*in vitro* or *in vivo*) for each PPRE (as reported in the literatures) are displayed in the output. The output we obtained shows that the database identifies BMPR2 to contain a putative PPRE in its promoter region with a high binding efficiency of 85% to PPARγ. Thus it could be a direct target of the transcriptional factor, PPARγ.

3.5.2. Knockdown of BMPR2 inhibits the migration and overexpression of BMPR2 restores the migratory potential of gastric cancer cells.

Although the role of BMPs in bone formation has been well-studied, little is known about their influence on tumor cells. Recent studies indicate that BMPs are associated with increased migration and invasion of tumor cells. A recent report has shown that BMP-9 could induce EMT in hepatocellular carcinoma cells [220]. Yet another study suggested that inhibition of BMP-2 could suppress migration and invasion of lung cancer cells [221]. BMPR2 was also found to be highly expressed in two prostate cancer cell lines that had the ability to form osteoblastic lesions *in vivo* [158]. Clinical study data obtained from our collaborator's lab also confirmed that BMPR2 had an inverse correlation to EMT in gastric cancer patient samples. Hence, we first proceeded to investigate if knockdown of BMPR2 could inhibit the migration of gastric cancer cells. Gastric cancer AGS cells were transfected with siBMPR2 and western blot analysis was performed to confirm the knockdown of BMPR2 as evidenced by Fig. 3.5.2(B). Then a wound healing assay was performed, and the gap difference was compared between cells transfected with control siRNA and those transfected with siBMPR2. As shown in Fig. 3.5.2(A), cells with lower expression of BMPR2 migrated at a much slower rate than control cells.

Next we investigated if over-expression of BMPR2 protein could increase the migratory properties of gastric cancer cells. Gastric cancer AGS cells were transfected with BMPR2 and overexpression was confirmed using a western blot assay (Figs. 3.5.2(C) and 3.5.2(F)). A wound healing assay was performed and the comparison of the gap difference between control cells and

those with forced overexpression of BMPR2, as shown in Fig. 3.5.2(D), led us to conclude that cells with higher expression of BMPR2 could migrate faster than control cells.

Overall the results of the above experiments clearly indicate that BMPR2 has an important role in modulating the migration of gastric cancer cells. With our previous experiments suggesting that BMPR2 might be a possible putative target of PPARγ, we next proceeded to analyze if a specific PPARγ agonist, troglitazone could modify the expression of BMPR2 and other EMT regulatory genes in gastric cancer.

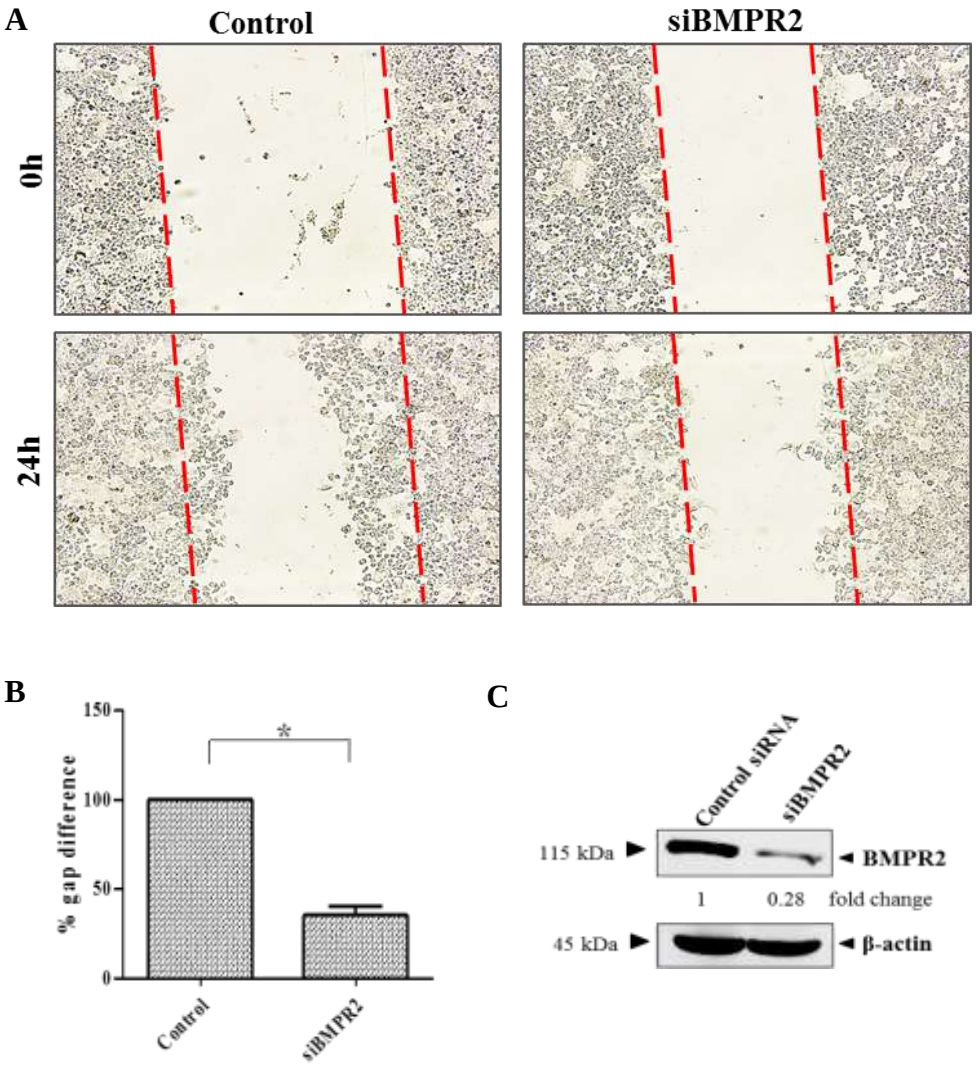


Fig 3.5.2: Knockdown of BMPR2 inhibits the migration of gastric cancer cells.

(A) AGS cells were seeded and transfected with either control siRNA or BMPR2 siRNA. 48 hours after transfection, a wound was created with a pipette tip and cells were allowed to migrate for 24 hours. Gap difference between control cells and BMPR2 knockdown cells was observed and images were recorded. (B) The percentage of the migratory cells of the treated group was normalized against the untreated group. The values are the means ± SD of three independent experiments. * indicates p value < 0.05 as compared to control. (C) Cells were later harvested, whole cell extract was obtained and probed for BMPR2 expression. β-actin was used as a loading control. Data representative of two independent experiments are shown. Densitometry analysis was performed to determine differences in fold change in protein bands between treated and control groups.

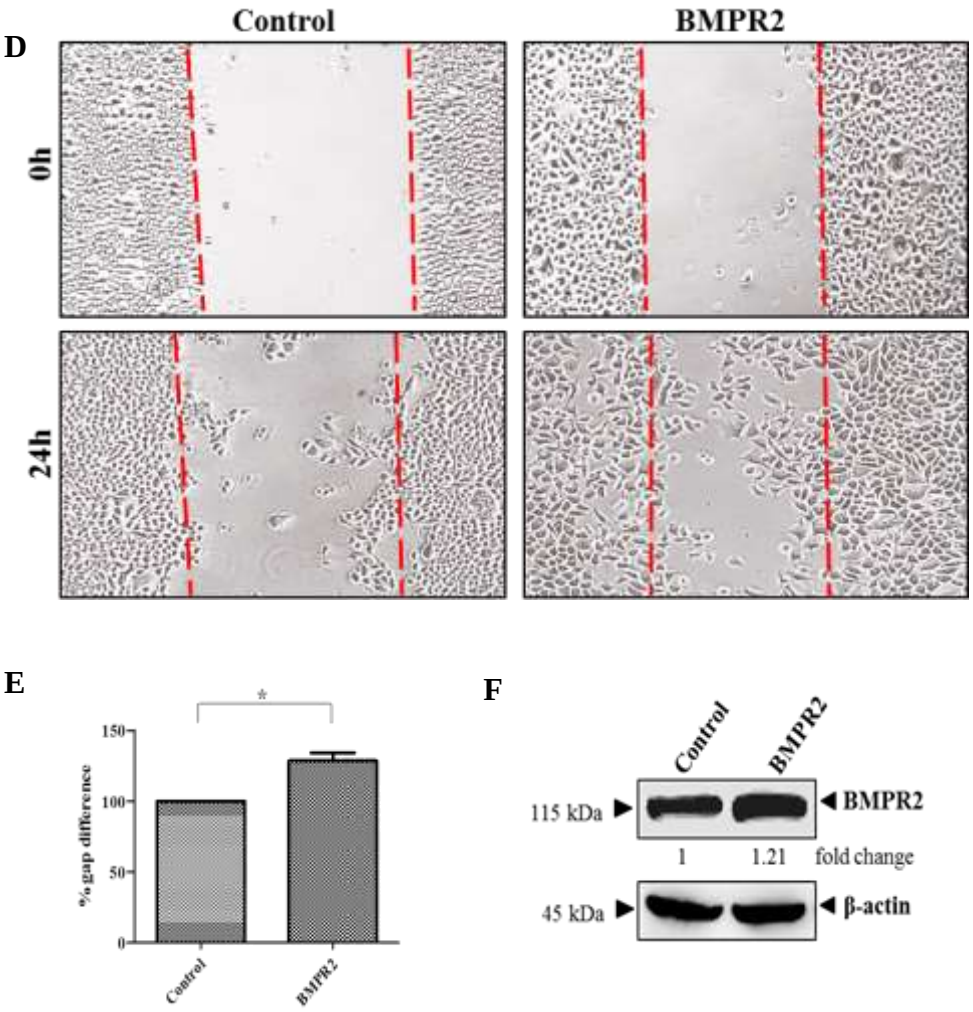


Fig 3.5.2: Overexpression of BMPR2 induces the enhanced migration of gastric cancer cells.

(D) AGS cells were seeded and BMPR2 was over-expressed. 48 hours after transfection, a wound was created with a pipette tip and cells were allowed to migrate for 24 hours. Gap difference between control cells and BMPR2 knockdown cells was observed and images were recorded. (E) The percentage of the migratory cells of the treated group was normalized against the untreated group. The values are the means ± S.E. of three independent experiments. * indicates p value < 0.05 as compared to control. (F) Cells were later harvested, whole cell extract was obtained and probed for BMPR2 expression. β-actin was used as a loading control. Data representative of two independent experiments are shown. Densitometry analysis was performed to determine differences in fold change in protein bands between treated and control groups.

3.5.3. Knockdown of BMPR2 reverses epithelial to mesenchymal transition of gastric cancer cells.

Receptor knockdown studies have been routinely used in research to identify and validate the function of a specific protein [222, 223]. To understand the function of BMPR2 in gastric cancer cells, we next logically proceeded to knockdown the expression of BMPR2 using small interfering RNA (siRNA) and analyze the difference in the levels of EMT genes from the non-transfected control cells. A recent study showed that siRNA-mediated knockdown of BMPR2 in Smad4 negative cells in colorectal cancer cells could lead to a decrease in their invasive ability [224]. Therefore we next elucidated the exact role of BMPR2 in EMT process of gastric cancer cells through a RNA interference methodology.

AGS cells were transfected with the most efficient concentration of siRNA and harvested for western blot analysis after 48 hours. The results as shown in Fig. 3.5.3, revealed that knockdown of BMPR2 could reverse EMT, as shown by the up-regulation in the expression of E-cadherin and down-regulation in the expression of N-cadherin thereby suggesting that BMPR2 has an important role to play in promoting the EMT of gastric cancer cells.

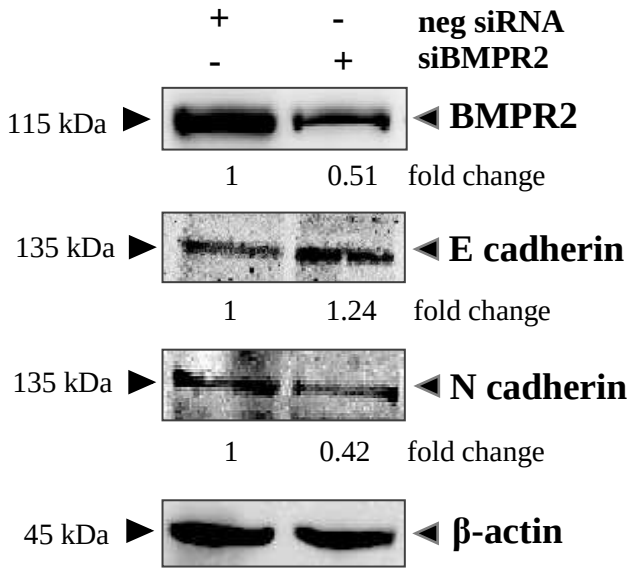


Fig 3.5.3: Knockdown of BMPR2 reverses epithelial to mesenchymal transition of gastric cancer cells.

AGS cells were seeded and transfected with either control siRNA or BMPR2 siRNA. 48 hours after transfection, cells were harvested and whole cell extract was obtained. Post western blot analysis, the expression of BMPR2, E cadherin and N cadherin was studied. β-actin was used as a loading control. Data representative of two independent experiments are shown. Densitometry analysis was performed to determine differences in fold change in protein bands between treated and control groups.

3.5.4. Ligand induced activation of PPARγ reverses EMT in gastric cancer cells

PPARγ agonists have been well studied for their ability to suppress proliferation, inhibit metastasis and induce apoptosis [225]. However, their role as inhibitors of epithelial to mesenchymal transition has not been explored extensively. A research group recently showed that curcumin, a polyphenolic natural compound, could counteract TGF-β1-induced EMT in renal tubular epithelial cells via ERK- and PPARγ- dependent pathway [226]. Yet another study reported that troglitazone, a synthetic PPARγ agonist, could significantly prevent TGFβ-2 induced epithelial-mesenchymal transition of retinal pigment epithelium cells [227]. Hence, we proceeded to analyze next whether troglitazone (structure shown in Fig. 3.5.4(A)) and isorhamnetin could modulate the expression of BMPR2 and other epithelial to mesenchymal transition markers in gastric cancer cells.

Gastric cancer AGS cells were treated with increasing doses of troglitazone and the expression level of BMPR2 and N-cadherin proteins was determined using western blot analysis. The results as shown in Fig. 3.5.4(B), showed that troglitazone could down-regulate the levels of BMPR2. It could also increase the expression of E-cadherin in a time-dependent manner. Isorhamnetin could also decrease BMPR2 levels in a dose-dependent manner. Overall, our experiments so far have indicated that ligand induced activation of PPARγ could inhibit EMT in gastric cancer cells. We next proceeded to analyze if this observed inhibitory effect was indeed primarily mediated through a PPARγ dependent pathway.

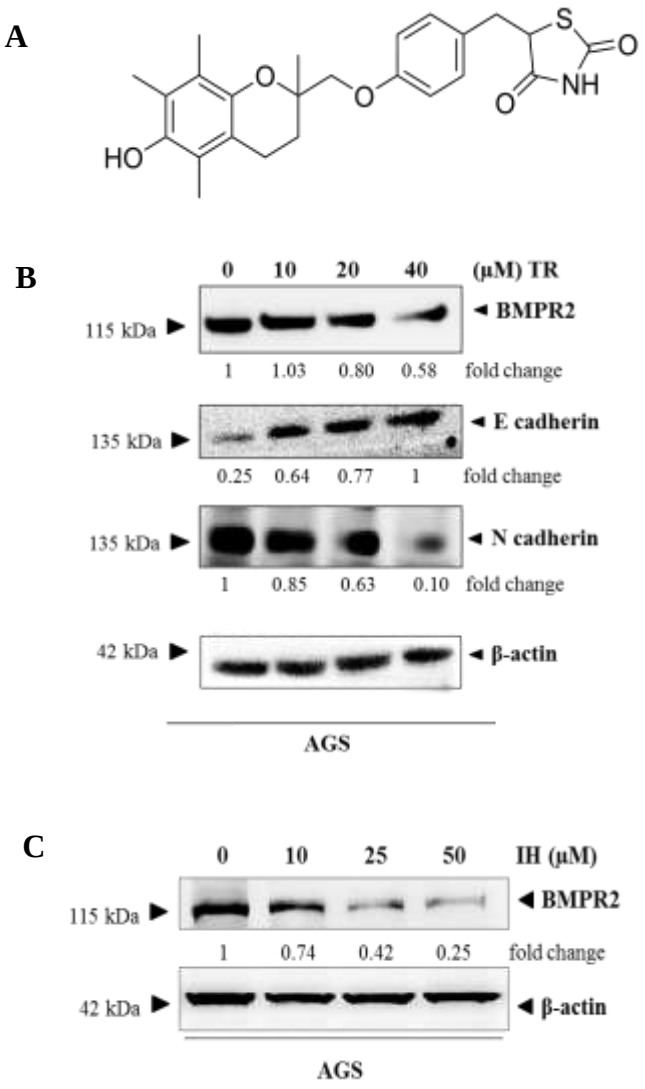


Fig 3.5.4: Ligand induced activation of PPARγ reverses EMT in gastric cancer cells.

(A) Chemical structure of troglitazone. (B) AGS cells were seeded and treated with increasing doses of troglitazone (10, 20 and 40 μM). Cells were then harvested, whole cell lysate was prepared and western blot analysis was done to detect the expression of BMPR2, E cadherin and N cadherin. β-actin was used as a loading control. Data representative of two independent experiments are shown. (C) AGS cells were treated with 0, 10, 25 and 50 μM of isorhamnetin for 24 hours. Cells were then harvested, whole cell lysate was prepared and western blot analysis was done to detect the expression of BMPR2 protein. Experiment was performed twice, and representative image is shown. Densitometry analysis was performed to determine differences in fold change in protein bands between treated and control groups.

3.5.5. Inhibition of EMT by isorhamnetin is partially reversed by a dominant negative form of PPARγ

As explained earlier, PPARγ dominant negative mutant is a DNA-binding defective form of PPARγ that suppresses its ability to bind to PPRE without affecting its ligand binding capacity. These mutants have been regularly used by researchers to understand if a specific mechanism is directly mediated through a PPARγ dependent pathway [228, 229]. Therefore, we next proceeded to investigate if PPARγ dominant negative mutant could inhibit the ability of isorhamnetin to modulate BMPR2 and markers of EMT in gastric cancer cells.

Gastric cancer AGS cells were treated with isorhamnetin for 24 hours after transfection with empty pCMX vector or pCMX DN mutant followed by western blot analysis to study the expression of EMT proteins. As shown in Fig.3.5.5, treatment of the cells with isorhamnetin could down-regulate the expression of BMPR2 protein. This was reverted back to its normal levels when a mutant was used. Isorhamnetin substantially increased the levels of E cadherin but this effect was less evident in the presence of a dominant negative mutant. Isorhamnetin also decreased the expression of N cadherin, but this effect seems to be consistent even in the presence of a mutant receptor. In other words, abolishing PPARγ activity did not affect the expression of N cadherin. Overall, using a PPARγ dominant negative mutant, we demonstrate that inhibition of EMT by isorhamnetin is at least partially mediated through PPARγ dependent pathway.

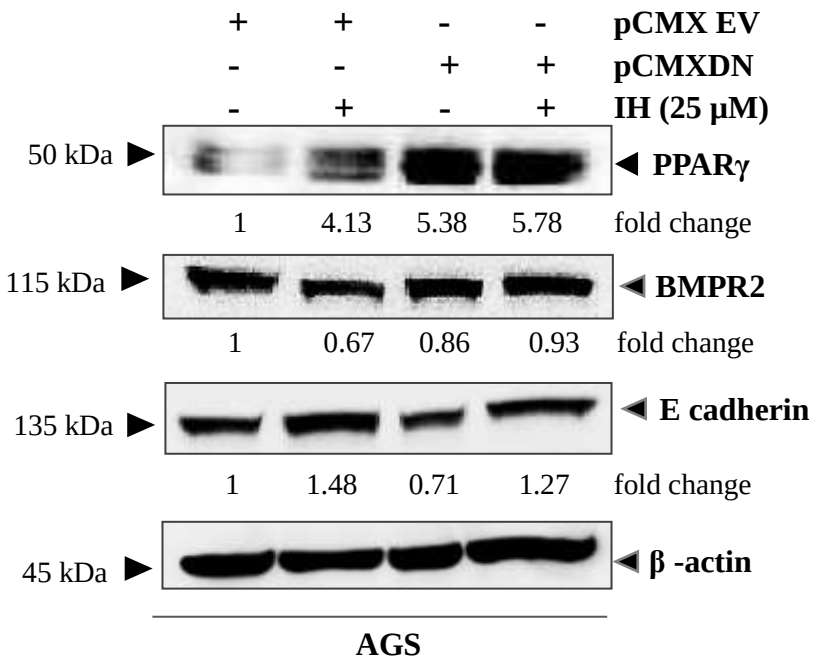


Fig 3.5.5: Inhibition of EMT by isorhamnetin is partially reversed by a dominant negative form of PPARγ.

AGS cells were seeded and transfected with either empty pCMX vector or pCMX dominant negative mutant. 48 hours post transfection, the cells were harvested, whole cell lysate was prepared and western blot analysis was done to detect the expression of BMPR2 and E cadherin. β-actin was used as a loading control. Data representative of two independent experiments are shown. Densitometry analysis was performed to determine differences in fold change in protein bands between treated and control groups.

3.5.6. Isorhamnetin does not modulate the expression of BMP4, the ligand of BMPR2

BMPs are secreted growth factors, belonging to the TGF-β superfamily, that exert their effects by binding to the BMP receptors. BMP4 and BMP7 have been found to be strongly expressed in primary and metastatic melanomas and contribute to their enhanced migration and invasion during tumor development [230]. A few studies have also suggested a possible link between PPARγ activation and down-regulation of BMP4 protein. For example, a report showed that increase in PPARγ signaling altered the expression of *Shh*, *FGF*, *Wnt*, and *BMP4* genes that are important for epithelial–mesenchymal crosstalk during the early lung development process [231].

To elucidate whether the observed inhibitory effects of isorhamnetin on EMT were specifically mediated via BMPR2, we next analyzed if treatment of gastric cancer cells with isorhamnetin could modulate the expression of BMP4, ligand of BMPR2. Two mesenchymal gastric cancer cells, AGS and AZ521 were treated with increasing concentrations of isorhamnetin and the expression of BMP4 protein was studied by western blot analysis. Our results, as shown in Fig.3.5.6, indicate that isorhamnetin could not substantially affect the expression of BMP4 protein in gastric cancer cells thereby confirming its specificity towards BMPR2.

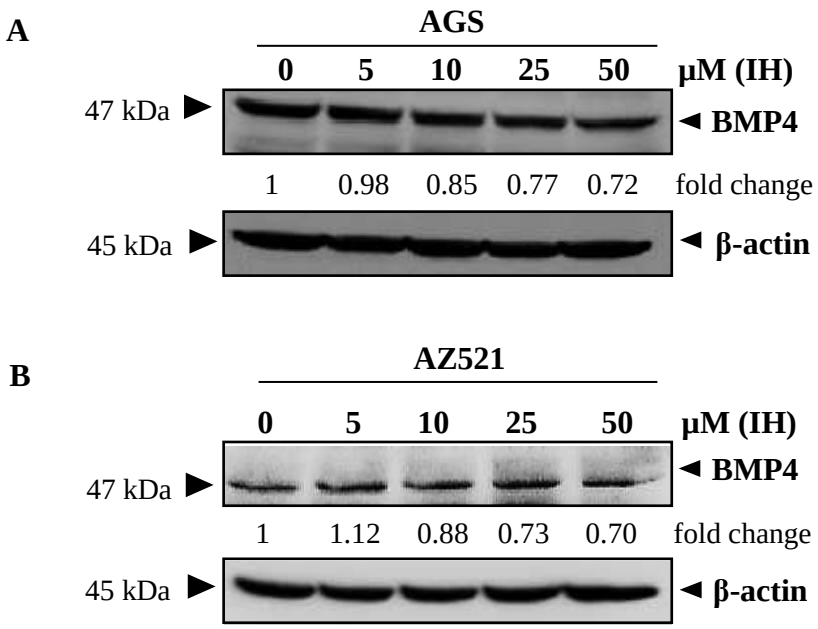


Fig 3.5.6: Isorhamnetin does not affect the expression of BMP4 in gastric cancer cells.

(A) AGS cells were treated with 0, 5, 10, 25 and 50 μM of isorhamnetin for 24 hours. Whole cell extracts were prepared, and lysates were subjected to western blot analysis using antibodies against BMP4. β-actin was used as a loading control. Data representative of two independent experiments are shown. (B) AZ521 cells were treated with 5, 10, 25 and 50 μM of isorhamnetin for 24 hours. Whole cell extracts were prepared, and lysates were subjected to western blot analysis using antibodies against BMP4. β-actin was used as a loading control. Data representative of two independent experiments are shown. Densitometry analysis was performed to determine differences in fold change in protein bands between treated and control groups.

3.6. Identification of isorhamnetin as a novel chemo-sensitizing agent in gastric cancer

3.6.1. Isorhamnetin enhances the cytotoxic effects of chemotherapeutic agents against gastric cancer cell lines

The use of a combination regimen of chemo-therapeutic drugs has been found to be superior in comparison to the effect of these agents used alone in the treatment of cancer. Research suggests that combined chemo-therapy improves local tumor control, reduces the rate of recurrence in patients without systemic disease, and eliminates residual primary tumor cells as a source of potential subsequent metastases [232]. Recent studies show that natural compounds could also work effectively in combination with existing anticancer therapies to combat cancer [233]. Our group has published a study in which we found that gamma-tocotrienol, a novel Vitamin E analogue, could chemo-sensitize gastric cancer cells to capecitabine in a xenograft mouse model [234]. Hence, our first aim was to analyze if isorhamnetin could act in combination with chemo-therapeutic drugs to inhibit gastric cancer cell viability. Therefore, we examined using the MTT assay whether isorhamnetin at a suboptimal concentration could enhance the cytotoxic effects of chemotherapeutic agents commonly employed for gastric cancer treatment. Growth inhibition rate was obtained and calculated as the percentage of dead cells versus control. Following a 24 hour treatment, it was found that isorhamnetin could significantly enhance the cytotoxicity of two chemotherapeutic agents; namely, capecitabine, and 5-fluorouracil (Fig.3.6.1).

Our results showed that 10 µM of isorhamnetin could indeed significantly enhance the cytotoxic effects of 5-fluorouracil and capecitabine in gastric cancer cells, while no significant effect was observed in combination with cisplatin.

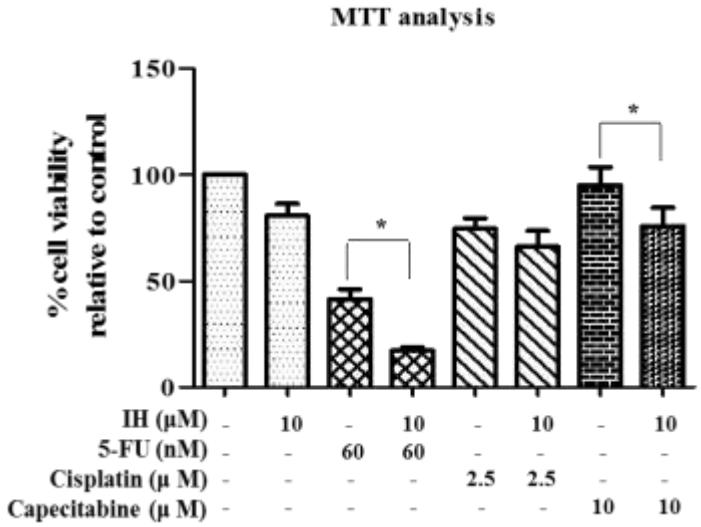


Fig 3.6.1: Isorhamnetin inhibits growth of gastric cancer cells in combination with chemotherapeutic agents.

AGS gastric cancer cells ($5x10^3$) were seeded in a 96 well plate and treated either alone with 10 µM of isorhamnetin, 60 nM of 5-fluorouracil, 2.5 µM of cisplatin and 10 µM of capecitabine or in a combination of each of the agents with 10 µM of isorhamnetin to investigate if it could enhance their cytotoxic effects. At the end of treatment, 20 µL of MTT solution was added and cells were incubated in the dark at 37°C for 4 hours. Cell viability was measured at 570nm at the end of treatment using a Tecan plate reader (Durham, NC, USA).Values are means ± SD of three independent experiments. * indicates p value < 0.05 as compared to control.

3.6.2. Isorhamnetin augments the apoptotic effects of capecitabine in gastric cancer cells *in-vitro*

Capecitabine, an oral fluoropyrimidine precursor, was first approved in 2001 for the treatment of metastatic colon cancer and has been used interchangeably with parenteral 5-fluorouracil in the treatment of upper gastrointestinal (GI) cancers [29]. Clinical trials show that outcome of patients receiving capecitabine is significantly better as compared to patients receiving 5-fluorouracil [235]. However, despite its efficacy, capecitabine has been linked to increased chemo-resistance in cancers. A study stated that a significant proportion of locally advanced rectal cancer patients do not respond well to standard treatment with fluoropyrimidines (capecitabine or 5-fluorouracil), requiring radical surgical resection, accompanied by a significant chance of relapse [236]. Yet another study showed that clinical refractoriness to nucleoside analogs, such as capecitabine, could be one of the main reasons underlying the extremely poor prognostic state of pancreatic cancer [237].

Therefore, we next proceeded to investigate if isorhamnetin at a sub-optimal dose could potentiate the effects of capecitabine and overcome chemo-resistance in gastric cancer cells. We employed an esterase staining assay (live/dead assay) to confirm whether isorhamnetin can significantly augment the apoptosis induced by capecitabine. As shown in Fig. 3.6.2, the sub-optimal doses of isorhamnetin (10 μM) or capecitabine (10 μM) had little effect on apoptosis alone but produced substantial enhancement of apoptosis when used in combination. Taken together, these data suggest that isorhamnetin can sensitize gastric cancer cells to chemotherapy.

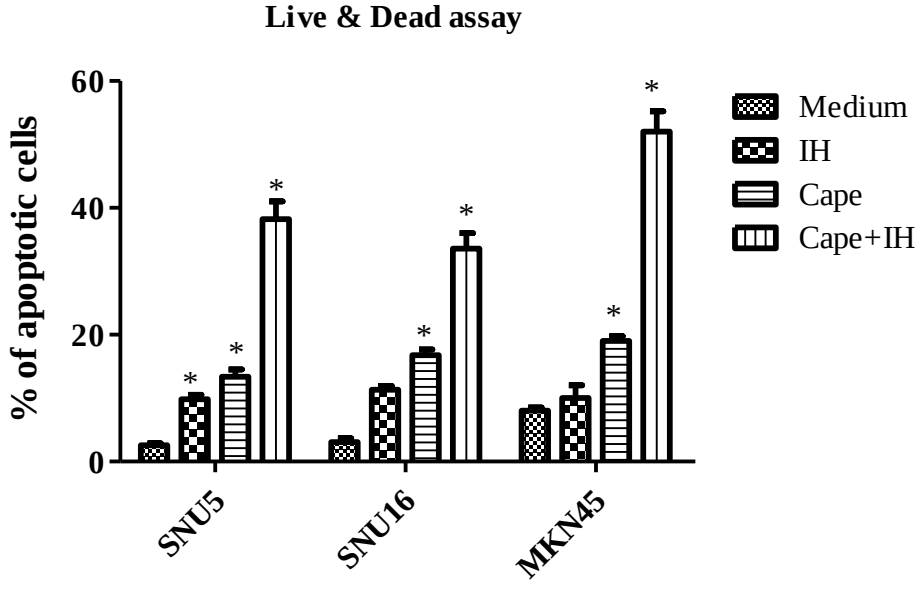


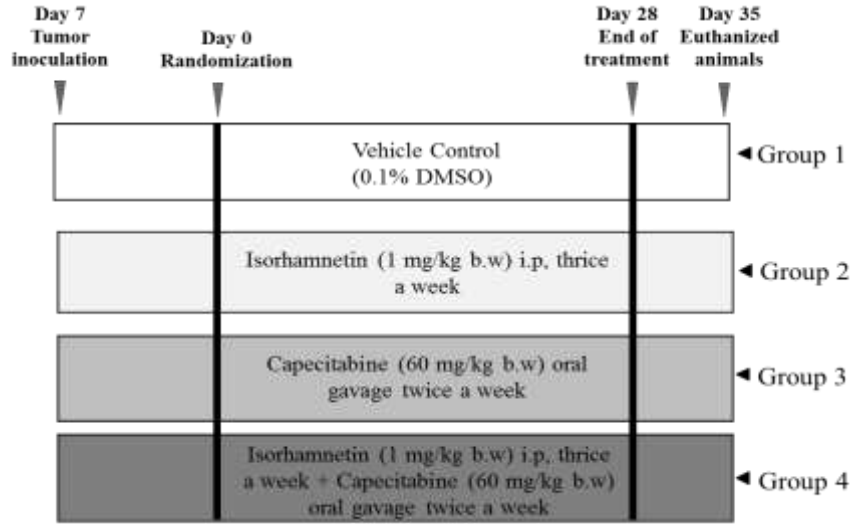
Fig 3.6.2: Isorhamnetin enhances the apoptotic effects of capecitabine in gastric cancer cells.

SNU-5, SNU-16 and MKN-45 cells were treated either alone with 10 µM of isorhamnetin or 10 µM of capecitabine, or a combination of both to assess the capacity of these agents to induce apoptosis either alone or in combination. Cells were treated with LIVE/DEAD® Cell Viability Assay reagents (Life technologies), mounted on a slide and analyzed under a fluorescence microscope as described under "Materials and Methods". Two independent experiments were performed and bar graph representing the mean + standard deviation is shown. The combination of both agents could significantly enhance apoptosis in three different gastric cancer cells. * indicates p value < 0.05 as compared to control.

3.6.3. Isorhamnetin enhances the inhibition of tumor growth induced by capecitabine *in vivo*

In-vivo evaluation of any anti-cancer drug is a critical step in predicting its efficacy before it can be tested under clinical settings. A variety of tumor systems are available, however, scientists typically employ subcutaneously growing tumor models because they can be predictive when performed under controlled conditions [238]. Therefore, we developed a human gastric cancer xenograft model in nude mouse by implanting gastric cancer SNU-5 cells. Thereafter, we analyzed the therapeutic potential of isorhamnetin and capecitabine either alone, or in combination, on the growth of subcutaneously implanted human gastric cancer cells in nude mice. The schematic representation of the experimental protocol is depicted in Fig. 3.6.3(A). SNU-5 cells were implanted subcutaneously in the right flank of nude mice. When tumors reached 0.25 cm in diameter after a week, the mice were randomized into 4 groups and exposed to chemo-therapeutic agents as per the experimental protocol. The treatment was continued for 4 weeks, and animals were sacrificed after 5 weeks. Interestingly, we noted that isorhamnetin or capecitabine alone when given at 1 mg/kg body weight produced a dramatic decrease in tumor volume as compared to control group (Fig, 3.6.3[D]). In the combination treatment group, significant reduction in tumor volume was observed when compared to control group or capecitabine alone group ($p < 0.001$) on day 35 (Fig. 3.6.3[B]).

A



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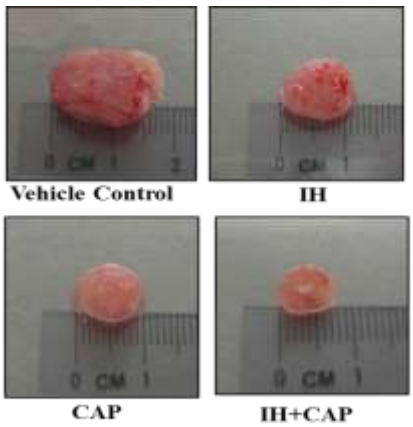
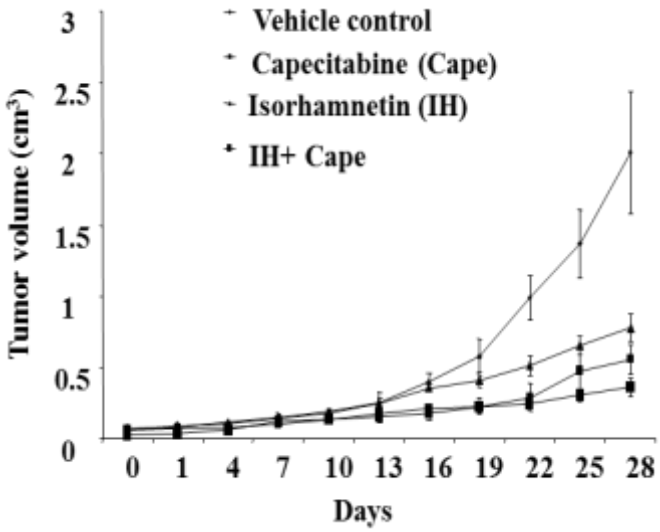


Fig 3.6.3: Isorhamnetin enhances the anti-tumor effect of capecitabine in gastric cancer xenograft mouse model.

(A) A schematic representation of the *in vivo* experimental protocol. Group 1 mice were injected with vehicle control (corn oil), Group 2 mice were given isorhamnetin (1 mg/kg body weight) through an intra-peritoneal injection, while Group 3 mice were given capecitabine (60 mg/kg body weight) through oral administration. Group 4 consisted of mice given a combination of isorhamnetin and capecitabine. (B) SNU-5 cells were injected subcutaneously into the right flank region of each nude mouse. When tumor reached 0.25 cm in diameter, the mice were randomized into 4 treatment groups, each n=5. After 5 weeks of treatment, mice were sacrificed and tumor volume was measured. Representative pictures of tumors isolated from different treatment groups are shown.

C



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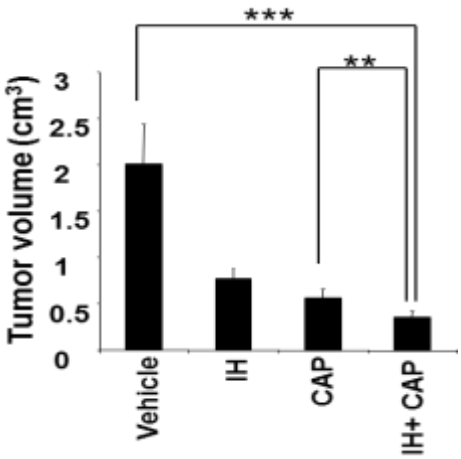


Fig 3.6.3: Isorhamnetin enhances the inhibitory effect of capecitabine in gastric cancer *in-vivo* (contd.).

(C) Therapeutic regimen of isorhamnetin and capecitabine either alone, or in combination, was administered to nude mice (previously injected with gastric cancer cells) for 4 weeks, and the animals were euthanized on the final day of the experimental protocol. The final tumor volume was measured as $V=4/3\pi r3$, where r is the mean radius of the three dimensions (length, width, and depth). Data are represented as mean + SD (n=5). (D) Data are represented as mean + SD (n=5). ***indicates $p<0.001$ and ** indicates $p<0.01$ as compared to the control group.

3.6.4. Isorhamnetin in combination with capecitabine inhibits expression of proliferative and angiogenic biomarkers in gastric cancer tissues

Ki-67 is a protein that is found to be absent in quiescent cells (G0) and universally expressed in proliferating cells [239]. It has been shown to be of prognostic influence in non-small cell lung cancer with a high index pointing toward poor prognosis [240]. A recent study found that the Ki-67 expression rate was 70.6% in the well- and medium-differentiated gastric cancer, 90% in the poorly differentiated gastric cancer and 90.9% in the non-infiltrated tissues suggesting that its expression is closely related to the clinical pathological characteristics of gastric cancer [241]. On the contrary, CD31 expression is related to neovascularization and may be associated with the clinical course of the cervical tumor [242]. Prior preclinical studies on gastric cancer have focused on analyzing the expression of CD31 to understand the anti-cancer and anti-angiogenic effects of various pharmacological drugs [243, 244].

Considering the clinical importance of Ki-67 in proliferation, and CD31 in angiogenesis of gastric cancer, we proceeded to determine if isorhamnetin in combination with capecitabine could modulate their expression in gastric tumor tissues. Fig. 3.6.4(A) shows that isorhamnetin and capecitabine alone downregulated the expression of Ki-67 in gastric tumor tissues and the combination of the two was most effective ($p<0.001$). Similarly, when examined for CD31, we found that both agents significantly reduced the CD31 expression (Fig. 3.6.4(B)) as compared to control group and were most effective when used together in treatment ($p<0.001$).

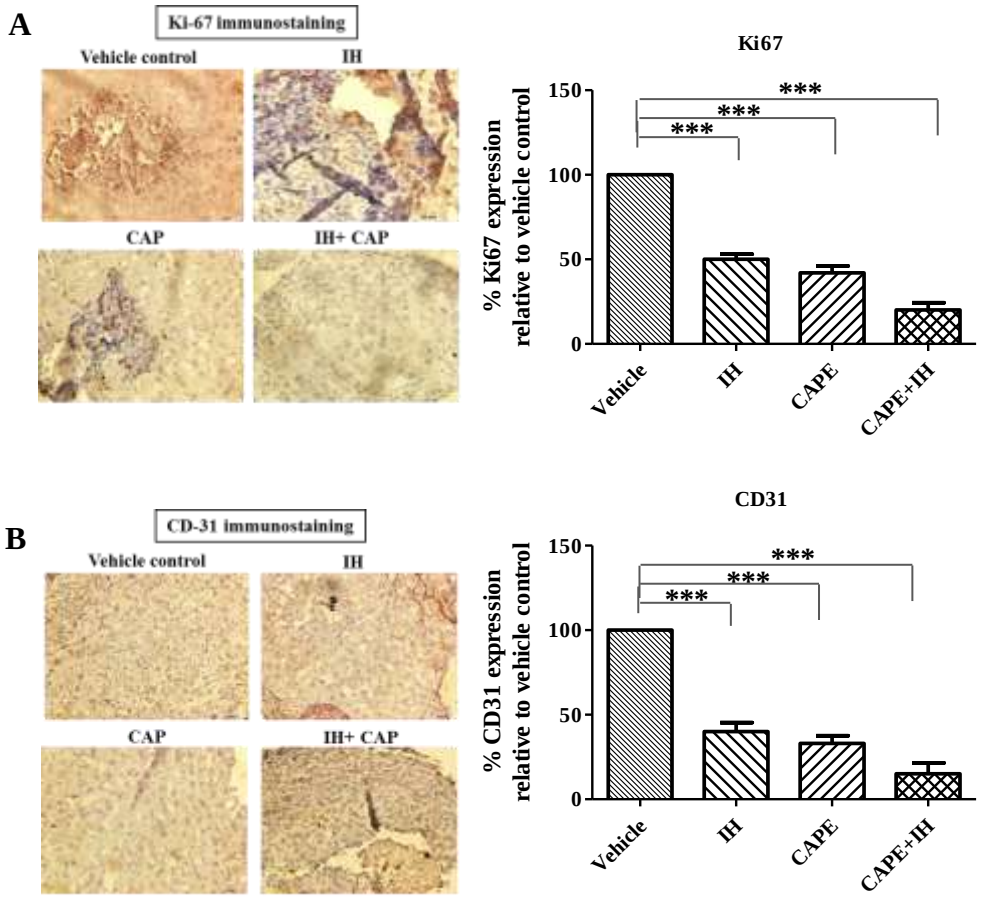


Fig 3.6.4: Isorhamnetin in combination with capecitabine inhibits the expression of Ki-67 and CD-31 in a gastric cancer xenograft model.

(A) Tumor tissues obtained after 35 days of treatment were subjected to immunohistochemistry analysis using Ki-67 specific antibody as described under "Materials and Methods" section. Images were taken using an Olympus BX51 microscope (magnification ×40). Representative image for each group is shown above. Positive cells (brown) were quantitated using the Image-Pro plus 6.0 software package (Media Cybernetics, Inc.). (B) Tumor tissues were subjected to immunohistochemistry using CD31 specific antibodies as described under "Materials and Methods" section. Images were taken using an Olympus BX51 microscope (magnification × 40). Representative image for each group is shown above. Positive cells (brown) were quantitated using the Image-Pro plus 6.0 software package (Media Cybernetics, Inc.). *** indicates $p<0.001$ as compared to the untreated group.

3.6.5. Isorhamnetin negatively regulates the expression of various oncogenic proteins involved in gastric cancer progression in tumor tissues

We observed using our *in-vitro* assays that isorhamnetin could modulate the expressions of various oncogenic proteins involved in survival, angiogenesis and metastasis. Therefore, we next proceeded to analyze if isorhamnetin alone or in combination with capecitabine could affect the expression of VEGF, COX-2 and MMP-9 in gastric tumor tissues. Vascular endothelial growth factor (VEGF) is one of the most commonly studied angiogenic molecule and is considered as an important biomarker in gastric cancer progression [245]. It has been reported as one the most potent and specific promoter of tumor angiogenesis and correlates with poor prognosis in gastric cancer patients [246]. Matrix metalloproteinases (MMPs) are enzymes that play an important role in tumor growth, invasion and metastasis [247]. Cyclooxygenase-2 (COX-2) has also been reported to be involved in gastric cancer invasion and its inhibition can suppress both angiogenesis and tumor growth [248]. Hence, we used an immunohistochemistry assay to analyze if isorhamnetin alone and/or in combination with capecitabine could affect the expression of these oncogenic proteins in tumor tissues. As shown in Fig.3.6.5, the expression of all these proteins was significantly downregulated in gastric tumor samples treated with isorhamnetin in combination with capecitabine. The downregulation was more than modest with either IH or capecitabine alone.

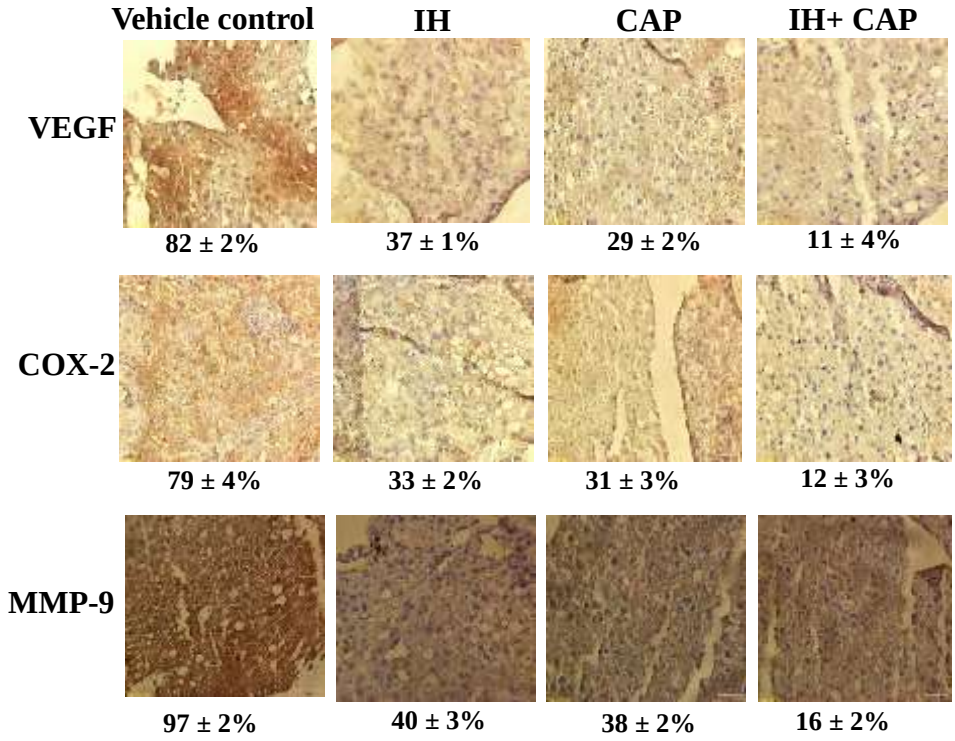


Fig 3.6.5: Isorhamnetin alone or in combination with capecitabine modulates the expression of various genes involved in gastric cancer progression in tumor tissues

Tumor tissues obtained after 35 days of treatment with either corn oil, isorhamnetin alone, capecitabine alone and a combination of isorhamnetin and capecitabine were subjected to immunohistochemistry analysis using VEGF, COX-2 and MMP-9 specific antibodies as described under "Materials and Methods" section. Images were taken using an Olympus BX51 microscope (magnification ×40). Representative image for each group is shown above. Positive cells (brown) were quantified using the Image-Pro plus 6.0 software package (Media Cybernetics, Inc.).

3.6.6. Isorhamnetin modulates the expression of BMPR2 and PPARγ in gastric tumor tissues

As mentioned earlier, BMP contribution in cancer is being extensively studied, considering the fact that they possess both, pro- and anti-tumorigenic activities in different cancers [249, 250]. Since our preliminary *in-vitro* data showed that isorhamnetin could increase the activation of PPARγ and inhibit the expression of BMPR2, we next proceeded to investigate if similar effects could be observed under *in-vivo* settings.

Hence, we used an immunohistochemistry assay to analyze if isorhamnetin alone and/or in combination with capecitabine could affect the expression of these two proteins in gastric tumor tissues. As shown in Fig.3.6.6, isorhamnetin could significantly downregulate the expression of BMPR2 and upregulate the expression of PPARγ in tumor tissues, thereby reemphasizing our central hypothesis that anticancer effects of isorhamnetin are partially mediated through the positive regulation of PPARγ signaling cascade.

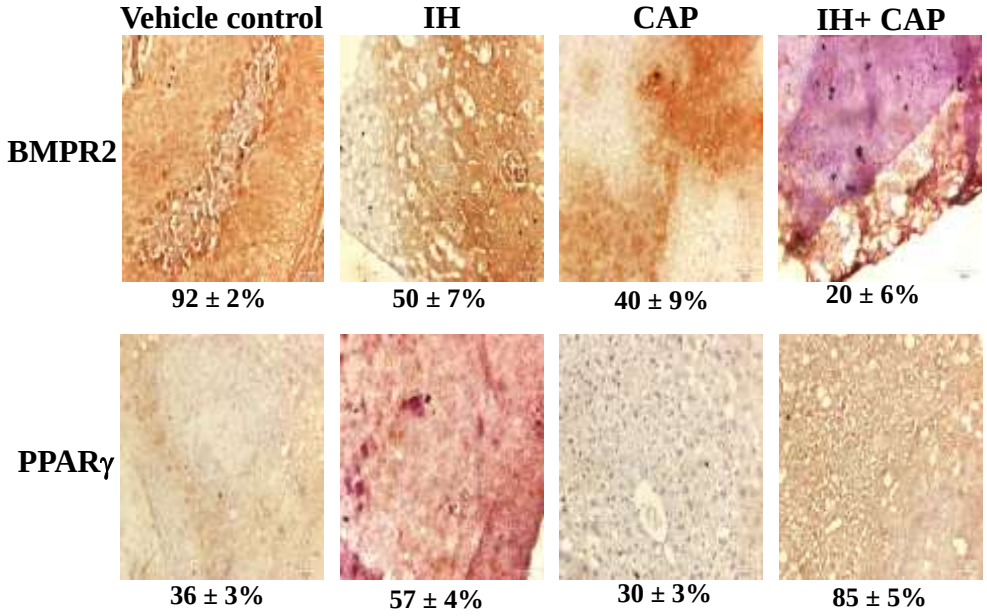


Fig 3.6.6: Isorhamnetin alone or in combination with capecitabine modulates the expression of BMPR2 and PPARγ in gastric tissues

Tumor tissues obtained after 35 days of treatment with corn oil, isorhamnetin, capecitabine or a combination of isorhamnetin and capecitabine, were subjected to immunohistochemistry analysis using BMPR2 and PPARγ specific antibodies as described under "Materials and Methods" section. Images were taken using an Olympus BX51 microscope (magnification ×40). Representative image for each group is shown above. Positive cells (brown) were quantified using the Image-Pro plus 6.0 software package (Media Cybernetics, Inc.).

4. DISCUSSION

4.1. Gastric cancer incidence and treatment options

Gastric cancer remains the fifth leading cancer worldwide in incidence, and it is the second leading cause of cancer-related mortality. It arises primarily through a cascade of events; namely non-atrophic gastritis, atrophic gastritis, gastric intestinal metaplasia (IM), gastric dysplasia, and, ultimately, gastric cancer [251]. According to the Singapore Cancer Registry Interim Report, gastric cancer is the 5th commonest cancer in males and 7th commonest cancer in females in Singapore. The exact mechanism(s) underlying this fatal disease is/are still being studied, but several prior studies have linked it to *Helicobacter pylori* infection [252].

Surgical and endoscopic resection remains to be the standard care for treatment of localized cancers of the gastro-intestinal tract while chemotherapy is primarily used in the patients with advanced tumors [253]. Chemotherapeutic treatments are primarily based on platinum and fluoropyrimidine combinations, or in three-drug regimens including taxanes or anthracyclines [254]. Palliative chemotherapy prolongs survival and improves cancer-related symptoms in patients with primary metastatic disease [255]. However, despite the availability of novel targeted therapies, gastric cancer remains significantly incurable with patients either developing chemo-resistance or relapsing after an initial response [256]. Moreover, majority of the drugs currently in use have severe side-effects and/or dose limiting toxicity [257], thereby indicating an urgent need to identify novel pharmacological agents that could mitigate the drawbacks associated with existing drugs.

4.2.Anti-cancer activity of natural compounds

The role of natural products as potent anti-cancer agents has been widely studied by various research groups. Till today, the interest in herbal compounds has been immense, with various groups engaged in active research to identify anticancer efficacy of plant derived agents. A recently published review article showed that natural compounds may also exhibit their anticancer effects by overcoming apoptotic resistance in pancreatic cancer cells [258]. These compounds have been extensively studied, owing to their effectiveness in treatment, relatively lower cost, and minimal side effects. Also, many synthetic drugs have the basic structure of natural compounds as their scaffold, and the quality of these leads have been found to better and often more biologically active as compared to their synthetic counterparts [259].

Natural products have also been shown to be highly effective in anti-ulcer treatment owing to their gastro-protective properties [260]. Flavonoids, in particular, have been extensively studied for their anti-oxidant and anti-inflammatory actions on various metabolic diseases as well as cancer [261]. Isorhamnetin is one such flavonoid that has been gaining attention for its chemo-preventive activity, as evident by the increasing research interest on this novel compound in the last few years [134, 262, 263].

4.3. Isorhamnetin: A novel natural agent

4.3.1. Investigating the cytotoxic effects of isorhamnetin against tumor cells

The major hurdles faced in the development of an anti-cancer agent drug are primarily related to the complexity of tumor cells and their associated microenvironment, as well as their similarity to the normal cells [264]. Since the 'hallmarks of cancer' defined by the renowned scientist Dr. Robert Weinberg include the ability of cancer cells to sustain proliferative signaling, evade cell death and induce metastasis [6], we aimed to identify a pharmacological agent that possessed the ability to overcome these pivotal characteristics of tumor cells. The most fundamental trait of a cancer cell remains to be its ability to sustain proliferation, as opposed to normal cells, that have a tightly controlled mechanism(s) to maintain homeostasis inside the cell. Thus, our initial experiments focused on assessing the cytotoxic effect of isorhamnetin on various gastric cancer cells.

We observed a significant decrease in the viability of various gastric cancer cells *in vitro* after treatment with isorhamnetin. Lauren's classification divides gastric carcinoma into diffuse and intestinal subtypes and thus we selected three different gastric cancer cell lines; SNU5 is a diffuse type cell line whereas AGS and MKN45 are intestinal type cancer cell lines [265]. As shown in the results (Fig. 3.2.1), we observed that isorhamnetin could significantly inhibit the cytotoxicity of different gastric cancer cells in a dose- and time-dependent manner. These results are concurrent with other studies which show that isorhamnetin can act as a potent anti-proliferative agent against skin and colorectal cancer cells [136, 139].

Current treatment for advanced gastric cancer consists of a combination of cytotoxic drugs; cisplatin as well as targeted agents such as trastuzumab had superior effects in randomized trials when compared to other agents like oxaliplatin, oral fluoropyrimidines and irinotecan that exhibited relatively less toxic results [266]. However, treatment with these agents is characterized by resistance, which is both acquired and intrinsic. This resistance could be caused by numerous cellular adaptations, including inactivation by glutathione and other anti-oxidants, as well as a rise in the levels of DNA repair or DNA tolerance [267]. Cancer drug resistance, therefore, is a complicated process and overcoming it is an important trait of an effective drug [268]. Our results indicated that isorhamnetin treatment could also significantly inhibit the viability of oxaliplatin-resistant NUGC3 cells and cisplatin-resistant AZ521 cells as seen in Fig. 3.2.1.1, thereby indicating that isorhamnetin could be used as a potent chemosensitizer of tumor cells.

When a drug is successful in one clinical setting, it encourages clinicians to further explore its efficacy for the therapy of other cancers as well. A classic example is imatinib, a tyrosine kinase inhibitor, which was initially discovered for the treatment of chronic myeloid leukemia and was then employed for the treatment of other tumors such as gastrointestinal stromal tumors and epithelial ovarian cancer [269]. Isorhamnetin was therefore tested on other tumor cell types as well, and as shown in Fig. 3.2.1.2, it could significantly inhibit the viability of MDA-MB-231 breast cancer cells, HepG2 liver cancer cells, DU145 prostate cancer cells and CAL27 head and neck cancer cells. These finding suggests that anticancer effects of

isorhamnetin are not restricted only to gastric cancer cells, but applicable on other solid tumors as well. The similarity of cancer and normal cells in the body, as described above, deem it important to analyze if the observed anticancer action of a drug is specific to tumor cells and it does not significantly affect the viability of non-transformed cells. As evident from Fig. 3.2.1.3, our results showed that isorhamnetin did not significantly alter the viability of HFE145 normal gastric epithelial cells when treated at various doses and time points.

Overall, the results of the above experiments indicate the potential effectiveness of isorhamnetin as an anti-proliferative agent and its specificity towards cancer cells. Also, the ability of isorhamnetin to sensitize drug resistant gastric cancer cells shows that it could potentially be used as a chemo-sensitizing agent to overcome drug resistance.

4.3.2. Isorhamnetin induces apoptosis in gastric cancer cells

Apoptosis is a tightly programmed cell death that eliminates unwanted cells and maintains the healthy balance between cell survival and death. Cancer cells, however, evade apoptosis allowing them to survive over their intended lifespan, thereby accumulating genetic alterations that 'deregulate cell proliferation, interfere with differentiation, promote angiogenesis, and increase invasiveness during tumor progression' [270]. Considering the importance of apoptosis as a regulatory mechanism to limit cancer progression, we performed the next set of experiments to analyze the potential role of isorhamnetin as a pro-apoptotic agent. The induction of apoptosis can be assessed by the accumulation of the cells in the Sub G1 fraction of the cell cycle. Our results, as seen in Fig. 3.2.2.2, showed that isorhamnetin could increase the accumulation of gastric cancer cells in the Sub G1 phase in a time-dependent manner. We also utilized an annexin V assay to detect the loss of membrane symmetry, a phenomenon that occurs during apoptosis. Treatment of cells with isorhamnetin was able to increase the number of annexin V-positive cells in a time-dependent manner, thus indicating induction of early apoptosis (Fig. 3.2.2.1). The results were concurrent with a recent study which showed that isorhamnetin could induce cell death and facilitate cell cycle progression to G0/G1 phase in colorectal cancer cells [136].

Activation of poly (ADP-ribose) polymerase (PARP) has been studied as an important hallmark of apoptosis because of its involvement in various cellular and molecular processes, such as cell survival and DNA repair [271]. A study evaluated the effect of isorhamnetin on human lymphoblastoid cells and found that it could increase PARP cleavage and induce apoptosis in the

cells [272]. Our study showed similar results, with isorhamnetin inducing PARP cleavage in two different gastric cancer cells in a time-dependent manner (Fig. 3.2.2.3). Along the same line, we also analyzed whether isorhamnetin modulated the expression of anti-apoptotic and anti-proliferative genes, such as *Bcl-2*, *Bcl-xL* and *Cyclin D1*. Our experiments confirmed that the flavonol could down-regulate the expression of these proteins in a time-dependent manner in two different gastric cancer cell lines (Fig. 3.2.2.4). This finding is in agreement with another study that indicated that isorhamnetin could modulate various oncogenic genes in favor of apoptosis in esophageal squamous carcinoma cells after 72 hours of exposure [273]. Isorhamnetin treatment also significantly down regulated the expression of various anti-apoptotic genes namely, *Bcl-2*, and *Bcl-xL* (Fig.3.2.2.5) thereby indicating that it can act as potent pro-apoptotic agent against cancer cells.

4.3.3. Potential molecular mechanism(s) of action of isorhamnetin

Our initial attempts to identify the potential molecular mechanism of anticancer actions of isorhamnetin led us to identify that it could bind to an important transcription factor called PPARγ. It is a nuclear receptor that is involved in the regulation of inflammation and in the processes of cellular proliferation, differentiation, and apoptosis [274]. In most cases, pharmacological agents with PPARγ-activating ability are found to be direct ligands of the receptor; physical binding to the receptor thus appears to be the most common mechanism for receptor activation. Our first step in this context, the computational docking of isorhamnetin with the crystal structure of

PPARγ, showed promising results as seen in Fig. S3.3.1. The complex of isorhamentin and PPARγ had interaction energy of -27.73 kcal/mol. The flavonol was found to have interactions with 5 polar residues and 9 non-polar residues in the receptor. Among these interactions, Cys 285 and Ser 289 were previously found to be essential for PPARγ ligand binding and activity [275, 276]. (Please refer to Appendix 2: data kindly provided by collaborator Dr. Chun Wei Yap, NUS School of Pharmacy).

We further validated the computational docking data by using the LanthaScreen time-resolved fluorescence resonance energy transfer (TR-FRET) PPARγ competitive binding assay (Life Technologies, Carlsbad, CA). This assay quantitatively determines the ability of novel test compounds to bind to purified hPPARγ LBD *in vitro*. The assay showed that isorhamnetin could indeed bind competitively to PPARγ with an IC50 of 12.18 μM. The fact that competitive binding is purely a bio-chemical and not cell-based assay might explain the lower IC50 value we obtained as compared to that observed previously in cell viability assays.

We utilized a combination of experimental techniques to ascertain PPARγ activation as the primary mode of action of isorhamnetin. As described earlier, PPARs are a family of transcription factors that have three sub-types; PPARα, PPARδ/β and PPARγ. Structure based studies have revealed that the three PPARs share a similar structure but differ in their spectrum of activity [277]. PPARα has not been explored extensively in cancer; only a few studies exist that indicate its role as an anti-cancer target upon activation [190, 278]. However, the role of PPARδ/β and PPARγ as potential anti-cancer targets has been well analyzed. Overexpression and/or ligand activation of PPAR-β/δ has

been shown to inhibit relative breast cancer tumorigenicity [279] and reduce invasion in pancreatic cancer [280]. It was thus important for us to investigate if isorhamnetin could activate PPARδ/β and PPARγ subtypes in gastric cancer cells. Therefore, cells were transfected with either GAL4-*m*PPARγ LBD or GAL4-*m*PPARδ LBD and analyzed for isorhamnetin induced activity through a luciferase based reporter assay. As seen in Fig. 3.3.3.1, isorhamnetin could activate both PPARδ/β and PPARγ subtypes in gastric cancer cells. Interestingly, though isorhamnetin could activate PPARδ/β, pre-treatment with a specific PPARδ/β pharmacological blocker, GSK0660 could not reverse its effect. However, activation of PPARδ/β through a specific PPARδ/β agonist, GW0472 could be reversed by pre-treatment with GSK0660 as shown in Fig. 3.3.3.5. These results suggest that the activation of PPARδ/β by isorhamnetin is not mediated through its direct binding to PPARδ/β, but possibly through a non-specific mechanism. However, the mild activation observed could be beneficial, given the numerous anti-cancer effects of PPARδ/β.

Consequently, we employed a 3xPPRE-tk-luc reporter plasmid to further characterize the effect of isorhamnetin on PPARγ signal transduction cascade in gastric cancer cells (Fig. 3.3.3.2). Our results revealed that isorhamnetin-induced PPARγ activation was evident as early as 2 hours after treatment, and increased substantially in a time- and dose-dependent manner, reaching a peak after 8 hours of treatment. We also observed an up-regulation of PPARγ protein expression upon treatment with isorhamnetin in a time- and dose- dependent manner, a phenomenon commonly observed in cells being directly exposed to PPARγ agonists [281, 282] that enhances its anti-cancer effects. A large cohort study also showed that PPARγ expression is

independently associated with better prognosis in colorectal cancer [283] suggesting that over-expression of PPARγ could positively regulate its biological actions.

Our next aim was to determine if isorhamnetin-induced activation of PPARγ required direct ligand binding to the receptor. To study this, we utilized GW9662, a specific antagonist of PPARγ that has negligible effect on the activity of PPARα and δ. We observed that GW9662 could significantly attenuate ligand-dependent PPARγ-activation induced by both isorhamnetin and an endogenous ligand, 15d-PGJ2 that can activate PPAR response elements as shown in Fig. 3.3.3.3. Also, an Annexin-V staining analysis after pre-treatment of cells with GW9662 showed a partial reversal of isorhamnetin induced apoptosis as shown in Fig. 3.3.3.5. This further indicated that the observed apoptotic effects of isorhamnetin could be partially mediated through the direct binding and activation of PPARγ.

Like other steroid hormone nuclear receptors, PPARγ contains a ligand-binding domain (LBD); a trans-activating domain (activation function 2), which, when activated by a ligand, changes its conformation to induce transcriptional activation; and a DNA-binding domain, which interacts with specific PPAR response elements (PPRE) that is found in the promoter region of PPAR-regulated target genes [284]. Therefore, we used a dominant negative form of PPARγ (PPARγ $^{C126A/E127A}$) to confirm whether DNA-binding functional domain of PPARγ was also required for the observed effects of isorhamnetin. Our results as shown in Fig. 3.3.3.4 indicate that isorhamnetin induced activation of PPARγ was substantially reversed in the presence of the mutant receptor. Also, isorhamnetin induced apoptosis was

attenuated in the presence of a dominant negative receptor, as evidenced by partial reversal of PARP cleavage and no change in the expression of Bcl-2 (Fig. 3.3.3.4 (B)). These findings indicate that the DNA-binding function of PPARγ is necessary for isorhamnetin induced apoptosis; in other words, we show that the anticancer effects of isorhamnetin are at least partially mediated through PPARγ activation.

Upon literature review, we further noted that the key amino acid, Tyr473, required for the binding of full agonists to human PPARγ [275] is not required for isorhamnetin induced PPARγ activity. We also observed that the capacity of isorhamnetin to activate PPARγ is approx. 50% lesser as compared to 15d-PGJ2 [285], an established endogenous ligand of PPARγ. Moreover, use of a PPARγ antagonist and dominant-negative mutant could only partially rescue the effects of isorhamnetin, suggesting that it could be functioning as a 'partial PPARγ agonist'.

With the failure of current PPARγ agonists, thiazolidinediones in the clinic, our finding that isorhamnetin could be a partial PPARγ agonist comes as a 'blessing in disguise'. It has been suggested that the adverse effects of PPARγ activators could be mitigated through use of partial PPARγ agonists, which can maintain the efficacy of full PPARγ agonists while lacking their typical side effects, such as edema and weight gain [286]. Whether isorhamnetin could efficiently function as a partial PPARγ agonist under *in vivo* settings, without inducing the side effects normally observed while using full PPARγ agonists, was evaluated in the later part of the project.

Research has shown that PPARγ heterodimerizes with the retinoid X receptor (RXR), another nuclear receptor activated by its own ligand

(purportedly 9 *cis*-retinoic acid), to initiate transcription [284]. However, in our study, experiments were focused solely on PPARγ without any artificial modulation of its binding partner RXR in the heterodimer, in order to analyze the specificity of isorhamnetin towards the PPAR receptor. Nevertheless, given the involvement of RXR in classical PPARγ-mediated transcriptional regulation of target genes, further studies are required to gain a detailed understanding of the involvement of RXR in the observed significant increase in PPARγ activity induced by isorhamnetin.

4.3.4. Isorhamnetin overcomes epithelial to mesenchymal transition in gastric cancer cells

Epithelial to mesenchymal transition is described as a process in which cells lose epithelial characteristics and gain mesenchymal ones, accompanied by a loss of cell-cell cohesiveness and enhanced migratory capacity [146]. Increasing evidence(s) suggest that gastric tumor cells harness epithelial to mesenchymal transition to increase their migratory and invasive ability [287]. The stromal-derived factor-1 (SDF-1)/CXCR4 axis also known as the CXCL12/CXCR4 axis is deregulated in multiple human cancers and blockade of this axis has been shown to inhibit pancreatic cell migration and invasion *in-vitro* [288]. Therefore, we investigated if isorhamnetin could inhibit the migratory and invasive capacity of gastric cancer cells, in the presence and absence of CXCL12. As evidenced by Fig. 3.4.1 and Fig. 3.4.2, isorhamnetin could significantly inhibit the migration and invasion of gastric cancer cells. We also found that CXCL12 induced gastric cancer cell migration and

invasion and this effect was significantly reduced upon treatment with isorhamnetin.

Several prior studies have indicated that activation of an endogenous or ectopically expressed PPARγ can inhibit cancer cell migration and invasion [289]. To elucidate if isorhamnetin mediated inhibition of gastric cancer cell migration and invasion was mediated through the PPARγ pathway, we pre-treated the cells with a pharmacological inhibitor of PPARγ, GW9662 [290], followed by exposure of isorhamnetin. Our results show that isorhamnetin could inhibit the invasive capacity of gastric cancer cells and this effect was partially reversed in the presence of GW9662 as shown in Figs. 3.4.3(C) and 3.4.3(D). Overall, we found that isorhamnetin is an effective anti-migratory and anti-invasive agent, and this effect is mediated at least in part through the modulation of PPARγ activation pathway.

Induction of EMT by TGF-β represents one of the key cell biological processes that mediate pro-tumorigenic actions. However, it appears to have a paradoxical role in tumor environment. TGF-β usually performs the role of a tumor suppressor in normal cells, and in early-stage carcinomas possibly via its ability to induce cell cycle arrest and apoptosis. Interestingly, as carcinomas begin to evolve and ultimately acquire metastatic characteristics, TGF-β begins to function as an oncogene that stimulates carcinoma growth, invasion, and metastasis [291]. Therefore, we next investigated if TGF-β could induce EMT in two gastric cell lines; non-neoplastic gastric mucosa cell line HFE145 and gastric-epithelial-like cancer cell line, YCC1. We found that treatment with TGF-β could induce fibroblast-like properties in cells, and convert them from an epithelial state to a mesenchymal state, as observed

through the modulation of various EMT regulated proteins like E-cadherin, vimentin and gamma-catenin [292]. This induced EMT was found to be reversed upon treatment with isorhamnetin, as observed by the increase in the expression of E-cadherin, and decrease in the expression levels of N-cadherin, and Vimentin proteins. Moreover, our results indicated that isorhamnetin could increase the expression of E-cadherin and induce its relocalization to the region of cell-cell adherent junction as shown in Fig. 3.4.6. We also found that isorhamnetin could reverse EMT in mesenchymal gastric cancer cells, AGS and MKN28, which are highly invasive in nature. Research groups have previously reported the potential of several dietary flavonoids to act as anti-metastatic agents against various cancers [293], however, ours is the first to prove the effectiveness of isorhamnetin as a potent anti-invasive/ant-metastatic agent in gastric cancer.

TGF-β activates a type I TGF-β receptor (TβRI) which phosphorylates Smad3, a receptor-activated Smad protein. In advanced cancer, this TGF-β/SMAD pathway can act as an oncogenic factor driving tumor cell invasion and metastasis, and is considered to be a therapeutic target [294]. Our results show that isorhamnetin could inhibit the expression of p-Smad3 in a time- and dose-dependent manner, thereby indicating that it may negatively regulate EMT in gastric cancer through inhibition of Smad3 mediated TGF-β signaling.

Bone morphogenetic proteins (BMP) are members of the TGF-β family, that mediate a highly conserved signal transduction cascade through the type-I and type-II serine/threonine kinase receptors. Though BMP signaling has largely been regarded as tumor suppressive, studies are emerging that portray their role as tumor-promoters. An important report published

recently showed that systemic inhibition of the BMP pathway (which is active in both the tumor cells and the surrounding tumor microenvironment) may lead to anti-metastatic functions in mammary cancer [249]. Clinical data kindly provided by our collaborator, Prof. Jean Paul Thiery (Appendix 3), provided us with hindsight that BMPR2 might have a direct correlation with EMT. We also deciphered through the patient data that higher expression of BMPR2 showed poor prognosis in gastric cancer. Therefore, we hypothesized that BMPR2 could play a major role in gastric tumor progression. Hence we proceeded to analyze whether isorhmanetin could down-regulate the expression of BMPR2 and if this down-regulation was also mediated through the PPARγ activation pathway.

Investigation of the molecular mechanism of PPARγ has clearly indicated that upon activation by a ligand, it bound to a DNA response element, PPRE (peroxisome proliferator response element), which is a DR-1 direct repeat of the consensus sequence TGACCT × TGACCT [295]. Even though majority of studies have focused purely on PPRE sites containing the DR1 region, our group has shown previously that genes containing a DR2 (direct repeats separated by two nucleotides) sequence could also be a potential PPARγ target [219]. We therefore used a PPRE search database, with BMPR2 gene sequence as the input and search criteria to include DR2 regions, as shown in Fig. S3.5.1, and found that bone morphogenetic protein receptor2 could possibly be a direct target of PPARγ, since it contained a putative PPRE region.

Along this line, we first proceeded to investigate the role of BMPR2 in epithelial to mesenchymal transition. We proceeded to either knockdown or

over-express BMPR2 to observe its effects. Knockdown of BMPR2 significantly inhibited the migration of gastric cancer cells, while over-expression of BMPR2 could increase the number of migratory cells as shown in Fig. 3.5.2. We also found that knockdown of BMPR2 could at least partially reverse EMT in gastric cancer cells, observed by the up-regulation of E-cadherin and down-regulation of N-cadherin, seen in Fig. 3.5.3. Taken together with the clinical data, we show that BMPR2 has an important role in promoting migration and epithelial-to-mesenchymal transition in gastric cancer.

Upon understanding the role of BMPR2 in promoting gastric cancer progression, we next examined if its tumor-promoting role was PPARγ dependent. Hence our first step was to analyze if troglitazone, known to be a potent PPARγ agonist, and isorhamnetin, which we have shown to be a partial PPARγ agonist, could modulate the expression of BMPR2 in gastric cancer cells. As observed in Fig. 3.5.4, our results showed that both the therapeutic agents could indeed down-regulate the expression of BMPR2 as well as other EMT regulated genes. We also found that use of a PPARγ dominant negative mutant could partially reverse the inhibitory effects of isorhamnetin on BMPR2 and EMT, as shown in Fig. 3.5.5, confirming that this inhibition of BMPR2 expression could be partially mediated through the DNA binding function of PPARγ. Review of existing literature indicated us that only 12 studies so far have investigated the role of PPARγ in EMT. Ours is therefore possibly the first study to report that activation of PPARγ could modulate EMT genes in gastric cancer, possibly through the down-regulation of BMPR2.

TGFβ can function as either a tumor-suppressor or tumor-promoter and thereby regulate a variety of cellular processes in cancer [296]. The biological effects of TGF-β family members are highly contextual; their behavior may vary according to the local environments and stage of disease. In our study, we show that BMPs promote gastric cancer progression, and inhibition of BMPR2 can partially reverse this phenomenon. However, considering the role of various other factors, such as hetero-dimerization of BMPR2 with BMPR1 and involvement of Smad1/5/8 pathway in BMP signaling, further studies are required to completely validate its role in gastric cancer. Nevertheless, the finding that BMPR2 promotes EMT, and that its effect may be modulated by PPARγ, is an important step in the identification of potential targets in cancer therapy.

4.3.5. Isorhamnetin enhances the inhibition of tumor growth induced by capecitabine in a gastric cancer xenograft model

Scientists have become increasingly dependent on mouse models to provide a clearer, pre-clinical evaluation of their test compound, based on the opinion that use of cell lines alone in a study might not be entirely successful. This is because cell lines are believed to be constantly changing in culture, rarely retaining the tumor heterogeneity present in the primary cancer and/or containing the components of the classical tumor environment [297]. Xenograft models are the most popular pre-clinical models for evaluating the anti-cancer activity of anti-neoplastic agents. Transplanted tumors were initially rejected in mice, which was later improved by the use of nude mice

with absence of thymus, which had significantly reduced capacity to reject 'foreign' tissues [298].

We therefore developed a human gastric cancer xenograft model in athymic nude mice using SNU-5 cells to examine the effects of isorhamnetin under preclinical settings. These cells are highly invasive in nature [244], and our group has successfully implanted them in nude mice previously to develop a gastric cancer tumor model [234]. Capecitabine (Xeloda, Roche) is an orally administered fluoropyrimidine drug that is enzymatically converted to 5-flurouracil following absorption from the gastrointestinal tract [299]. However, patients develop chemoresistance over time due to several genetic and molecular alterations and no longer respond effectively to treatment. In such cases, patients are typically treated with a combination of agents to effectively overcome drug resistance [237].

Our *in-vitro* results clearly show that isorhamnetin could significantly enhance the cytotoxic effects of various chemotherapeutic agents, including capecitabine when used at a sub-optimal concentration in three different gastric cancer cell lines (Fig 3.6.2). To validate these findings *in-vivo*, we next analyzed the potential effect of isorhamnetin and capecitabine either alone, and/or in combination, on the growth of subcutaneously implanted human gastric cancer cells in nude mice. Interestingly, we found that isorhamnetin or capecitabine alone as well as the combination of the two agents could effectively reduce tumor volume in a xenograft model. Very few studies exist, to our knowledge, which have investigated the anti-cancer effects of isorhamnetin in mouse tumor models. One recent study showed that daily pre-treatment of rats with isorhamnetin (5 mg/kg, i.p.) could significantly reduce

doxorubicin-induced myocardial damage and suppress the activation of mitochondrial apoptotic and mitogen-activated protein kinase pathways [300]. Yet another study showed that isorhamnetin when administered at doses of (0.1 mg/kg and 0.5 mg/kg) significantly decreased tumor volume in C57BL/6 mice that were subcutaneously implanted with Lewis lung cancer cells [138]. These are in accordance with our study, in which we demonstrate that isorhamnetin could significantly potentiate the tumor growth inhibition induced capecitabine in a xenograft model.

As described above, Ki-67 is a well-established proliferation biomarker while CD-31 is a marker associated with increase in angiogenesis. These markers have been regularly assessed in diverse tumor tissues using immunohistochemical assays [301]. Our data showed that isorhamnetin indeed could inhibit the expression of both Ki-67 and CD31 either alone and/ or in combination with capecitabine. VEGF, COX-2 and MMP-9 are proteins that play an important role in gastric cancer progression and invasion, hence we next examined if there was an alteration in the expression of these proteins after treatment of isorhamnetin *in-vivo*. As shown in Fig. 3.6.4, isorhamnetin alone and/or in combination with capecitabine could down-regulate the expression of the various oncogenic proteins involved in gastric cancer progression.

We also noted that there was a significant increase in the expression of PPARγ protein (Fig.3.6.5) in the tumor tissues upon treatment with isorhamnetin, and this was further increased in the mice group exposed to the combination of both isorhamnetin and capecitabine. Also, we noted no significant weight gain in mice treated with isorhamnetin, a characteristic

side-effect observed with use of classical PPARγ agonists such as thiazolidinediones in cancer therapy [302]. Having identified BMPR2 as a potential target of PPARγ, we also determined whether its expression could be modulated *in-vivo*. Interestingly, we found that BMPR2 expression was significantly down-regulated in tumors treated with isorhamnetin as well as those exposed to the combination of both isorhamnetin and capecitabine.

The therapeutic efficacy of isorhamnetin in mouse tumor models has not been extensively studied, with few selective studies [134, 138, 139] that have attempted to understand its action *in-vivo.* Ours is therefore the first to analyze the effect of isorhamnetin in combination with chemotherapy in gastric cancer, and we show that isorhamnetin may have a significant potential for gastric cancer therapy which can be further enhanced by capecitabine treatment.

5. CONCLUSIONS

In our study, we report for the first time that isorhamnetin exhibits its anticancer effects in gastric cancer cells through the positive regulation of PPARγ signaling pathway. We observed that this flavonoid could reduce proliferation, induce apoptosis, inhibit epithelial to mesenchymal transition and chemosensitize gastric cancer cells to capecitabine in a xenograft mouse model.

We postulate that this action of isorhamnetin is mediated at least in part through the activation of PPARγ and support the same providing multiple mechanistic evidences, to prove that it can, not only induce PPARγ activity, but also modulate the expression of the PPARγ regulated genes in gastric cancer cells. Also, the increase in PPARγ activity by isorhamnetin could be partially reversed in the presence of PPARγ pharmacological blocker and a mutated PPARγ dominant negative plasmid, thereby indicating that isorhamnetin could act specifically towards activating PPARγ. We further observed that isorhamnetin could competitively bind to PPARγ and form interactions with 7 polar residues and 6 non-polar residues within the ligand-binding pocket of PPAR-γ that are reported to be critical for its activity [161]. This hypothesis was also supported by a virtual predictive tumor cell system, where 0.5μM and 5μM concentrations of isorhamnetin were seen to cause PPARγ activation.

To understand the possible molecular mechanism(s) of action of isorhamnetin, we evaluated its effect on the apoptosis in gastric cancer cells. We found that isorhamnetin was able to induce significant apoptosis concomitant with down-regulation in the expression of the various anti-

apoptotic/anti-proliferative proteins (Bcl-2, Bcl-xL, and Cyclin-D1), and increase in PARP cleavage in a time dependent manner in gastric cancer cells.

Cancer metastasis refers to the spread of cancer cells from the primary neoplasm to distant sites, where secondary tumors are formed, and remains the major cause of mortality in cancer patients. In this respect, our study is the first to demonstrate that isorhamnetin can indeed inhibit the migratory and invasive properties of gastric cancer cells in the presence of the chemokine, CXCL12. Along this line, we also show for the first time that isorhamnetin can inhibit EMT, which is an important phenomenon in angiogenesis, possibly via the abrogation of BMPR2 expression in gastric cancer cells. Ours is also the first study to suggest that BMPR2 is a possible target of PPARγ, and the inhibition of BMPR2 can significantly reduce migration and EMT in gastric cancer cells.

Whether these *in-vitro* observations with isorhamnetin have any relevance under *in-vivo* settings was also investigated. We used a xenograft model in which mice were treated either alone with isorhamnetin or capecitabine or with a combination of both agents. We noted that isorhamnetin could indeed chemosensitize gastric tumors to capecitabine, as observed by the effective down regulation of Ki-67, CD-31, VEGF, COX-2 and MMP-9 in tumor tumors. To the best of our knowledge, no prior studies with isorhamnetin in xenograft GC models have been reported so far, and our overall findings suggest that isorhamnetin has a tremendous potential in gastric cancer therapy.

Thus, overall, our experimental and predictive experiment results clearly indicate that anti-proliferative, pro-apoptotic and chemosensitizing

effects of isorhamnetin in gastric cancer are mediated at least in part through the activation of transcription factor PPARγ and provide a sound basis for pursuing the use of isorhamnetin further, either alone or in combination with existing therapy, to reduce the side effects and promote treatment efficacy for gastric cancer.

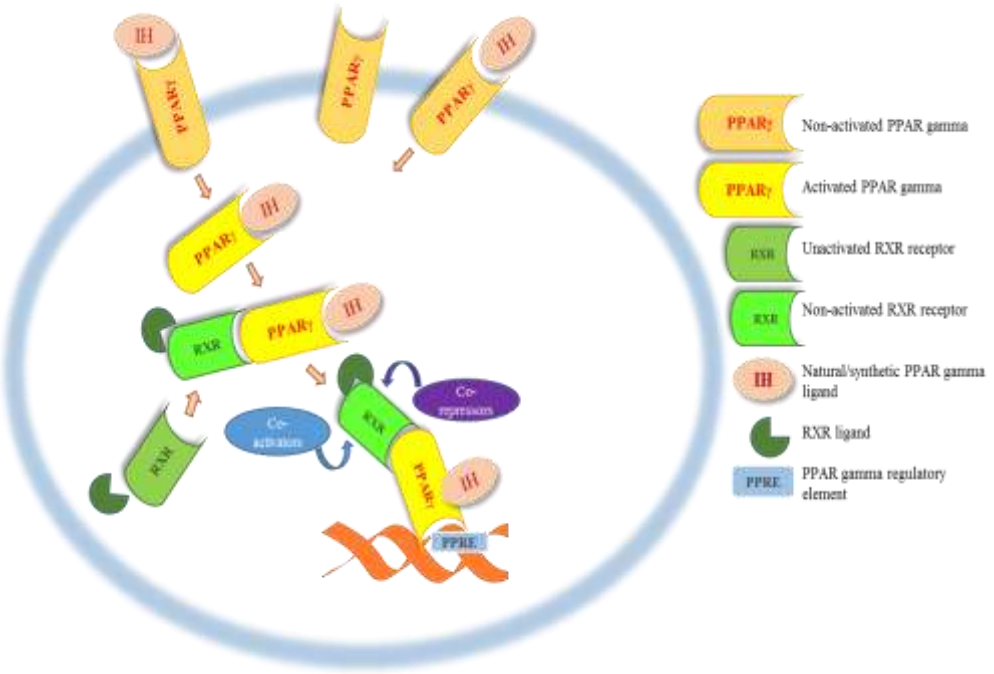
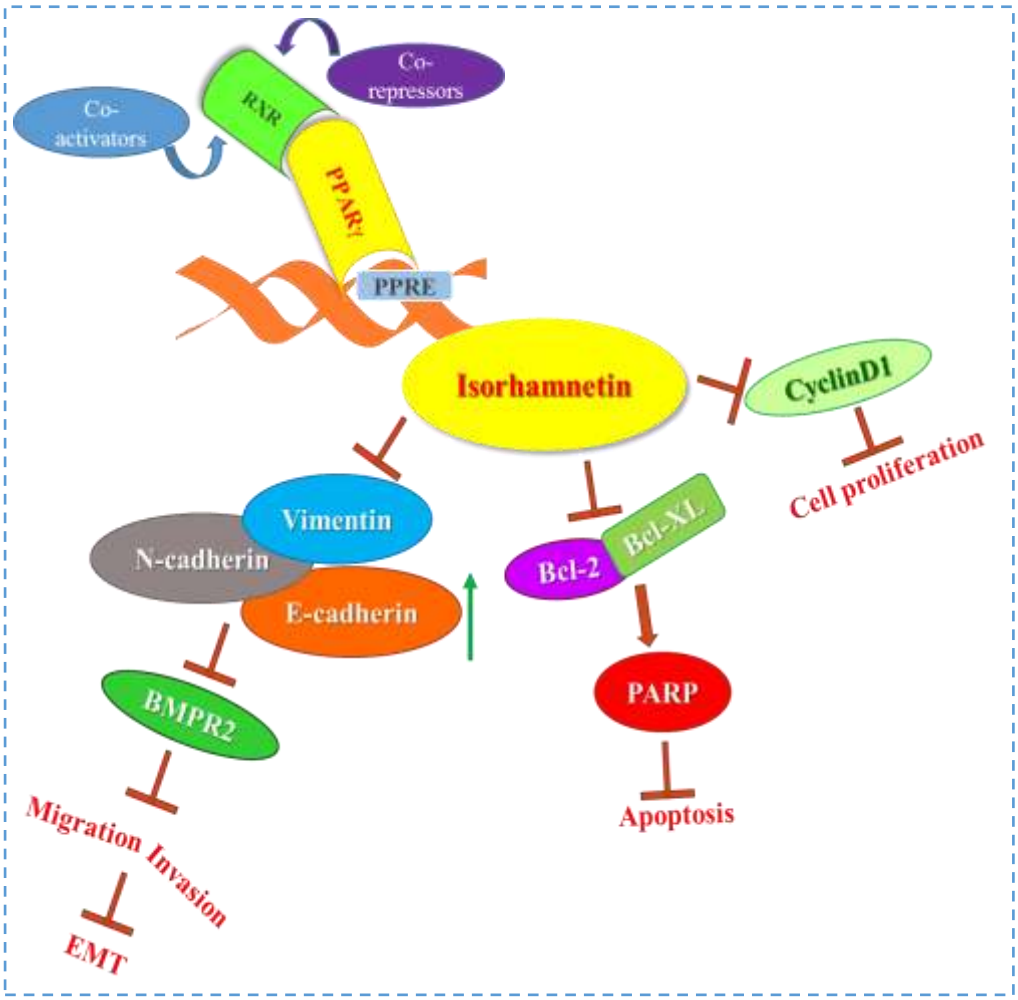


Figure 5.1. A schematic diagram representing the possible molecular mechanism of action of isorhamnetin in gastric cancer cells

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that were first discovered 20 years ago. PPARγ, an isoform of PPAR, is highly expressed in cancer cells and treatment with PPARγ ligands is shown to induce cell differentiation and apoptosis. Isorhamnetin acts as a partial ligand of PPARγ, by interacting via two key residues, Cys 285 and Ser 289. Activated PPARγ binds to RXR, and interacts with co-repressors or co-activators. The complex further binds to selected PPRE regions in the DNA to regulate various genes.

Figure 5.2. A sschematic diagram of the various therapeutic roles of isorhamnetin in gastric cancer



Isorhamnetin binds and activates PPARγ, which leads to modulation of various genes involved in proliferation, anti-apoptosis and angiogenesis. Through this figure, we show that isorhamnetin can inhibit proliferation by down-regulating *CyclinD1*, induce apoptosis by modulating the expression of *Bcl-2, Bcl-XL* and inducing cleavage of PARP, and finally inhibit epithelial-to mesenchymal transition via the down-regulation of EMT regulated genes and the expression of BMPR2. Thus, through the modulation of multiple oncogenic targets, isorhamnetin can act as an effective therapeutic agent in gastric cancer.

6. FUTURE DIRECTIONS

Our attempts to examine the effects of isorhamnetin are only the beginning of an elaborate research study to understand the anti-cancer effects of this promising metabolite. Even though our study has successfully demonstrated the preliminary anticancer mechanism(s) of isorhamnetin both *in-vitro* and *in-vivo*, it is still important to identify the co-repressors and co-activators of PPARγ that are required for the optimum activity of this flavonoid. Also, a comparative study that explores the detailed anti-cancer effects, pharmacokinetic properties and toxicological profiles of isorhamnetin and its parent compound, quercetin, side-by-side will definitely provide a clearer picture of the *in vivo* efficacy of the two drugs simultaneously. Lastly, our primary data reveals the potential of BMPR2 as a tumor-promoter and as a putative target gene of PPARγ. However, further knockdown studies are required to completely understand the crosstalk between these two proteins.

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8. APPENDIX-I

2.2. Methods

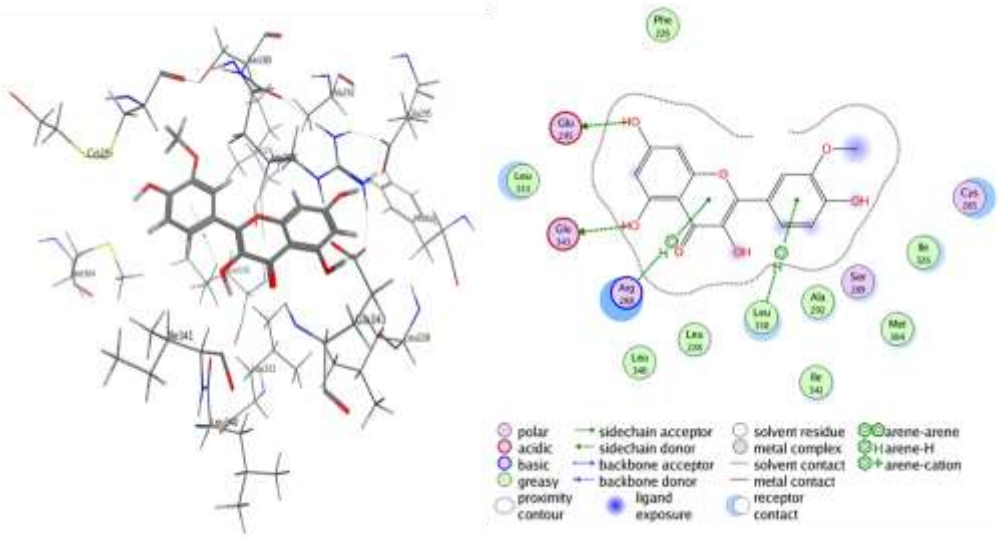
2.2.3. Molecular docking analysis

An X-ray crystallography structure of PPAR-γ (PDB ID: 2Q5S) was obtained from the RCSB Protein Data Bank. This structure was utilized in the study since it was found to have a relatively good resolution of 2.05Å, with R value of 0.199 and R free value of 0.245. The protein file contains two copies of PPAR-γ. The first copy, chain A was removed since it contained less residues than the second copy, chain B. The ligand associated with chain A and all water molecules were also removed. The remaining protein chain and its associated ligand were then processed using the default settings for the "Protonate 3D" feature in the software Molecular Operating Environment (MOE) to add hydrogen atoms and determine the ionization state of the residues. Molecular docking of IH to PPAR-γ was then performed using the Dock feature in MOE. The "Alpha PMI" algorithm was used to generate 250 different poses for IH. Alpha HB scoring function was used to rank these poses. The top 10 poses were retained and further refined by energy minimization. The MMFF94x forcefield using Reaction Field model was used for the energy minimization. Side chains of residues with 6 Å from the ligand were allowed to move during energy minimization. After energy minimization, the pose with the best interaction energy with the receptor was retained, as described previously [303].

3. RESULTS

3.3.1. Molecular docking of isorhamnetin with PPARγ

Fig.3.3.1 shows the ligand interaction map and pose of isorhamentin inside PPARγ. The complex of isorhamentin and PPARγ had interaction energy of -27.73 kcal/mol. Isorhamentin was found to have interactions with 5 polar residues and 9 non-polar residues. Among these interactions, Cys 285 and Ser 289 were previously found in earlier studies to be important for binding and activity.



Supplementary fig 3.3.1: Isorhamnetin interacts with PPARγ

The ligand interaction map of IH inside PPARγ (left) and 3D conformational structure of IH inside PPARγ (right). IH interacts directly with PPARγ at 7 polar residues and 6 non-polar residues out of which two residues, Cys285 and Ser289 were previously found to be important for its binding and activity.

9. APPENDIX-II

2.2. Methods

2.2.13 (A) Virtual predictive studies

Predictive analysis was performed using the Virtual Tumor Cell technology, Cellworks Group Inc, CA, USA which has been extensively validated and aligned with cancer physiology. The Cellworks Tumor cell platform provides a dynamic and transparent view of cancer disease cellular physiology at the functional proteomics abstraction level. The platform's open-access architecture provides a framework for different 'what-if' analysis and studies in an automated high-throughput methodology. The Cellworks platform is implemented using a three-layered architecture. The top later is a TUI/GUI (Text user interface/graphic user interface) driven user interface. The middle layer is the comprehensive representation of signaling and metabolic pathways covering all cancer phenotypes. The bottom layer is the computational backplane which enables the system to be dynamic and computes all the mathematics in the middle layer.

2.2.13 (B) Platform description

The virtual Tumor Cell Platform consists of a dynamic and kinetic representation of the signaling pathways underlying tumor physiology at the bio-molecular level. All the key relevant protein players and associated gene and mRNA species with regard to tumor related signaling are comprehensively included in the system with their relationship quantitatively represented. Pathways and signaling for different cancer phenotypes comprise 20000 plus crosstalk with more than 8000 intracellular molecules. The

platform includes important signaling pathways comprising growth factors like EGFR, PDGFRA, FGFR, c-MET, VEGFR and IGF-1R, cell cycle regulators, mTOR signaling, p53 signaling cascade, cytokine pathways like IL1, IL4, IL6, IL12, TNF; lipid mediators and tumor metabolism..Fig. S3.1 (A) shows the customized Cellworks Tumor Cell Platform that was created to align to AGS human gastric cancer cell line (KRAS mutant, PI3KCA mutant, RUNX3 deleted, β-catenin mutant and CDH1 deleted).

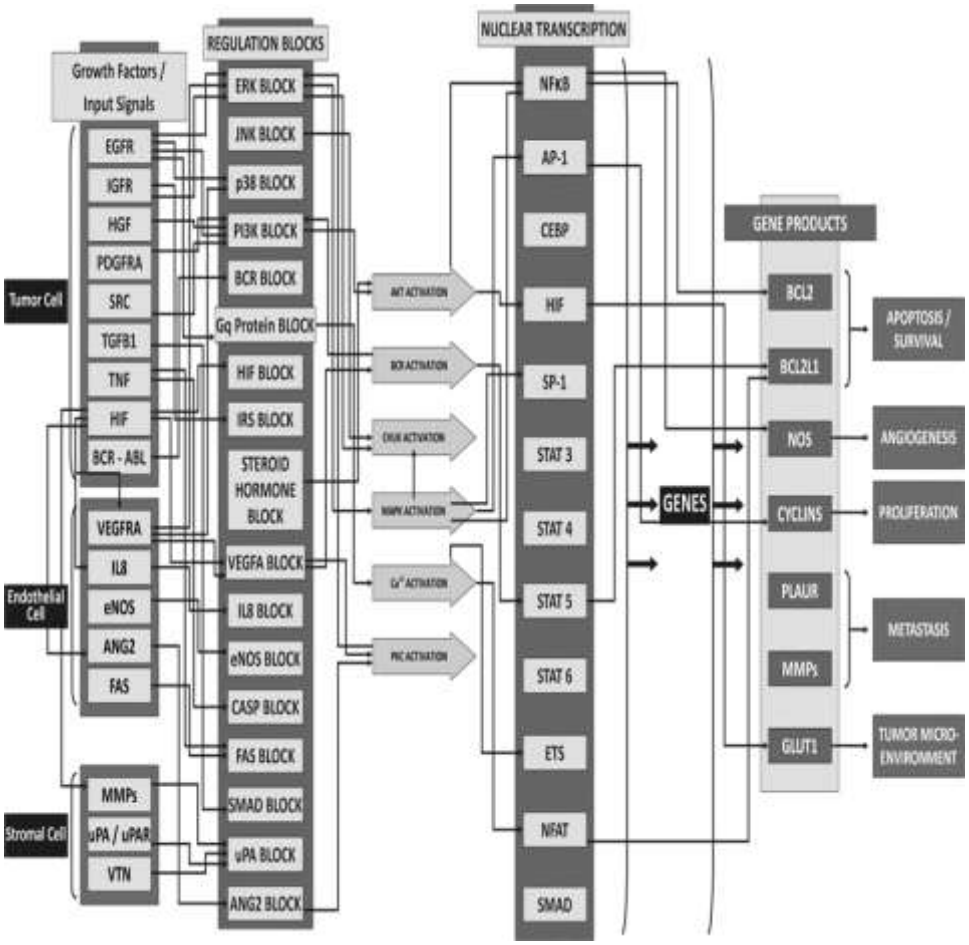
2.2.13 (C) Predictive Study Experimental Protocol

The virtual Tumor cell is simulated in the proprietary Cellworks computational backplane and initialized to a control state wherein all molecules attain the control steady state values, following which the triggers are introduced into the system. The virtual tumor cell technology allows the end user to align the system to a known cancer cell line with perturbations in known markers or mutations that can be used for further analysis. In this kinetic based virtual tumor cell platform, there is no statistical variation in the outputs. The system provides predictive semi-quantitative trends visibility into all phenotypes and bio-markers. The system predictions have been validated against a large number of retrospective and prospective studies and the accuracy of predictions is very high.

3. RESULTS

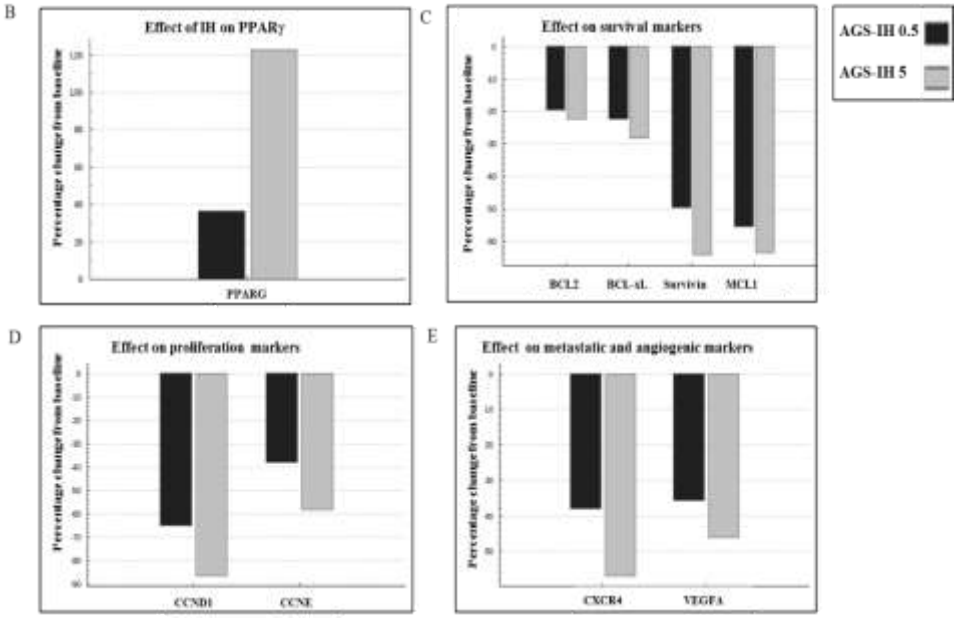
3.1. *In silico* analysis of anti-cancer effects of isorhamnetin

IH was observed to be an activator of PPAR gamma and tested at concentrations of 0.5μM and 5μM with a K_a of 1.19μM. (Fig. S3.1 (B)). Testing the effect of IH on anti-apoptotic genes such as *Bcl-2, Bcl-xl, survivin* and *Mcl-1* showed positive results with IH down-regulating expression of these genes (Fig. S3.1 (C)). Of the two proliferative markers tested, CyclinD1 was found to show a higher reduction when compared to Cyclin E on treatment with IH (Fig. S3.1 (D)). The impact of IH on angiogenic and metastatic markers VEGFA and CXCR4 was also studied and showed a reduction of ~55% and 45% with 5 μM of IH, respectively (Fig. S3.1 (E)). Caspases-9 and 3 were found to decrease significantly on treatment with IH (Fig. S3.1 (F)). Out of the apoptotic gene products, BAK showed a higher increase when compared to BAX (Fig. S3.1 (G)). Cleaved PARP1 was found to show a very high increase of ~2500% and ~5000% with 0.5 μM and 5 μM of IH (Fig. S3.1 (H)).

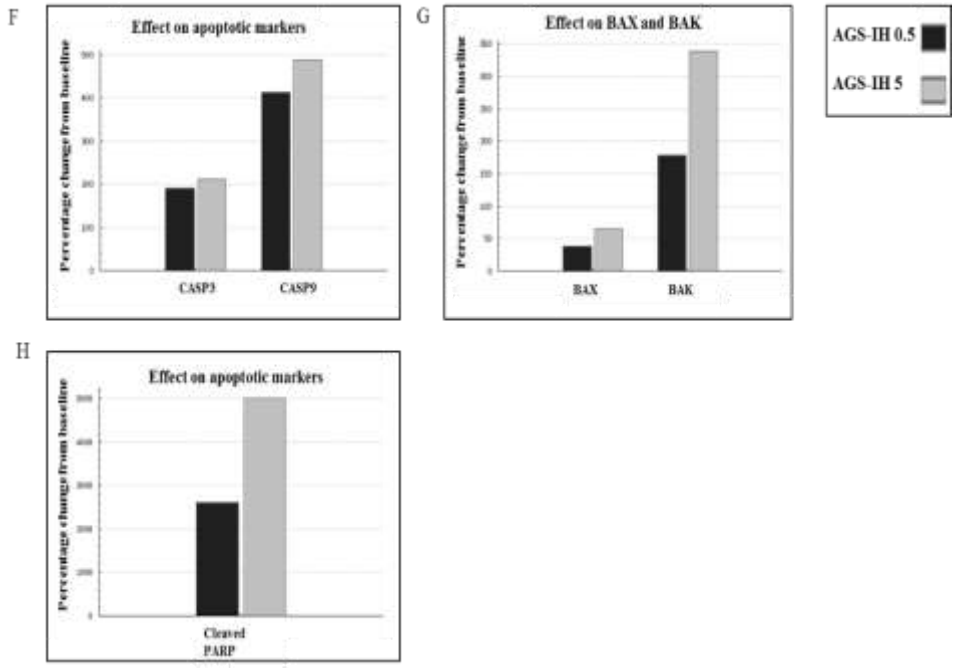


Supplementary fig 3.1: Predictive in silico Virtual Tumor Cell platform generated results:

(A) The figure illustrates a high-level view of the maze of interactions and cross-talks present in the Virtual Tumor Cell platform. The Cellworks virtual epithelial tumor cell platform on which predictive studies were conducted, is an integrated representation of the pathways in cancer that includes phenotypes of proliferation, apoptosis, angiogenesis, metastasis and conditions found within tumor microenvironment such as tumor-associated inflammation.



(B) Increase in PPAR Gamma activity upon treatment with IH. (C) The figure depicts the effect of IH on survival markers-BCL2, BCL-xl, Survivin and MCL1. (D) The figure depicts the impact of IH on Proliferative markers-CCND1 and CCNE. (E) The figure depicts the impact of IH on angiogenic and metastatic markers VEGFA and CXCR4.



(F) The impact of IH on apoptotic markers- CASP3 and CASP9. (G) The impact of IH on BAX and BAK levels. (H) The effect of IH on cleaved PARP1.

10. APPENDIX –III

2.2. Methods

2.2.16 (A) Data preprocessing of Affymetrix microarray gene expression

Microarray data GSE15460 [Ooi2009], a collection of human gastric cancer on Affymetrix U133Plus2 platforms were downloaded from Gene Expression Omnibus (GEO) and normalized using Robust Multichip Average (RMA).

2.2.16 (B) Epithelial-Mesenchymal Transition Scoring

The computation of EMT scores was performed using single sample GSEA (ssGSEA) [Verhaak2013] and a gastric-cancer specific EMT signature derived [Tan2014]. Briefly, the up-regulated genes in epithelial and mesenchymal states were obtained by comparing gastric carcinoma cell lines expression profiling. A BinReg model [Gatza2010] was then built based on these up-regulated genes to differentiate epithelial and mesenchymal gastric cell lines. Subsequently, the top 25% of the gastric cell lines with the highest probabilities for epithelial or mesenchymal phenotype were used to obtain the epithelial or mesenchymal specific gene list for the gastric cancer cell lines using Significance Analysis of Microarray (SAM) *q*-value =0 and ROC value of 0.85. The EMT score is defined as the normalized subtraction of the mesenchymal from epithelial enrichment score computed by ssGSEA. The EMT score ranges from -1.0 (fully epithelial) to +1.0 (fully mesenchymal).

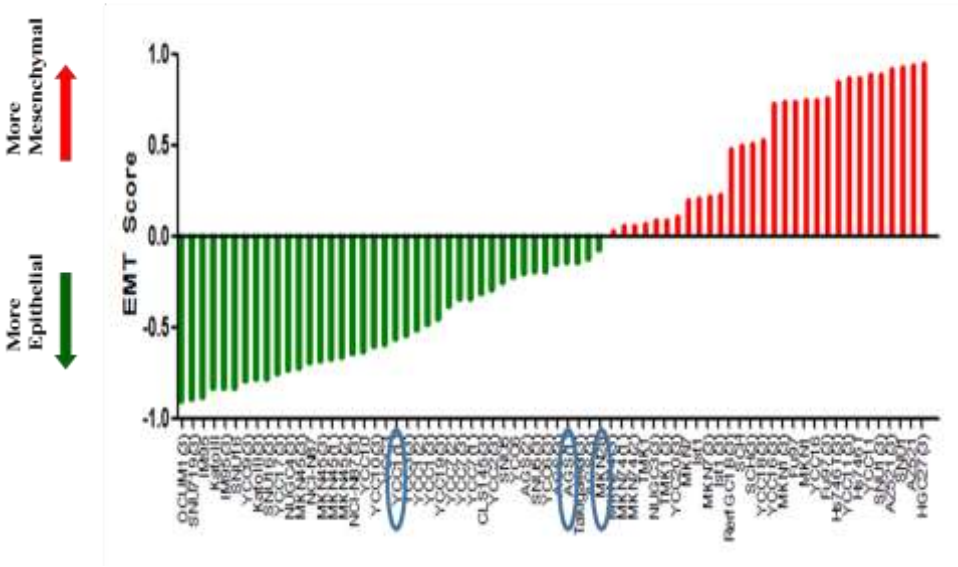
2.2.16 (C) Statistical analysis

Spearman correlation coefficient test was computed using Matlab®. Kaplan-Meier analyses were performed using Graphpad Prism ® version 5.0.

3. RESULTS

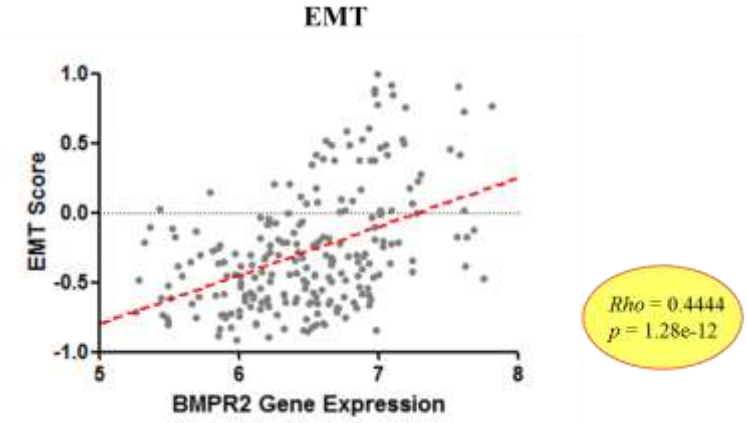
3.7. Co-relation between BMPR2 and EMT

As shown in Fig.3.7 (A), a comprehensive figure of gastric cell lines arranged according to EMT score was obtained through the analysis. They are shown to be arranged from the least epithelial to the most epithelial. We also observed that BMPR2 expression correlated positively to EMT score, indicating that higher expression of BMPR2 associates with higher possibility of EMT. Further, we observed that higher expression of BMPR2 had poor prognosis in gastric cancer survival as seen in Fig. 3.7 (C).



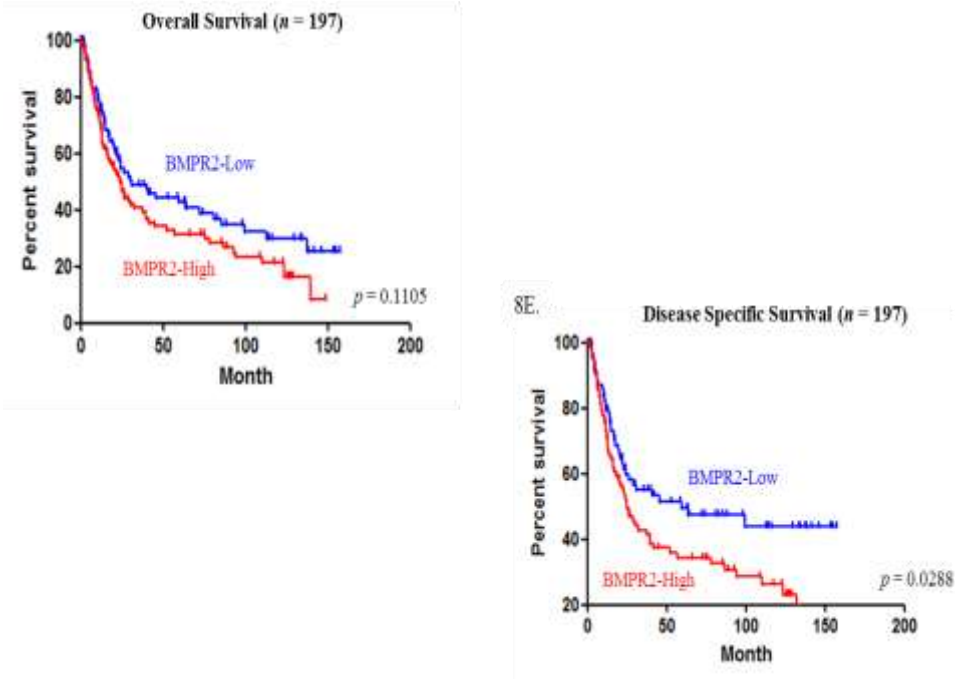
Supplementary fig. 3.7 (A): Gastric cell lines arranged according to EMT score

(A) EMT score (*y*-axis) of 39 gastric carcinoma cell lines (*n*=70; GSE15460) aligned from the most epithelial (green) to the most mesenchymal (red).



Supplementary fig. 3.7 (B): BMPR2 positively co-relates with EMT score

(B) Scatter plot of the EMT score (*y*-axis) and *BMPR2* gene expression (*x*-axis) of 231 gastric carcinoma from GSE15460 cohort. A linear regression (red, dotted) line shows correlation between EMT score and *BMPR2* expression. *Rho* and *p*-value are computed by Spearman correlation coefficient test.



Supplementary fig. 3.7 (C): Prognostic significance of BMPR2 in gastric cancer

Kaplan-Meier analyses of overall (C) and disease-specific survival (D) of gastric cancer patients stratified based on *BMPR2* gene expression. Patients with *BMPR2* gene expression < and ≥ median were grouped into BMPR2-low (blue) and BMPR2-high (red) respectively. The *p*-values were computed by log-rank test.