Regulation of Ras p21 and RalA GTPases Activity in Mammary Epithelial Cells by Quinine

By

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ABSTRACT

G protein-coupled receptors (GPCRs), a group of 7 transmembrane proteins, are the most targeted molecules in drug discovery studies. These proteins consist of an extracellular amino- and an intracellular carboxy-terminus and regulate various physiological processes in humans. Quinine as a bitter taste receptor agonist can activate G protein-coupled receptor family of proteins.

One of the most extensively studied small G protein is Ras p21. The Ras p21 proteins (H-ras, K-ras and N-ras) are important in cell proliferation and upon mutations can be oncogenic. There are other small G proteins in the Ras superfamily that have ~30-50% sequence similarity with Ras p21. Ral G proteins (RalA and RalB) have high homology (~50%) with Ras p21 and participate in various cellular functions. Results from a previous study by our group showed that quinine causes activation of RalA in CHRF cells and, that these cells express the bitter taste receptor, T2R4. Ral proteins can be activated directly or through an alternative pathway that requires Ras p21 activation resulting in the recruitment of RalGDS, a guanine nucleotide exchange factor for Ral. Using MCF10A (normal mammary epithelial cells) and MCF7 (non-invasive mammary epithelial cancer cells) cell lines we investigated the effect of quinine, a bitter compound, in the regulation of Ras p21 and RalA activity. Results showed that in the presence of quinine, Ras p21 is activated in both MCF10A and MCF7 cells; however, RalA was inhibited in MCF10A cells and no effect was observed in the case of MCF7 cells.

The downstream effector for Ras p21, MAP kinase, was activated in both the MCF10A and MCF7 cells. Western blot analysis confirmed expression of RalGDS in MCF10A cells that was higher than that observed in MCF7 cells. Although RalGDS was detected in MCF10A and MCF7 cells, it did not result in RalA activation due to Ras p21 activation suggesting that Ras p21-RalGDS-RalA pathway is not active. The possibility exists that quinine has a direct effect in the regulation of RalA activity in the MCF10A cells. Preliminary results of protein modeling showed that quinine can interact with RalA through R79 amino acid which is located in the switch II region loop of the RalA protein. It is possible that quinine causes a conformational change which results in the inhibition of RalA activation even though RalGDS is present in the cell. More studies are needed to elucidate the mechanism(s) that play a role in regulating Ral activity in mammary epithelial cells.

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LIST OF ABBREVIATIONS

AHL-12	AT-hook motif nuclear-localized protein 12
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CCR7	C-C chemokine receptor type 7
CXCR4	C-X-C chemokine receptor type 4
DMEM	Dulbecco's Modified Eagle Medium
EC50	Half maximal effective concentration
EDTA	Ethylene diamine tetra-acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FBS	Fetal Bovine Serum
GAP	GTPase Activating Protein
GDP	Guanosine Diphosphate
GEF	Guanine nucleotide Exchange Factor
GPCR	G Protein-coupled receptor
G Protein	Guanosine Nucleotide-binding Protein
GTP	Guanosine Triphosphate
HEK 293T	Human Embryonic Kidney Cells T-antigen
IC50	Half Maximal Inhibitory Concentration
kDa	Kilo Dalton

LPA	Lysophosphatidic acid
MEK/MAPKK	Mitogen-activated protein kinase kinase
PBS	Phosphate-buffered saline
PLC	Phospholipase C
PIP	Phosphatidylinositol phosphate
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PVDF	Polyvinylidene difluoride
RalBP1	Ral binding protein 1
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
T2R	Bitter Taste Receptor/Taste Receptor Type 2
T2R4	Bitter Taste Receptor/Taste Receptor Type 2 Member 4

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1. Introduction

1.1. Overview

A cell detects and responds to external stimuli, reacting to changes in the external environment which ultimately lead to alterations in cell metabolism and other cell properties, such as proliferation and differentiation. External information needs to be transduced to specific intracellular target proteins to accomplish these critical biological processes (Giamarellos-Bourboulis et al. 2006; Good, Zalatan, and Lim 2011). The signal transduction process is achieved through inhibiting or activating specific proteins in signaling pathways (Brown David I. and Griendling Kathy K. 2015).

Some proteins respond to these signals by translocating to different compartments within the cell, in fact, they can be activated by translocating to the cytosol while remaining inactive when associated with the plasma membrane or vice versa. Additionally, two other mechanisms also contribute to regulating cellular processes by being turned on or off during signal transduction. One way is to phosphorylate the target proteins, while the second is to exchange guanine nucleotide bound to a protein (Wheeler-Jones 2005; Lazer and Katzav 2011). My focus is on the role of guanine nucleotide binding proteins or also termed, GTP-binding or G proteins and how they participate in the signal transduction pathways.

1.2. Cell surface receptors

Membrane receptors also known as transmembrane receptors or cell surface receptors are embedded in the lipid bilayer plasma membrane of the cell and act in the cell signaling pathways by binding to extracellular ligands. Transmembrane receptors are divided into three parts consisting of extracellular domain, transmembrane domain, and intracellular or cytoplasmic domain. Membrane receptors are categorized into three different classes based on their structure and function; ion-channel linked receptors, enzyme-linked receptors, and G protein-coupled receptors which is the focus of this study (Kenakin and Miller 2010; Loh et al. 2018).

1.3. G protein-coupled receptors

The first crystal structure for a mammalian G protein-coupled receptor was characterized in 2000 and was related to bovine rhodopsin (IF88) (Palczewski et al. 2000). Later in 2007, the structure of an engineered human β2-Adrenergic GPCR was solved for the first time (Cherezov et al. 2007; Rasmussen et al. 2007; Rosenbaum et al. 2007).

Seven-(pass)-transmembrane domain receptors known as G protein-coupled receptors (GPCRs), 7TM receptors, or G protein-linked receptors (GPLR), form a large family of receptors that act as extracellular detector molecules which are able to activate intracellular signal transduction pathways. Ultimately, they activate cellular responses by coupling with G

proteins. They are referred to as seven-transmembrane receptors since they pass seven times through the plasma membrane (Trzaskowski et al. 2012).

GPCRs mediate their action through two principal signal transduction pathways namely, the phosphatidylinositol pathway, and the cAMP pathway (Alfred G. Gilman 1987; Brogi et al. 2014). In the presence of ligand, conformational changes make GPCRs act as a guanine nucleotide exchange factor (GEF) and consequently activate the related G protein by replacing the GDP bound to the Gα subunit for a GTP. The Gα subunit dissociates from Gβγ heterodimer in order to directly target functional proteins or affect signaling proteins downstream depending on the type of alpha subunit (Gαq/11, Gαs, Gα12/13, Gαi/o) (Wettschureck and Offermanns 2005). Over a hundred members of G protein-coupled-receptors are the target of one-third of FDA (Food and Drug Administration) approved drugs and the global sales for these drug targets is reported to be over 180 billion US dollars (Hauser et al. 2018). One group of GPCRs belonging to this family are called the, bitter taste receptors, and are the focus of my study.

1.4. Taste sensation and bitter taste receptors

Human beings are able to sense five primary tastes including sour, salty, sweet, bitter, and umami (Melis and Tomassini Barbarossa 2017). The latter is commonly referred to as taste for glutamic acid described as the taste of meat or broth. Taste receptors are the starting point for recognition of taste and are located on mucous of the tongue, throat, and palate sections. Each receptor cell can detect specific kind of taste and initiate the potential transmission of the taste sensation through different mechanisms. For instance, whereas

substances that are salty or those that are sour have links to ion channels, umami, sweet and bitter flavors are transmitted via receptors linked to G proteins, (A. Bachmanov et al. 2014; Bushman, Ye, and Liman 2015; Challis and Ma 2016; Drayna 2005; Kikut-Ligaj and Trzcielińska-Lorych 2015; Lee et al. 2017).

The five different taste sensations use secondary messenger systems to trigger neural signals. Examples of these messengers include, IP3 and cyclic AMP. IP3 acts by releasing calcium from the intracellular stores leading to depolarization of cells.

There are a group of 25 chemosensory receptors in humans known as bitter taste receptors (T2Rs) that belong to the GPCR superfamily and are responsible for signal transduction in the presence of various bitter agonists (Chandrashekar et al. 2000; Shaik, Medapati, and Chelikani 2019). Recently, it has been shown that bitter taste receptors are expressed in various extraoral tissues such as brain, respiratory system, airways, and reproductive tissues (Foster, Roura, and Thomas 2014). These receptors are proposed to play a protective role in humans by mediating various physiological functions in extraoral tissues (Deshpande et al. 2010).

In addition to the recent developments of the T2Rs functions in airway physiology, they also have an essential role in the pathophysiology of many cancers as well as developmental and metabolic disorders (Ansoleaga et al. 2015; A. A. Clark et al. 2015; Jeon et al. 2008; Kim et al. 2018; Shaik et al. 2016). For instance, it has been shown that in pancreatic cancer, a bacterial metabolite, known as AT-hook motif nuclear-localized protein 12 (AHL-12) can potentially cause T2R38 activation on phagocytes (Gaida et al. 2016). Furthermore, in another study T2R10 was shown to improve chemo-sensitivity in pancreatic

cell lines (Stern et al. 2018). In addition, it has been reported that T2R8 and T2R10 induce anti-invasive and anticancer responses in human neuroblastoma cells (Seo et al. 2017).

There are many environmental and chemical ligands that activate taste GPCRs. For instance, bitter and sweet taste modalities recognized by bitter taste receptors (T2Rs) and sweet taste receptors (T1Rs), are part of G protein-coupled receptors (Adler et al. 2000; Chandrashekar et al. 2000; Munk et al. 2016). Over 700 different bitter ligands have been found to activate bitter taste receptors which are classified into synthetic and natural substances (Meyerhof et al. 2010; Jaggupilli et al. 2016). Among all these structurally diverse ligands, quinine, a natural alkaloid, is one of the bitterest and most well studied compounds. Apart from its bitter taste, quinine has other properties such as acting as an anti-malaria, anti-inflammatory, anti-pyretic, and analgesic drug (Meyerhof et al. 2010; Sai P. Pydi et al. 2014). It has been shown that quinine can activate various T2Rs; however, its efficacy (EC50= 1mM) has only been determined for T2R4 (Meyerhof et al. 2010).

1.5. Bitter Taste receptor involvement in breast cancer

It is estimated that ~26% of women all over the world are diagnosed with breast cancer which is a leading cause of cancer-related mortality and 14% of them will die because of the condition (Pati et al. 2013). Therefore, there is a crucial need to find better diagnostic and treatment options to improve the prognosis and survival rate for breast cancer.

It has been shown in experimental and clinical data that GPCRs play a significant role in cancer progression and metastasis (Dorsam and Gutkind 2007; Nieto Gutierrez and

McDonald 2018). Thus, it makes GPCRs and their ligands prime targets for cancer therapies (Y. Liu et al. 2016; Nieto Gutierrez and McDonald 2018). In fact, GPCRs control various aspects of tumorigenesis, including cancer-related signaling pathways, cancer cell invasion, proliferation as well as migration (Dorsam and Gutkind 2007; Arakaki, Pan, and Trejo 2018).

Studies have shown that upregulation of many GPCRs occurs in breast tumor cells and tissues, such as chemokine receptors CXCR4 and CCR7 (Schmid et al. 2004; Cabioglu et al. 2005; Lokeshwar, Kallifatidis, and Hoy 2020), lysophosphatidic acid (LPA) receptors, and protease-activated receptors (PARs)(Simi and Gwendal 2007) (Simi and Gwendal 2007; Panupinthu, Lee, and Mills 2010). Also, it has been previously shown that quinidine and chloroquine, known as bitter taste agonist, caused apoptosis in MCF7 breast cancer cells via p53 dependent pathway (Q. Zhou, McCracken, and Strobl 2002)

Many of the receptors (T2Rs) for bitter taste agonists in breast cancer cells have been characterized *in vitro* (Singh et al. 2011). In 2014, a group characterized the expression of some of the bitter taste receptors such as T2R1, T2R4, T2R10, T2R38, and T2R49 in MCF10A (non-cancerous mammary epithelial cell line), MCF7 (poorly metastatic cell line), and MDA-MB-231 (highly metastatic breast cancer cell line) cell lines. Flow cytometry results showed that T2R4 was expressed in MCF10A cells at a significantly higher ($P < 0.001$) level than in the MDA-MB-231 and MCF7 cells while there was no significant difference in the other T2Rs' expression (Singh et al. 2014). Additionally, this study showed that T2R4 is expressed at a higher level in the breast epithelial cells relative to the other T2Rs.

There are many natural plant extracts or active constituents isolated from broccoli, Brussel sprouts, and bitter melon that have been shown to have an anti-cancer property,

specifically anti-breast cancer action (Levitsky and Dembitsky 2014; Muhammad et al. 2017). Many flavonoid compounds including, quercetin and apigenin, which have been shown to have an inhibitory effect on breast cancer, are also defined as T2R14 agonists (Jeong et al. 2009; Roland et al. 2013; Lin et al. 2015; Rivera et al. 2016; Bauer et al. 2017; Hariri et al. 2017).

In 2019 Martin et al. demonstrated that polymorphic variants of T2Rs such as those of TAS2R4 and TAS2R14 can potentially result in an increase in cancer risk (Carrai et al. 2011; Yamaki et al. 2017; Lambert et al. 2019).

In 2020, Singh et al. demonstrated that API and quinine are specific agonist for T2R4 and T2R14, respectively. They used normal and cancerous knockdown breast cells (shT2R4 and shT2R14) and results demonstrated a decrease in receptor responses to their corresponding agonists in knockdown cells compared to control cells (Singh et al. 2020). Taken together it is suggested that expression of T2Rs in cancer is functional and could be induced by exogenous ligands. However, there are not many studies that have characterized endogenous ligands for T2Rs activation (Lossow et al. 2016).

1.6. Guanine Nucleotide-binding Proteins (G Proteins)

Guanine Nucleotide binding proteins are a large group of enzymes that play a critical role in signal transduction pathways acting as molecular switches (Hurowitz et al. 2000; Batista and Helguero 2018). G proteins consist of two subgroups known as

heterotrimeric/large G proteins and monomeric/small G proteins (Konstantinopoulos, Karamouzis, and Papavassiliou 2007; X. E. Zhou, Melcher, and Xu 2019).

1.6.1. Heterotrimeric G proteins

Heterotrimeric G proteins act as molecular switches in order to turn on intracellular signaling cascades when an extracellular stimuli activate the corresponding G protein-coupled receptor (GPCR) (Oldham and Hamm 2008). Heterotrimeric G proteins affect many types of effector proteins including, ion channels, phosphodiesterase (PDE), adenylyl cyclase, phospholipase A2 (PLA2), and phosphoinositide-specific phospholipase C (PI-PLC) (Pandey et al. 2010).

Heterotrimeric G proteins which are located on cytoplasmic part of the plasma membrane, consist of an α, β, and γ subunit which have molecular masses of 41-45, 35, and 8 kDa, respectively (Table 1). They are responsible for responding to extracellular signals initiated by activated 7-transmembrane receptors (Milligan and Kostenis 2006; Koehl et al. 2019). The α, β, and γ subunits form a GDP bound Gαβγ complex in the resting state, which happens in the absence of receptor stimulation. However, in the presence of a ligand, GPCR is activated and acquires GEF (guanine nucleotide exchange factor) ability, causes the G protein activation by exchanging GDP on the alpha subunit for GTP. The binding of GTP causes G_{α} dissociation from the $G_{\beta\gamma}$ complex due to a conformational change in the alpha subunit (Figure 1) (A G Gilman 1987; Galés et al. 2006; Denis et al. 2012).

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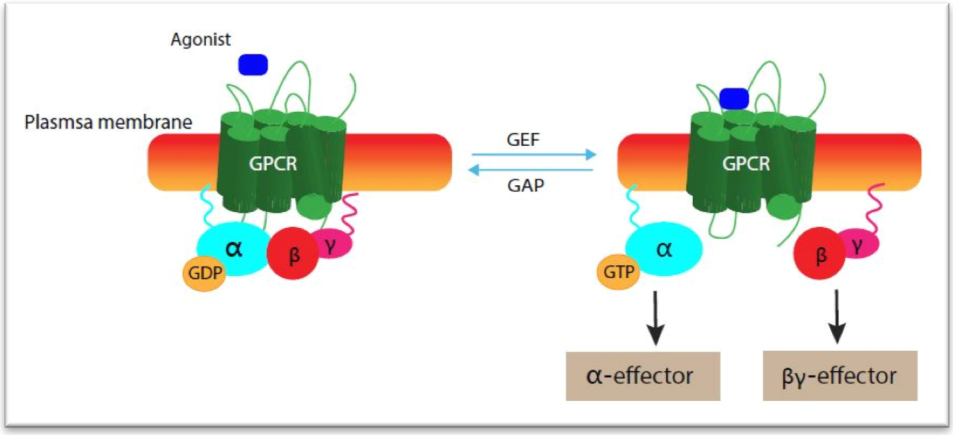

Figure 1. Heterotrimeric G proteins; the activation/deactivation cycle. GEF acts by converting GDP-G protein alpha subunit to GTP-G protein alpha and results in dissociation of Gαβγ complex.

The activated G protein then dissociates into an α and a βγ complex. GTP bound Gα is active and interacts with the appropriate effector protein(s). βγ complex also activates its downstream effectors upon dissociation from the α subunit. Intrinsic GTPase activity leads to the inactivation of the G protein and GDP bound Gα re-associates with a βγ complex to form the inactive G protein that can again associate with a resting receptor (Oldham and Hamm 2008).

1.6.2. Monomeric/small G proteins

Small G proteins have high similarity with the G_{α} subunit of heterotrimeric G proteins; however, their molecular mass is different ranging from 20 to 30 kDa, and they do not have $G_{\beta\gamma}$ subunit (Table 1) (Yang 2002; García-Nafría et al. 2018). Small G proteins play a pivotal role in downstream signal transduction pathways controlling cellular functions, such as

proliferation, cell division, cellular motility, and apoptosis (Csépányi-Kömi, Lévay, and Ligeti 2012). Small G proteins, similar to the heterotrimeric G proteins, cycle between the active (GTP-bound form) and the inactive (GDP-bound form) acting as molecular switches while interacting with effectors in a variety of signal transduction pathways (Takai, Sasaki, and Matozaki 2001; Dohlman and Campbell 2019).

G Proteins	Superfamily	Subfamily	Examples
Small G protein	RAS Superfamily	Ras	K-Ras, H-Ras, N-Ras, R-Ras, RalA, RalB, Rap1A
		Rho	RhoA, RhoB, RhoC, RhoD, Rac1, Rac1B, Rac2
		Ran	Ran, TC4
		Rab	Rab1A, Rab1B, Rab2, Rab3A, Rab3B, Rab3C, Ram
		Sar1/Arf	ARF1, ARF2, ARF3, ARF4, ARLs, TRIM23, ARFP
Heterotrimeric G protein		Gs	Gs, G_{olf}
		Gi	$G_{i/o}$, G_t, G_{gust}, G_z
		Gq	G_q, G_{11}, G_{14}, G_{15}, G_{16}
		G12	$G_{12/13}$

Table 2. Classification of G proteins. There are different subfamilies of small and large G proteins categorized based on their functional and structural similarities.

1.7. Ras p21

Ras p21 proteins are a member of low molecular weight GTPases superfamily and has been conserved from yeast to humans (Wennerberg, Rossman, and Der 2005; McCormick 2019). Earlier studies discovered Ras p21 genes as retroviral oncogenes hijacked by the host genome from Kirsten (v-Ki-Ras) and Harvey (v-Ha-Ras) rat sarcoma viruses (Chang et al. 1982; Tsuchida, Murugan, and Grieco 2016; Murugan, Grieco, and Tsuchida 2019). Ras

oncogenes have now been discovered in the genome of human tumors identified later as mutated and constitutively active Ras p21 proteins and this form of Ras p21 is present in one-third of all human cancers (Karnoub and Weinberg 2008).

There are four related 21kDa Ras p21 proteins in mammalian cells named H-Ras, K-Ras4A, K-Ras4B, and N-Ras and are encoded by *H-Ras*, *K-Ras*, and *N-Ras* genes (Barbacid 1987; G. J. Clark and Der 1995; Takács et al. 2020). Different forms of Ras p21 proteins demonstrate ~85% amino acid sequence similarity and are expressed in all types of cells (Bahrami et al. 2018).

Like other small GTPases, Ras p21 proteins are active when they are bound to GTP, and inactive in the GDP-bound form. Exchange of GTP for GDP on Ras p21 proteins causes conformational changes allowing them to interact with various downstream effectors resulting in their activation (Downward 2003; Wennerberg, Rossman, and Der 2005; Buday and Vas 2020). Indeed, membrane-bound Ras GTPases are considered essential intermediates, acting as transmitters responsible for mediating extracellular signals through various intracellular signaling pathways, ultimately influencing cellular function (Downward 2003; Veleri et al. 2018; Clark and Der 1995).

Inside the cell, there are two different classes of regulatory proteins acting as GDP/GTP nucleotide exchange factors. Guanine nucleotide exchange factors (GEFs) are responsible for enhancing GDP to GTP exchange. On the other hand, GTPase-activating proteins (GAPs) causes GTP hydrolysis to GDP resulting in inactivation of Ras p21 protein as illustrated in Figure 2 (Downward 2003; Liu, Yan, and Chan 2017; Wilson and Tolias 2016).

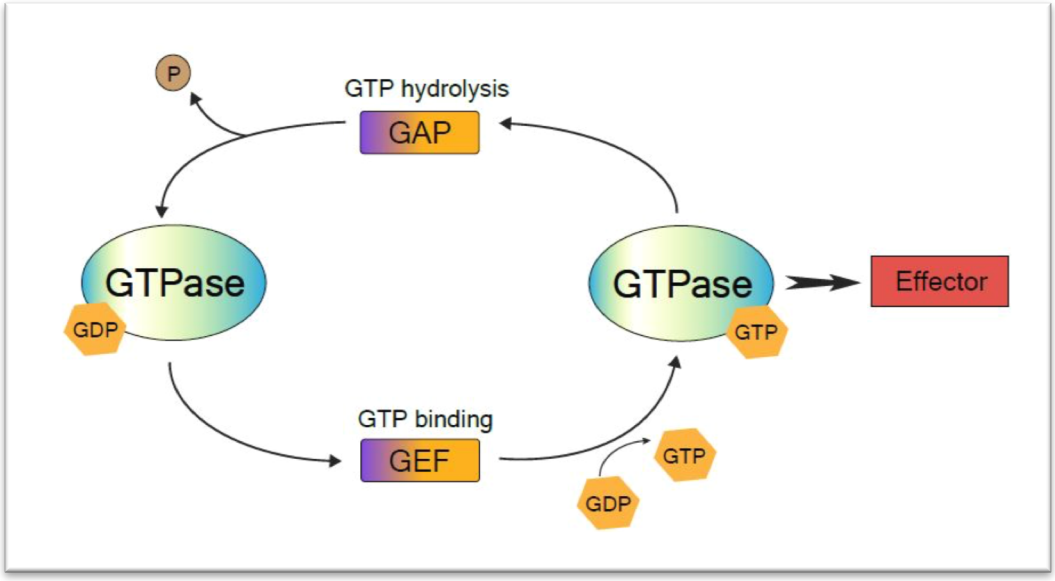

Figure 2. Small GTPases activation and deactivation cycles. GDP-bound small GTPases such as Ras are activated by GEFs (guanine nucleotide-exchange factors), which facilitate GDP to GTP exchange. GAP (GTPase-activating protein) act to enhance GTP hydrolysis which results in GTPase activation.

So far, there are many GEFs identified such as, SOS, which are responsible for facilitating the Ras p21 activation. SOS, as a ubiquitously expressed RasGEF, mediates Ras p21 activation through different mechanisms including, receptor tyrosine kinases such as epidermal growth factor receptors (EGFRs), G protein-coupled receptors (GPCRs), and tyrosine kinase-coupled receptors (Downward 2003; Young, Lou, and McCormick 2013; Wilson and Tolias 2016).

1.8. Ras p21-related signaling pathways

There are myriads of mechanisms responsible for the deregulation of the Ras p21 signaling pathway in cancer. These mechanisms consist of Ras p21 gene mutations or Ras p21 upstream or downstream proteins modifications, highlighting why understanding Ras p21 signaling pathways is essential. Active GTP-Ras p21 is able to bind to and activate multiple downstream effectors to influence various cellular processes such as proliferation, migration, cell survival, cytoskeletal reorganization, and other targets contributing to cellular transformation (Shields et al. 2000a; Downward 2003; Takács et al. 2020).

There are two most critical downstream effectors of Ras p21, namely, Raf family, and PI3 kinase. It has been shown that the Raf serine/threonine kinases family consists of Raf-1, B-Raf, and A-Raf, which are structurally similar to each other (Shields et al. 2000b; Downward 2003; Keeton, Salter, and Piazza 2017). Ras p21 activation results in cytosolic Raf translocation to the plasma membrane and resulting in its activation by interacting with Ras p21 (Hibino et al. 2011; Khanal et al. 2010; Santarpia, Lippman, and El-Naggar 2012). Activation of Raf leads to phosphorylation (activation) of MEK1 and MEK2, which are also known as MAP/Erk kinases or mitogen-activated protein kinase kinase (MAPKK) 1 and 2, which ultimately phosphorylate and activate ERK1/2 extracellular signal-regulated kinases, also known as mitogen-activated protein kinase (MAPK) 1 and 2 (Downward 2003; Shields et al. 2000a; Tian et al. 2018). Finally, activated MAPKs are able to translocate to the nucleus, and consequently phosphorylate and regulate various transcription factors in the nucleus such as c-Fos, Elk1, c-Jun (Li et al. 2018). These regulatory interactions affect cyclin D-1 expression, which consequently leads to cell cycle progression (Pruitt and Der 2001; Hirayama et al. 2020).

After the Ras/Raf/MEK/ERK pathway, PI3K (phosphatidylinositol-3 kinase) is the second-best characterized pathway downstream of Ras p21. Ras p21-GTP is responsible for promoting PI3Ks translocation to the plasma membrane. It has been shown that PI3Ks have a P85 regulatory subunit and a P110 catalytic subunit. Activated Ras affect the regulatory subunit by causing a conformational change, which ultimately activates the catalytic subunit of PI3Ks (Pacold et al. 2000; Pruitt and Der 2001; Zand et al. 2011). PI3 kinase causes PIP2 phosphorylation, which leads to generating PIP3. PIP3 can bind to several proteins via pleckstrin homology (PH) domains, including 3-phosphoinositide-dependent protein kinase-1 (PDK1) as well as Akt serine/threonine kinase, also known as protein kinase B (PKB) (Shields et al. 2000b; Jhanwar-Uniyal et al. 2019). It has been shown that PDK1 is responsible for activation of several protein kinases including, Akt, which in turn phosphorylates diverse protein targets, leading to various signaling cascades, including the cell survival promotion (Cantley 2002; Roy et al. 2010; Duncan et al. 2020). The third downstream pathway of Ras p21 is the RalGEF-Ral pathway which is discussed in part 1.11.

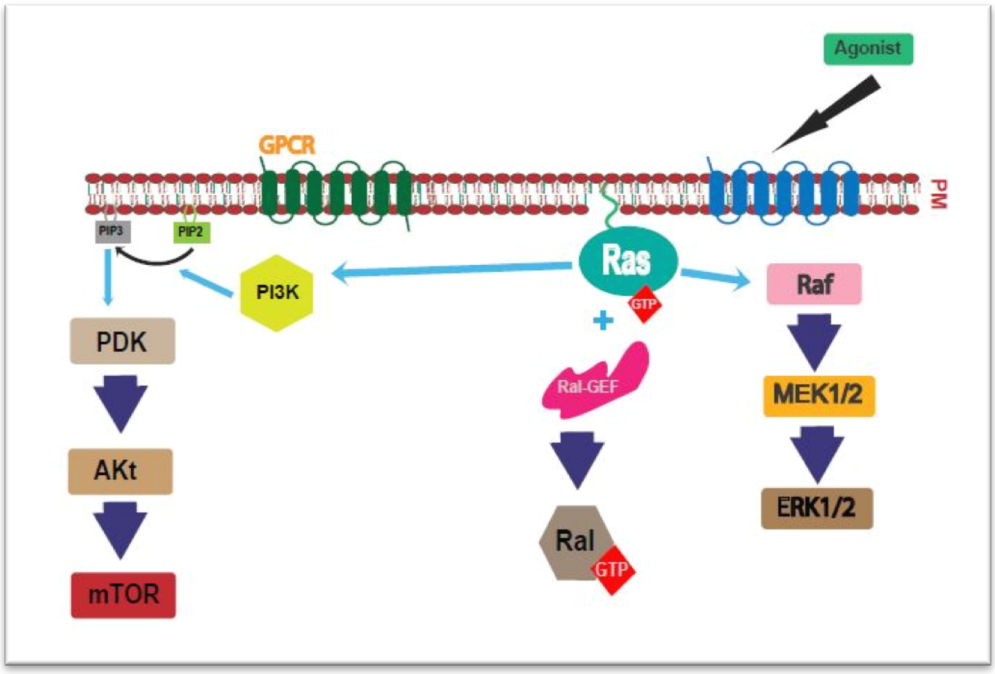

Figure 3. Ras p21 signaling pathways. GTP-bound Ras stimulates the catalytic activity of several families of effector proteins. Three of the most important ones are shown here. Activation of Raf protein kinases results in ERK activation through initiating the MAPK (mitogen-activated protein kinase) cascade. PI3Ks (phosphoinositide 3-kinases) function to generate second-messenger lipids including phosphatidylinositol-3, 4, 5-trisphosphate in order to activate survival signaling kinase including, Akt. Ral pathway is the other downstream pathway of Ras p21 which is initiated by RalGEF (guanine nucleotide exchange factors) activation.

1.9. Improper Ras p21 Signaling and Cancer

Previous studies have demonstrated that mutations are responsible for inhibiting the intrinsic GTPase activity of Ras p21. Ultimately, chronic Ras p21-GTP maintains downstream effectors in the active state, leading to cellular transformation (Downward 2003; Ho et al. 2016). Statistically, the most frequent mutations in Ras p21 occur in K-Ras with 85%, followed by N-Ras with 15%, and H-Ras, which is reported to be less than 1% of total Ras mutations. Clinical data showed that different isoforms of Ras are mutated in distinct tissues. For instance, 90% of all pancreatic adenocarcinomas, 45% of colorectal cancers, and 35% of

lung adenocarcinomas are often related to K-Ras mutations (Downward 2003; Xu et al. 2018). However, N-Ras mutation is reported in 30% of all acute myelogenous leukemia patients and liver cancer cases, in addition to 15% of melanoma cases (Downward 2003). H-Ras is also reported in 10% of all kidney and bladder cancer cases (Downward 2003; Xu et al. 2018).

In addition, improper receptor activation and Ras p21 signaling can be caused by mutation, amplification, or overexpression of its upstream receptor tyrosine kinases and mutated-Ras p21 effectors results in their over-stimulation (Bugaj et al. 2018). In fact, Ras-GAP deletion or silencing reduces GTP hydrolysis to GDP, which consequently results in negative Ras p21 signaling regulation, increased level of active Ras p21 (GTP-bound). One of the most well-known example of Ras-GAP deletion is related to neurofibromatosis type I disorder, which is caused by neurofibromin loss encoded via the NF1 gene located on chromosome 17 (Walker and Upadhyaya 2018). Neurofibromatosis type I is an inherited syndrome causing tumor development in neural crest origin tissues. Ras activation and, consequently, malignant tumor formation occurs in the absence of both copies of NF1 genes. (Ho et al. 2016; Walker and Upadhyaya 2018; Mund et al. 2020). Promoter hyper-methylation of RASSF1A is a Ras-GAP silencing example that has been reported in different primary types of tumors such as kidney, lung, liver, pancreas, and breast, among others. (Dammann et al. 2005; Tong et al. 2010).

As mentioned before, Ras p21 proteins activate various downstream effectors and initiate multiple signaling cascades including Raf/MEK/ERK , PI3-kinase/Akt, and RalGEF-Ral (Shields et al. 2000b; Degirmenci et al. 2020) as depicted in Figure 4. There are various activating mutations and gene amplifications reported in the PI3-kinase and Raf pathways in cancer patients. B-Raf mutations, which happen in the kinase domain causing constant

activation of the B-Raf, have been reported in roughly 70% of melanomas and 15% of colorectal cancer cases (Downward 2003; Roskoski 2018).

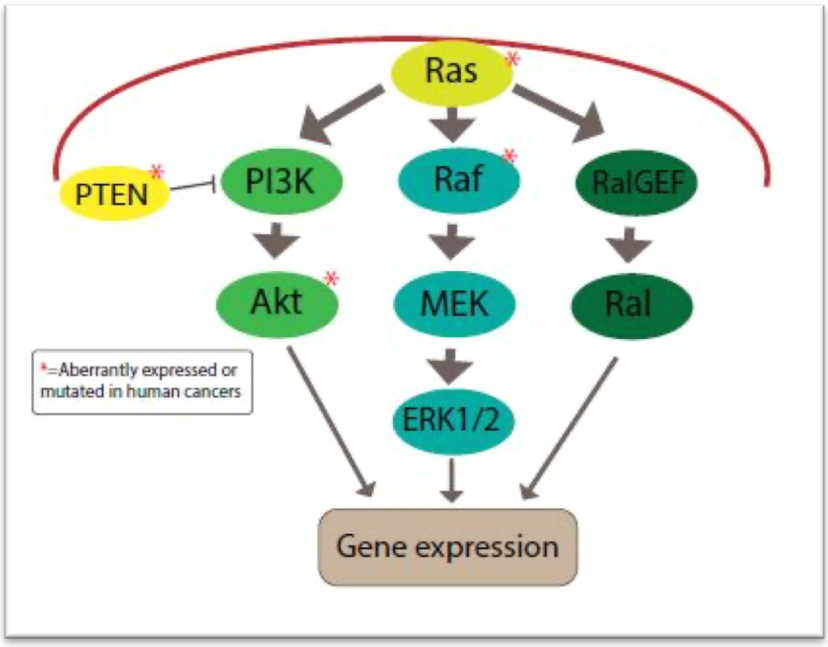

Figure 4. Improper activation of Ras p21 signaling pathway. Three different Ras p21 effectors are characterized downstream of Ras p21 including PI3 lipid kinases, Raf serine/ threonine kinases, and RalGEF.

In addition, the PI3 kinase pathway's aberrant activation has been reported through PI3-kinase amplification in ovarian cancer patients and Akt2 amplification in breast and ovarian cancers (Chen et al. 2016; Noorolyai et al. 2019). Moreover, the lack of PTEN as a tumor suppressor and metabolic regulator causes the frequent activation of PI3 kinase pathway (Eng 2003; Roskoski 2018). Deletion of PTEN, which is a negative regulator of PI3-kinase/Akt pathway, has been reported in 30-40% of cancer patients (Simpson and Parsons 2001; Ghoneum and Said 2019).

However, mutations that lead to RalGEF/Ral pathway activation and tumor development have not been well characterized compared to the Raf and PI3-kinase pathways.

In RalGEF/Ral signaling pathway, mutations were identified by large-scale tumor genomic screens and shown to occur in several RalGEFs, including Rgr, Rgl1, Rgl2, and RalGDS in lymphoma, breast, skin, and colon cancers, respectively (Bodemann and White 2008; Kelly et al. 2020). However, RalGEFs' functional relevance in cancer remains unclear.

1.9.1. Ras p21 in Breast Cancer

Mutated active Ras p21 leads to immortalized human mammary epithelial cell transformation. Also, expressing transgenic Ras p21 leads to development of a mammary tumor in mice (Dimri, Band 2005; Güran and Safali 2005; Fonti et al. 2019). Moreover, it has been shown that the ectopic expression of Ras p21 occurs in MCF7 cells line, as estrogen-dependent cells promotes estrogen-independent growth; however, there is limited evidence regarding endogenous Ras p21 activation in human breast tumors (Kasid and Lippman 1987; Kasid et al. 1985; Sukocheva et al. 2020).

Studies showed that overexpression of EGFR and HER2 growth factor receptors because of aberrant activation could result in downstream activation of Ras p21. It is also reported that in breast tumors and cell lines with EGFR and HER2 overexpression, there is an increased level of activated Ras p21 (Lintig et al. 2000; Eckert et al. 2004; Maennling et al. 2019). Therefore, aberrant activation of Ras p21 might not be the only cause of Ras p21 activation in breast tumors. Since Ras p21 proteins' overexpression is reported in 20-50% of breast cancer cases, another potential mechanism by which Ras p21 activation occurs is the result of reduced expression of Ras-GAP as a Ras p21 suppressor protein (Palaskas et al. 2011; Bellazzo and Collavin 2020).

The H-Ras silencing itself has sufficiently decreased mammosphere formation, tumor formation, and metastasis (Yu et al. 2007). Therefore, H-Ras inhibition in breast cancer stem cells introduces a promising therapeutic approach; however, further studies are needed to elucidate these mechanisms.

Since Ras p21 plays an undeniable role in human cancers, substantial effort has been made to develop pharmacologic agents blocking Ras p21 functions and/or other associated signaling pathways for cancer treatment.

1.10. Ral GTPases

Ras-like GTPase proteins are categorized under Ras subfamily of small G proteins and includes RalA and RalB, which were isolated more than 20 years ago (Neel et al. 2011). Ral GTPases are reported to have ~58% identity to Ras p21 GTPases and is mostly related to the regions interacting with guanine nucleotides (Guin and Theodorescu 2015). RalA and RalB sequences demonstrate around 85% similarity at the amino acid level and like other GTPases act as molecular transmitters in cell signaling pathways by cycling between the active (GTP-bound) and inactive (GDP-bound) forms (Takai et al. 2001; Csépányi-Kömi et al. 2012). This GDP-GTP exchange cycle in Ral proteins is mediated by RalGEF family of proteins. Ral-GTP (active form) binds to downstream effectors leading to their effects on cells (Feig 2003; Bodemann and White 2008; Seibold et al. 2019). GTP-hydrolysis results in Ral signaling pathway inactivation due to the associated intrinsic GTPase activity and is enhanced by GAPs (Figure 2) (Bodemann and White 2008; Shirakawa and Horiuchi 2015).

1.11. RalGDS-Ral pathway

There are two most critical downstream effectors of Ras p21, namely, Raf family, and PI3 kinase. Apart from Raf family, and PI3 kinases, there is another downstream effector reported for Ras p21 known as Ral-GEFs family consisting of RalGDS (Ral guanine nucleotide dissociation stimulator), Rgl (RalGDS-like gene), Rgl2/Rlf, and Rgl3, and all these possess a C-terminal RBD (Ras p21 Binding Domain) (Feig 2003; Masuda et al. 2012).

The RalGDS belongs to the RalGEF group of proteins (also known as RIP1) acts as a GEF for Ras-like small GTPases RalA and RalB. Active GTP-bound Ras p21 binds to the Ras p21 binding domain of RalGEFs and can result in the activation of RalA and RalB. It has been shown that PDK1 can also bind to RalGDS, which is not PDK1 kinase-activity-dependent, to promote the nucleotide exchange activity of RalGDS by decreasing the N-terminal auto-inhibitory effect of PDK1 on its catalytic domain. Various cellular processes are affected by Ral signaling pathway activation, such as polarized vesicle trafficking, cell morphology changes, growth factor receptor endocytosis, and several transcription factors' regulation (Feig 2003; Bodemann and White 2008; Moghadam et al. 2017).

1.12. Ral-GTPase involvement in cancer progression

Following the discovery of the significant role of RalGDS effectors on Ras p21-related oncogenic mechanisms in addition to Raf/MEK/ERK and PI3k pathways, Ral GTPases became an interesting target in cancer studies (Feig 2003; Ghoroghi et al. 2021). Besides, Ral

proteins can also be activated by RalGDS and GEFs lacking Ras p21-binding domains such as RalGPS1 and RalGPS2 and independently of Ras p21 signaling pathways. It is also reported that Ral can be mobilized by second messengers of phosphatidylinositide via a C17 terminal PH (pleckstrin homology) domain (Bodemann and White 2008; Feig 2003). These mechanisms of Ral activation are still to be elucidated (Feig 2003; Bodemann and White 2008; Shirakawa and Horiuchi 2015).

Although Ral and Ras p21 proteins show high sequence similarity to each other, Ral proteins act through a different set of downstream proteins and have diverged from effector binding regions of Ras p21 at some point (Bodemann and White 2008). These Ral effectors consist of Ral binding protein1 (RalBP1), which is also known as RLIP76, and function as Rac/Cdc42 GAP *in vitro* (Neel et al. 2011; 2012; Yan and Theodorescu 2018) , filamin as an actin binding protein (Gentry et al. 2014), Sec5 and Exo84 as members of exocyst (a multi-subunit complex) (Moskalenko et al. 2003; Kidd et al. 2010), and ZO-1–associated Y-box factor ZONAB (Frankel et al. 2005; Kidd et al. 2010).

Moreover, RalA and RalB constitutively bind to phospholipase D1(Bernfeld et al. 2018) . Ral-GTPase binding to its effector results in linking Ral to various cellular processes such as polarized vesicle trafficking, actin cytoskeletal remodeling, and receptor-mediated endocytosis (Bernfeld et al. 2018).

In addition, RalA may regulate both endocytosis and exocytosis at nerve terminals in a Ca^{2+}-dependent manner. *In vitro* studies have shown that Ral is activated by the Ca^{2+}/calmodulin (CaM) complex in response to elevated levels of Ca^{2+} (Wang and Roufogalis 1999; Park 2001; Evans et al. 2018). Of relevance to exocytosis in platelets, Ral is activated

by factors that stimulate secretion by platelets and α-thrombin activates Ral A via the Ca^{2+} signaling pathway (Clough et al. 2002; Walsh et al. 2019; Wolthuis et al. 1998).

1.13.Breast cells; Normal and Cancerous

1.13.1 MCF10A: Brief History

MCF10A cells originated from fibrocystic breast tissue of a 36-year-old patient and are spontaneously immortalized as non-transformed mammary epithelial cells. MCF10A cells do not show features of breast cancerous cells such as tumorigenicity in nude mice, anchorage-independent growth. However, with a stable-near-diploid karyotype, they express specific breast antigen that can be detected by MC5 and MFA monoclonal antibodies (Soule et al. 1990, 10; Yoon et al. 2002; Daly et al. 2018). They have also been shown to have genetic modifications that are typical in culture-adapted breast epithelial cells, such as loss of p16 locus (Yaswen and Stampfer 2002; Debnath et al. 2003; Daly et al. 2018).

1.13.2 MCF7 cell line as a breast cancer model system

MCF7 as an epithelial cancer cell line is an invasive breast ductal carcinoma line of cells derived from breast adenocarcinoma. In 1973, it was first isolated from the pleural effusion of a patient at the Michigan Cancer Foundation. MCF7 cell line has several characteristics such as estrogen responsiveness which makes them an ideal tool for using in *in-vitro* breast cancer studies (Schnelzer et al. 2000; Guttilla et al. 2012). Although the MCF7 cell line originates from an advanced metastatic tumor, it is noninvasive and is one of the most common xenograft models for breast cancer. MCF7 cell line is identified as an early-stage

cancer model since it has a functional ER, and they are dependent on estrogen for their growth both *in vitro* and *in vivo* (Welsh 2013).

From the above review, role of Ras p21 in cancer is clear and bitter taste receptors can potentially play a role in breast cancer. In addition, Ral GTPases are likely to play a role in cancer as their activity can be regulated by Rasp21 via the RalGDS. This led us to investigate the connection between bitter taste receptor, and regulation of Ras p21 and RalA activity by using quinine as a bitter taste receptor agonist.

2. Hypothesis

Bitter taste receptor activation in response to the agonist, quinine, regulates Ras p21 and RalA activity in MCF10A and MCF7 mammary epithelial cells.

3. Objectives

3.1. Characterize the effect of quinine on the activity of Ras p21 in MCF10A and MCF7 cells

3.2. Characterize the effect of quinine on the activity of MAPK pathway in MCF10A and MCF7 cells

3.3. Characterize the effect of quinine on RalA activity in MCF10A and MCF7 cells

3.4. Detection of RalGDS expression in MCF10A and MCF7 cells.

4. Materials and Methods

4.1. Materials

Product name	**Supplier**	**Cat #**
DMEM, high glucose	Thermo Fisher	11965118
TC10 System sample slide Dual chamber	Bio-Rad	1450011
Immobilon-P PVDF Membrane	Sigma aldrich	IPVH00010
Gibco™ Trypsin-EDTA (0.5%), No Phenol Red	Thermo Fisher	15400054
Hydrocortisone	SIGMA	H0888-1G
Insulin, human recombinant, zinc solution	Thermo Fisher	12585014
Horse Serum	Sigma	H1270-500ML
4x Laemmli Sample Buffer	Bio-Rad	1610747
Restore™ Western Blot Stripping Buffer	Thermo Fisher	21059
Epidermal growth factor (EGF), Human recombinant	Sigma-Aldrich	01-107
Reagent Alcohol, Certified, 70% (v/v), LabChem™	Fisher Scientific	LC222105
Methanol (Peroxide-Free/Sequencing)	Fisher Scientific	6000978

Corning® Small Cell Scraper	corning	3010
Precision Plus Protein™ Dual Color Standards	Bio-Rad	1610374
Clarity™ Western ECL Substrate	Bio-Rad	1705060
Sodium Dodecyl Sulfate (SDS)	Fisher scientific	BP166-500
TGX Stain-Free™ FastCast™ Acrylamide Kit, 12%	Bio-Rad	1610185
Insulin	Sigma	I5500
Millipore Sigma™ Millex™-GP Sterile Syringe Filters	Millipore Sigma	SLGP033RS
Pierce™ Bovine Serum Albumin Standard Ampules,	Thermo Fisher	23209
TGX Stain-Free™ FastCast™ Acrylamide Kit, 12%	Bio-Rad	1610185
Temed	Bio-Rad	1610801
Penicillin-Streptomycin	Sigma	P4333-100ML
Phosphatase Inhibitor Cocktail 3	Sigma	P0044-1ML
Raf-RBD beads	Cytoskeleton	RF02
His-Ras control protein	Cytoskeleton	RS02
Cell Lysis Buffer	Cytoskeleton	CLB01-S
Wash Buffer	Cytoskeleton	WB01-S 1

GTPγS stock: (non -hydrolysable GTP analog)	Cytoskeleton	BS01
GDP stock	Cytoskeleton	GDP01
Protease Inhibitor Cocktail	Cytoskeleton	PIC02
Active Ral Affinity beads	Cytoskeleton	RL07
Anti-Pan Ras monoclonal antibody	Cytoskeleton	AESA02
Anti-RalA monoclonal antibody	Cytoskeleton	ARL01
Anti-rabbit IgG, HRP-linked Antibody	cell signaling	7074S
Peroxidase AffiniPure Goat Anti-Mouse IgG + IgM (H+L)	Jackson Labs	115-035-068
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody	Cell Signaling	9101
p44/42 MAPK (Erk1/2) (L34F12) Mouse mAb	Cell Signaling	4696
RALGDS Polyclonal Antibody	Thermo Fisher	PA5-49099
Ral A constitutively active control protein	Cytoskeleton	RL23
Quinine HCL	Sigma	Q1125
DC Protein Assay Reagents Package	Bio-Rad	5000116
Fetal Bovine Serum	Invitrogen	26140079

4.2. Methods

4.2.1 Cell culture

Human mammary epithelial cell lines MCF10A and MCF7 were kind gifts from Dr. James Davie and Dr. Etienne Leygue, Research Institute in Oncology and Hematology (RIOH), University of Manitoba, Winnipeg, Canada. MCF7 epithelial cancer cell line was maintained in DMEM with 10% FBS and 1% penicillin-streptomycin, whereas MCF10A was maintained in DMEM supplemented with 5% horse serum, hydrocortisone (0.5 μg/ml), insulin (10 μg/ml), epidermal growth factor (10 ng/ml), and 1% penicillin-streptomycin. Adherent MCF10A and MCF7 cells were seeded at $1x10^6$ cells. At day 4, cells reached ~70% confluency and were transferred to 150 mm culture dishes. 5 days later, confluency reached ~60% and cells were placed in starvation medium. For MCF7 cells, it was growth medium excluding FBS. MCF10A cell's starvation medium consisted of the medium deprived of serum, insulin and EGF.

4.2.2 MCF10A and MCF7 Lysate collection

After serum starvation for 24 hrs, MCF10A and MCF7 cells were treated with 1 mM quinine or left untreated and lysate prepared as below.

Media from culture plates containing control or quinine (1 mM for varying times of 5, 10, and 15 minutes) treated cells was aspirated and rinsed twice with ice cold PBS. The cells

were detached from the plates using a scraper. 1.2 ml ice cold lysis buffer (supplemented with 1x protease inhibitors and 1% v/v phosphatase inhibitors) was added to the culture dish. Consequently, lysates were transferred into ice cold pre-labeled 2 ml micro-centrifuge tubes and immediately centrifuged at 10,000 rpm at 4˚C for 2 min. 100 µl of each sample was saved for protein quantification and the remaining was snap frozen and kept at -80˚C.

4.2.3 Pulldown Assay for Active Ras p21 and RalA GTPases

To confirm that Raf-RBD and RIP1-RBD beads are able to pull-down active Ras p21 and RalA respectively, 300 µl (300 µg of protein) of cell lysate was loaded with GTPγS. Briefly, 1/10th volume of loading buffer provided in the Ras p21 or RalA activation assay kit was added to cell lysate mentioned above followed immediately by the addition of 1/100th volume of GTPγS (200 µM final concentration). The mixture was incubated at 37˚C for 30 minutes with gentle rotation. After 30 minutes incubation, microtube was transferred to 4˚C and 1/10th of STOP buffer was added to the mixture. The samples were used immediately in a pull-down assay as described in section 4.2.4. In case of RalA, volume of GTPγS added was 1/50th (400 µM final concentration), 30 µl of pull-down beads (Raf-RBD or RalBP1-RBD) were added to each sample and incubated at 4˚C for 1 hour on a rotator and centrifuged for 1 min at 5000 x g at 4˚C. Afterward the supernatant was carefully discarded, beads were pelleted by centrifugation at 5000 x g at 4˚C for 3 min and washed twice with 500 µl of wash buffer. Finally, 25 µl of 4x Laemmli buffer was added to each sample and heated at 100˚C for 2 minutes. Western blot analysis was carried out on samples using antibody against Ras p21 or RalA.

4.2.4 Active Ras p21 and RalA pulldown assays after treatment with quinine

After 24 hours starvation, quinine (1 mM final concentration, dissolved in double distilled water) was added to the cells for 0, 5, 10, and 15 minutes, respectively. 300 µg of each sample (0, 5, 10 and 15 minutes treated samples with quinine) were used in Ras p21 and RalA pull-down experiments. 30 µl of pull-down beads were added to each sample and incubated at 4˚C for 1 hour on a shaker and centrifuged for 1 min at 5000 x g at 4˚C. Afterward, the supernatant was carefully discarded, and the beads were washed with 500µl of wash buffer. After lysis, Raf-RBD (beads were used to pull down active Ras p21) and RalBP1-RBD (beads were used to pull down active RalA) beads were pelleted by centrifugation at 5000 x g at 4˚C for 3 minutes. Finally, 25 µl of 4x Laemmli buffer was added to each sample and heated at 100˚C for 2 minutes. Western blot analysis was carried out on samples using the appropriate antibody (Ras p21 for Raf-RBD and RalA for RalBP1-RBD).

4.2.5 Active ERK1/2 analysis

In order to measure the active (phosphorylated) level of ERK1/2, 25 µg of each sample (0, 5, 10, and 15 minutes treated samples with quinine) was used for Western blot analysis. The cells were lysed in the presence of phosphatase inhibitor cocktail.

4.2.6 His-Ras p21 protein control

3 ng of the recombinant His-Ras control was run on the SDS-PAGE gel as a positive control and as a guide for endogenous Ras p21 which has a molecular weight of approximately 21 kDa compared to 25 kDa for His-Ras p21.

4.2.7 RalA constitutively active control protein

3 ng of recombinant His-RalA protein was run on SDS-PAGE gel as a positive control and as a guide for endogenous RalA. Although Ral superfamily of proteins have a molecular weight of 27 kDa, the His-tag on the recombinant RalA results in running slightly higher on the SDS-PAGE gel.

4.2.8 Western blot protocol

Protein lysates (samples and controls) were quantified using Bio-Rad DC (detergent compatible) protein assay and run on the 12% SDS-PAGE gel at 300 voltage for 25 minutes for lower molecular weight proteins in this study (Ras p21 and RalA) and 35 minutes for ERK1/2 which molecular weight of 42-44 kDa.

4.2.9 RalGDS expression in MCF10A and MCF7 cells

35 µg of MCF10A and MCF7 protein lysates were loaded on 8% SDS-PAGE gel along with 35 µg of total protein lysate from HEK293 cells. Western blot analysis was carried out using the RalGDS polyclonal antibody according to manufacturer's instructions.

4.2.10 Statistical analysis

The bands were quantified using ImageJ software and the results for each sample was normalized by using the respective total proteins (in this study total Ras p21, RalA, and ERK1/2). Normalized data was analyzed by using one-way ANOVA program of PRISM6-Graphpad software and $P<0.05$ was considered as significant difference compared to control.

4.2.11 Molecular docking of Quinine ligand with human RalA

The three-dimension structure of the human RalA-GDP was obtained from the RCSB Protein Data Bank (http://wwww.rcsb.org) using PDB ID: 2BOV (Bum-Erdene et al. 2020; Yan et al. 2014). The crystal structure was retrieved and prepared using the Protein Preparation Wizard workflow in Maestro12.6 (Schrodinger, New York, NY, 2020). Missing side chains and loops were added with the Prime module. Further, protein structure was protonated at pH 7.0 using PROPKA and Epik, respectively. The model was energy minimized, and the quality was checked using Procheck (https://servicesn.mbi.ucla.edu/PROCHECK/). The Ramachandran plot showed >95% of

residues are in the favorable and allowed regions. Quinine is a known bitter taste compound, and the structure was retrieved from Pubchem (CID:8549) as .sdf file (https://pubchem.ncbi.nlm.nih.gov/compound/8549#section=2D-Structure).

Before docking quinine to RalA structure, we performed binding site prediction analysis using the SiteMap module of Schrodinger. The binding sites were identified by overlaying a three-dimensional grid around the region. Each point of the grid (site point) is evaluated using van der Waals energies. Points are linked together to form the putative binding site. Each site is evaluated based on its ability to bind a ligand (SiteScore) and its druggability (DrugScore). SiteMap module considers all ligands as drugs and predicts where they can be druggable. Both SiteScore and DrugScore use the weighted sums of three parameters, namely the (i) number of site points in the binding site; (ii) enclosure score that is a measure of how open the binding site is to solvents; and (iii) hydrophilic character of the binding site (hydrophilic score). Unlike DrugScore, SiteScore limits the impact of hydrophilicity in charged and highly polar sites. A binding site with SiteScore and DrugScore of 0.8 is considered to fit a small molecule ligand. SiteScore and DrugScore values closer to 0.8 are considered ‘difficult’ to the drug, while binding sites with SiteScore and DrugScore closer to 1.1 are classified as highly ‘druggable’ (Halgren 2007)

The ligprep module was performed to prepare the ligand from two dimensions to three dimensions and to optimize for docking and receptor grid generation to bind the ligand to the protein. The optimized ligands were docked into the RalA protein structure using the Glide (extra precision XP) module. Based on the Glide score, the best poses were selected, and compare with SiteMap prediction. The energy minimized and stimulated complex of RalA docked with quinine was analyzed to study the interactions.

5. Results

5.1 Raf-RBD and RalBP1-RBD beads pulldown assays

In order to confirm that the beads can pull-down the active form of Ras p21 and RalA from MCF10A and MCF7 cell lines, Ras p21 Binding Domain (RBD) region of Ras p21 protein effector (Raf kinase) and Ral-BP1, RBD region of Ral protein effector were used as a tool for affinity purification of GTP-Ras p21 and GTP-RalA from cell lysates. In this step, 300 µg of cell lysates were loaded with GTPγS (Fig. 5a and 5b, lane 2) or GDP (Fig. 5a and 5b, lane 3). Results demonstrate that GTP-Ras p21 (Fig. 5a, lane 2) was pulled out and the 21 kDa band for Ras p21 protein (Fig. 5a) and 27 kDa band for RalA proteins are shown (Fig. 5b) in MCF10A cell lysates. Similar results were obtained when MCF7 cell lysate was used in the pull-down assay (results not shown).

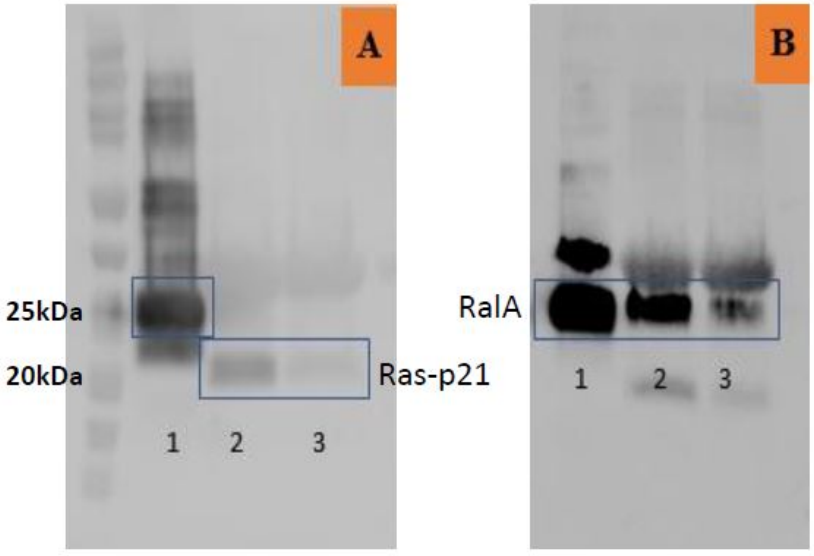

Figure 5. Affinity-binding protein pulldown assay. Part A is related to Ras p21 protein pulldown experiment and Ras-GTPγS and Ras-GDP results show 21 kDa band, considered as positive control and negative control, respectively (part A, lanes 2 & 3). Lane 1 shows 3 ng of recombinant His-Ras control protein. Part B is related to RalA protein pulldown experiment and shows a 27 kDa band. Lane 1 represents 3 ng of RalA constitutively active control protein. Lane 2 & 3 show RalA-GTPγS and RalA-GDP, respectively. The final Raf-Ras p21

binding domain and RalBP1-RalA binding domain bead pellets were suspended in 25 µl 4X Laemmli's sample buffer and heated at 100°C for 2 min. Western blots were performed by using Anti-Pan Ras monoclonal antibody (1:250 v/v) and Anti-RalA monoclonal antibody (1:500 v/v) for Ras p21 and RalA analysis, respectively.

5.2 Effect of quinine on activity of Ras p21 in MCF10A cells

In order to evaluate the effect of quinine on Ras p21, MCF10A cells were treated with 1mM quinine for 0, 5, 10, and 15 minutes, respectively. Active Ras p21 was pulled-down using Raf-RBD. Representative results indicated a significant increase in the activation of Ras p21 after 10 minutes incubation compared to control (Fig. 6).

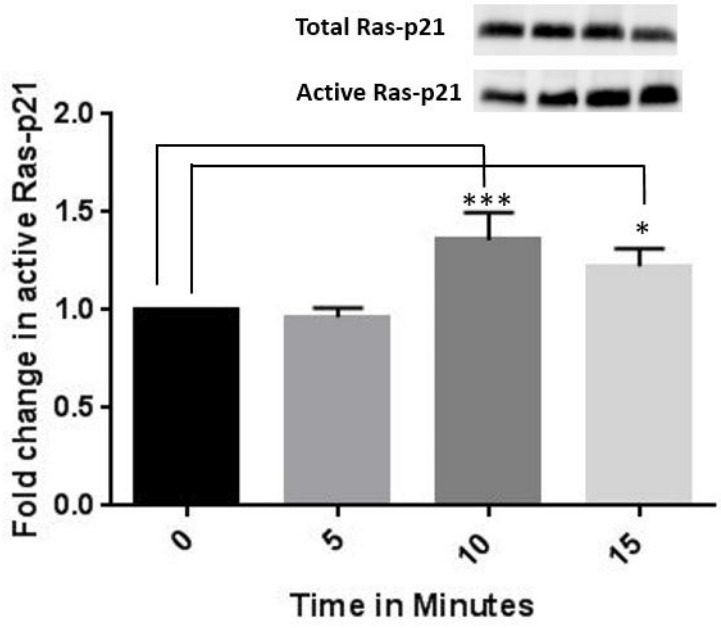

Figure 6. Effect of quinine on Ras p21 activity in MCF10A cells. MCF10A cells were cultured in DMEM high glucose (containing 4.0 mM L-glutamine) media supplemented with 5% horse serum, 1% penicillin streptomycin, 0.5 µg/ml hydrocortisone, 10 ng/ml EGF, and 10µg/ml insulin in 5% CO2 at 37˚C. When cells reached 60-70% confluency, they were serum starved for 24 hours in starvation media which is the same as non-starvation media deprived of serum, insulin and EGF. The cells were then treated with quinine (1 mM) for 0, 5, 10, and 15 minutes and lysed in lysis buffer. Active form of Ras p21 was pulled down using Raf-RBD (Ras p21 binding domain) after 1hour incubation of the lysates with beads at 4˚C with constant rocking. After washing

the beads with wash buffer, beads were pelleted down by centrifugation at 5,000 x g at 4˚C for 3 minutes. The final bead pellet was suspended in 25 µl 4X Laemmli's sample buffer and heated at 100˚C for 2 min. Western blot analysis was performed by using Anti-Pan Ras monoclonal antibody (1:250 v/v). The bands were quantified using ImageJ program. Each result was normalized against the total Ras p21 in the corresponding sample. The statistical significance of the data was analyzed using one-way ANOVA in Graphpad Prism6 software and values with *p-value < 0.05, ***p-value <0.001. The experiment was repeated a minimum of three times, and a representative blot is shown above.

5.3 Effect of quinine on activity of RalA in MCF10A cells

Since quinine caused activation of Ras p21 in MCF10A and this can cause activation of RalA via the Ras p21-RalGDS pathway, we investigated if quinine regulates RalA activity. Therefore, to understand the effect of quinine on RalA activation, MCF10A cells were treated with 1 mM quinine for different time points as follows, 0, 5, 10, and 15 minutes. Representative results show that there is a drastic decrease in the GTP-bound form of RalA in the presence of quinine in this study (Fig. 7).

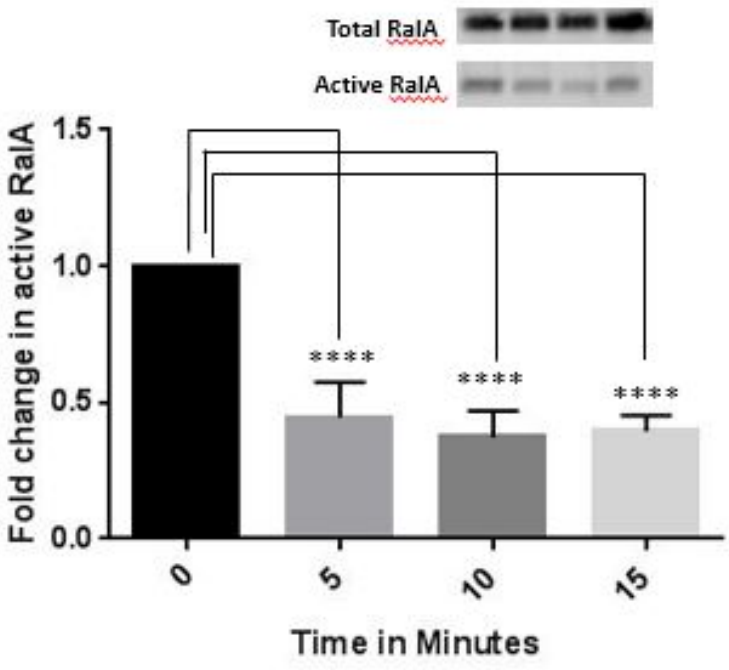

Figure 7. Effect of quinine on RalA activity in MCF10A cells. MCF10A cells were cultured in DMEM high glucose (containing 4.0 mM L-glutamine) media supplemented with 5% horse serum, 1% penicillin streptomycin, 0.5 µg/ml hydrocortisone, 10ng/ml EGF, and 10µg/ml insulin in 5% CO2 at 37˚C. When cells reached 60-70% confluency, they were serum starved for 24 hours in starvation media which is the same as non-starvation media deprived of serum, insulin and EGF. The cells were then treated with quinine (1 mM) for 0, 5, 10, and 15 minutes and lysed in lysis buffer. Active form of RalA was pulled down using RalBP1-RBD (RalA binding domain) after 1hour incubation of the lysates with beads at 4˚C with constant rocking. After washing the beads with wash buffer, beads were pelleted down by centrifugation at 5,000 x g at 4˚C for 3 minutes. The final bead pellet was suspended in 25 µl 4X Laemmli's sample buffer and heated at 100˚C for 2 min. Western blot was performed by using Anti-RalA monoclonal antibody (1:500 v/v). The bands were quantified using ImageJ program. Each result was normalized against the total RalA in the corresponding sample. The statistical significance of the data was analyzed using one-way ANOVA in Graphpad Prism6 software and values with ****p-value <0.0001. The experiment was repeated a minimum of three times, and a representative blot is shown above.

5.4 Effect of quinine on phosphorylation of ERK1/2 in MCF10A cells

The downstream effector in the Ras p21 pathway is the MAPK. Thus, we investigated if quinine mediated activation of Ras p21 resulted in ERK1/2 phosphorylation. Representative results show a significant increase in activation of ERK1/2 after 15 minutes incubation with quinine (Fig. 8).

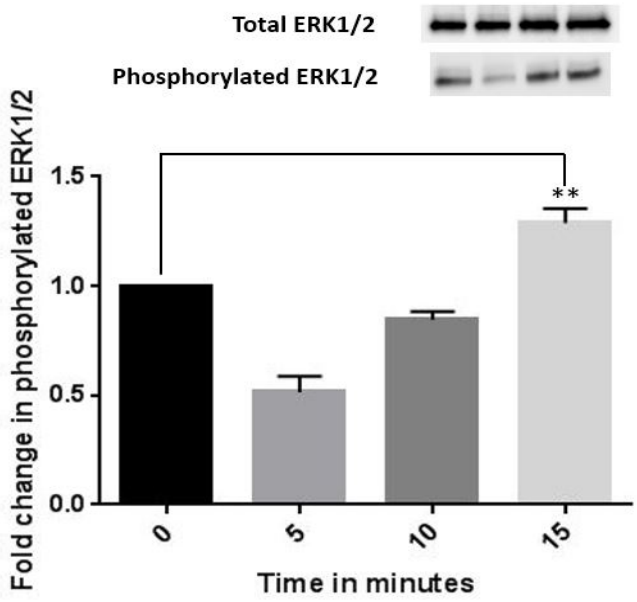

Figure 8. Effect of quinine on ERK1/2 phosphorylation in MCF10A cells. MCF10A cells were cultured in DMEM high glucose (containing 4.0 mM L-glutamine) media supplemented with 5% horse serum, 1% penicillin streptomycin, 0.5 µg/ml hydrocortisone, 10ng/ml EGF, and 10µg/ml insulin in 5% CO2 at 37˚C. When cells reached 60-70% confluency, they were serum starved for 24 hours in starvation media which is the same as non-starvation media deprived of serum, insulin and EGF. The cells were then treated with quinine (1mM) for 0, 5, 10, and 15 minutes, and lysed in lysis buffer. 20µg sample from each time point (0, 5, 10, and 15 minutes) treated with quinine were suspended in 10 µl 4X Laemmli's sample buffer and heated at 100°C for 2 min. Western blot analysis was performed by using p44/42 MAPK(Erk1/2) (L34F12) (1:2000 v/v) and Phospho-p44/42 MAPK(Erk1/2)(Thr202/Tyr204) (1:1000 v/v) monoclonal antibodies for quantifying total ERK1/2 and phosphorylated ERK1/2, respectively. The bands were quantified using ImageJ program. Each result was normalized against the total ERK1/2 in the sample. The statistical significance of the data was analyzed using one-way ANOVA in Graphpad Prism6 software and values with *p-value < 0.05, **p-value <0.01, and ****p-value <0.0001. The experiment was repeated a minimum of three times, and a representative blot is shown above.

5.5 Effect of quinine on activity of Ras p21 in MCF7 cells

In order to determine the effect of quinine on activation of Ras p21 in MCF7 cells (cancerous cell line), cells were incubated with 1 mM quinine for four different time point as for MCF10A cells (0, 5, 10, and 15 minutes). The results demonstrated a significant increase in active Ras p21 after 10 minutes incubation with 1 mM quinine (Fig. 9).

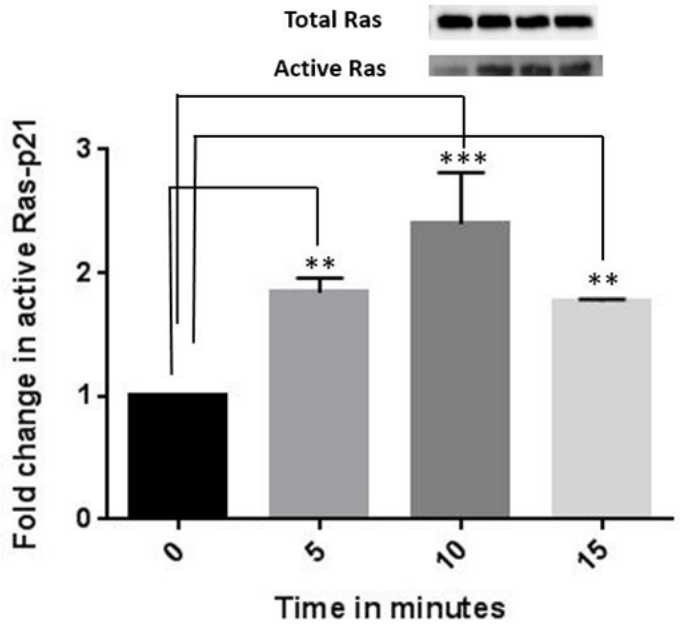

Figure 9. Effect of quinine on Ras p21 activity in MCF7 cells. MCF7 cells were cultured in DMEM high glucose (containing 4.0 mM L-glutamine) media supplemented with 10% FBS, 1% penicillin streptomycin in 5% CO_2 at 37˚C. When cells reached 60-70% confluency, they were serum starved for 24 hours in starvation media which is the same as non-starvation media deprived of serum. The cells were then treated with quinine (1mM) for 0, 5, 10, and 15 minutes and lysed in lysis buffer. Active form of Ras p21 was pulled down using Raf-RBD (Ras p21 binding domain) after 1hour incubation of the lysates with beads at 4˚C with constant rocking. After washing the beads with wash buffer, beads were pelleted down by centrifugation at 5000 x g at 4˚C for 3 minutes. The final bead pellet was suspended in 25 µl 4X Laemmli's sample buffer and heated at 100°C for 2 min. Western blot analysis was performed by using Anti-Pan Ras monoclonal antibody (1:250 v/v). The bands were quantified using ImageJ program. Each result was normalized against the total Ras p21 in the sample. The statistical significance of the data was analyzed using one-way ANOVA in Graphpad Prism6 software and values with **p-value < 0.01, ***p-value <0.001. The experiment was repeated a minimum of three times, and a representative blot is shown above.

5.6 Effect of quinine on activity of RalA in MCF7 cells

The activation of RalA in response to quinine was measured in MCF7 cells. The representative results demonstrated that quinine did not cause significant activation of RalA in MCF7 cells (Fig. 10).

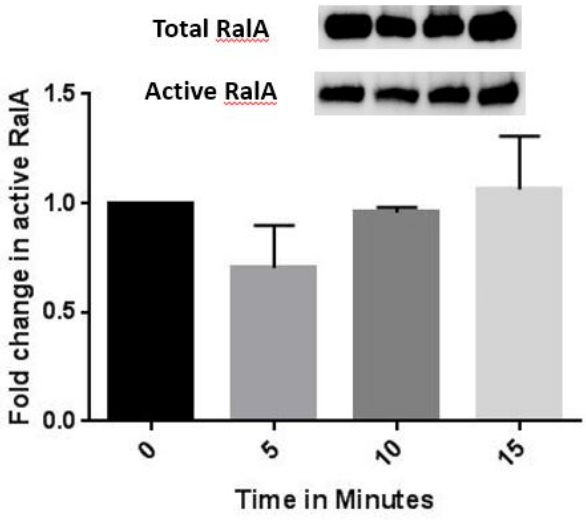

Figure 10. Effect of quinine on RalA activity in MCF7 cells. MCF7 cells were cultured in DMEM high glucose (containing 4.0 mM L-glutamine) media supplemented with 10% FBS, 1% penicillin streptomycin in 5% CO2 at 37˚C. When cells reached 60-70% confluency, they were serum starved for 24 hours in starvation media which is the same as non-starvation media deprived of serum. The cells were then treated with quinine (1mM) for 0, 5, 10, and 15 minutes and lysed in lysis buffer. Active form of Ras p21 was pulled down using RalBP1-RBD (RalA binding domain) after 1hour incubation of the lysates with beads at 4˚C with constant rocking. After washing the beads with wash buffer, beads were pelleted down by centrifugation at 5000 x g at 4˚C for 3 minutes. The final bead pellet was suspended in 25 µl 4X Laemmli's sample buffer and heated at 100˚C for 2 min. Western blot was performed by using Anti-Pan Ras monoclonal antibody (1:250 v/v). The bands were quantified using ImageJ program. Each result was normalized against the total Ras p21 in the corresponding sample. The statistical significance of the data was analyzed using one-way ANOVA in Graphpad Prism6 software and values with **p-value < 0.01, ***p-value <0.001. The experiment was repeated a minimum of three times, and a representative blot is shown above.

5.7 Effect of quinine on phosphorylation of ERK1/2 in MCF7 cells

Since quinine caused activation of Ras p21 in MCF7 cells, we investigated if this resulted in phosphorylation of ERK1/2. The results demonstrated a significant increase in ERK1/2 phosphorylation in response to quinine (Fig. 11).

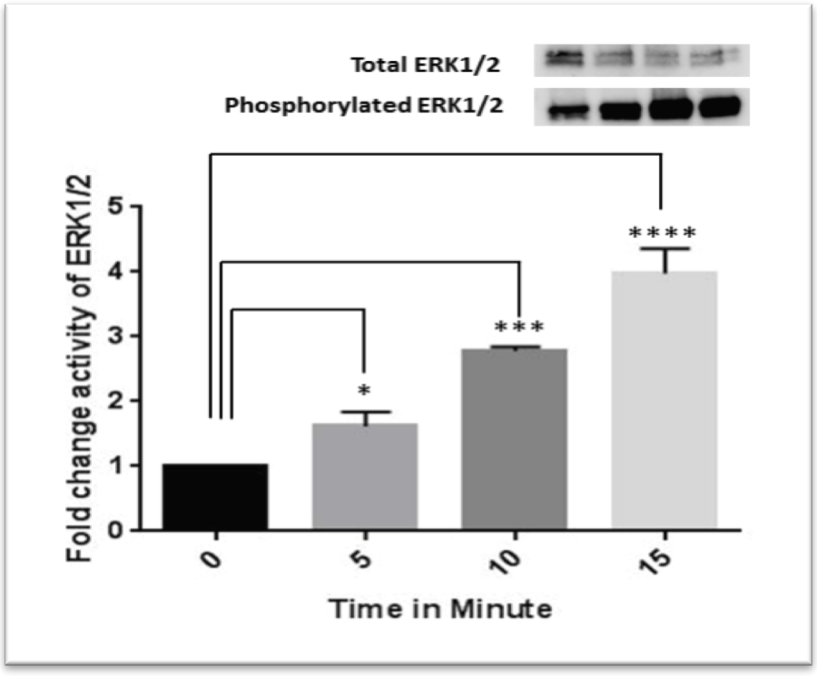

Figure 11. Effect of quinine on ERK1/2 phosphorylation in MCF7 cells. MCF7 cells were cultured in DMEM high glucose (containing 4.0 mM L-glutamine) media supplemented with 10% FBS, 1% penicillin streptomycin in 5% CO2 at 37˚C. When cells reached 60-70% confluency, they were serum starved for 24 hours in starvation media which is the same as non-starvation media deprived of serum. The cells were then treated with quinine (1mM) for 0, 5, 10, and 15 minutes and lysed in lysis buffer. 20µg of each time point samples (0, 5, 10, and 15 minutes) treated with quinine were suspended in 10 µl 4X Laemmli's sample buffer and heated at 100˚C for 2 min. Western blot was performed by using p44/42 MAPK(Erk1/2) (L34F12) (1:2000 v/v) and Phospho-p44/42 MAPK(Erk1/2)(Thr202/Tyr204) (1:1000 v/v) monoclonal antibodies for quantifying total ERK1/2 and phosphorylated ERK1/2, respectively. The bands were quantified using ImageJ program. Each result was normalized against the total ERK1/2 in the corresponding sample. The statistical significance of the data was analyzed using one-way ANOVA in Graphpad Prism6 software and values with *p-value <0.05, ***p-value <0.001, and ****p-value <0.0001. The experiment was repeated a minimum of three times, and a representative blot is shown above.

5.8 RalGDS expression in MCF10A and MCF7 cells

In order to identify the the presence of RalGDS in the mammary epithelial cell lines, the expression level of RalGDS was measured in both MCF10A and MCF7 cells. Representative results show RalGDS (95 kDa protein) is expressed in HEK293 cells as a positive control (Fig. 12, lane 1) and MCF10A cells (Fig. 12, lane 2); however, a weak band for RalGDS is observed in the MCF7 cell lysate (Fig. 12, lane 3).

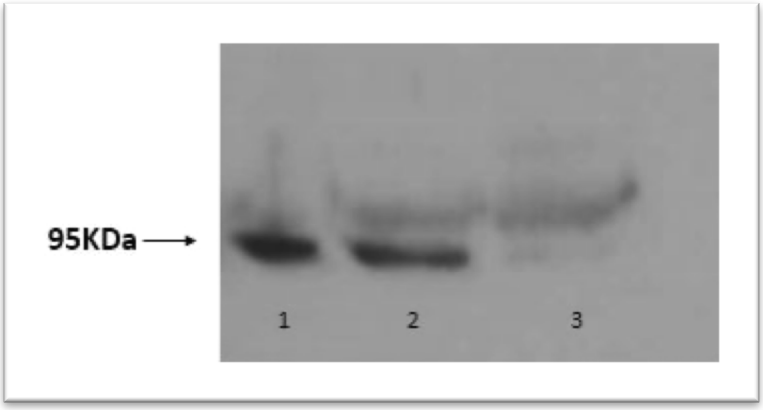

Figure 12. RalGDS expression level. 35 µg of HEK293 (positive control based on RalGDS antibody instruction, lane 1) MCF10A (lane 2) and MCF7 (lane 3) protein lysate were suspended in 10 µl 4X Laemmli's sample buffer and heated at 100˚C for 2 mins and were loaded on 8% SDS-PAGE gel. Western blot analysis was performed using RALGDS polyclonal antibody (1:500 v/v).

5.9 Molecular docking of Quinine interaction with RalA results

To explore the interaction of quinine with RalA, we performed molecular docking. The RalA is formed by the switch-II region (Ral70-Ral77), helix α2 (Ral78-Ral85) and one face of helix α3. This site forms the GDP binding pocket to RalA and putative site for

compounds. The crystal structures used in this study was of RalA-GDP (PDB code 2BOV, Fig. 13 a, b).

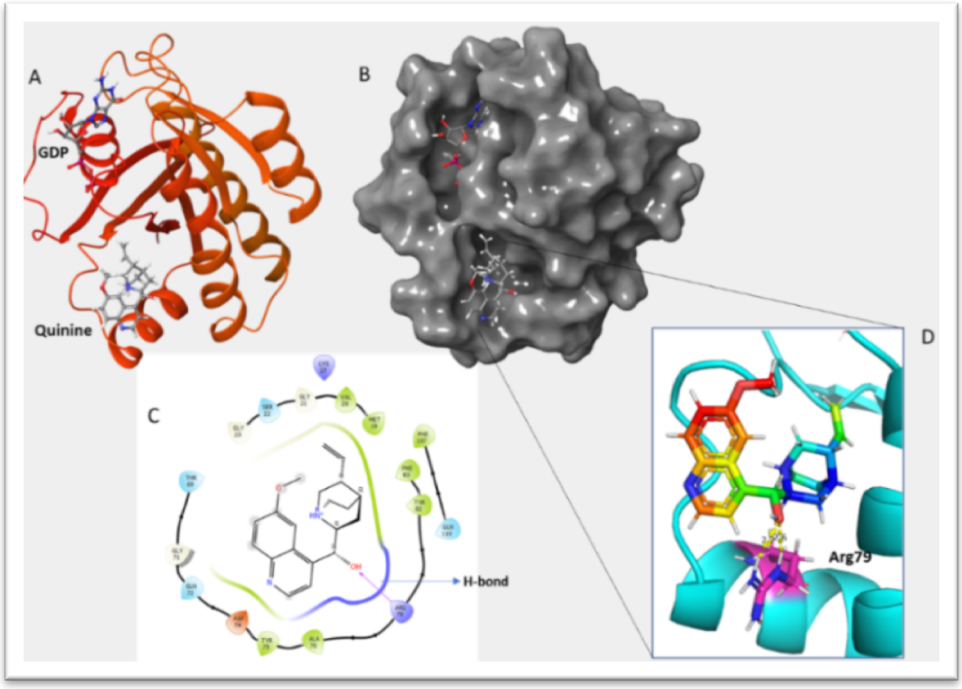

Figure 13. Crystal structure of RalA docked with ligand Quinine. Maestro v12.6 was used to perform molecular docking of quinine with RalA-GDP. The complex structure was generated with published structure of RalA-GDP (PDB: 2BOV) A. Ribbon model showing GDP and Quinine binding site. Both ligands are shown as stick representation B. Surface structure representation of docked complex showing the orientation of binding pockets C. Two- dimensional interaction map of Quinine with residues of RalA protein and arrangement of other amino acids around ligand D. Blown out image of Quinine and interacting residue Arginine at location 79 by H-bond.

The SiteMap module analyzed the druggable binding site with drug score of 0.7521 A^{o} and site score 0.8043 A^{o}. The area volumes for the quinine binding site were calculated 152.98 A^{3} (Fig. 14). The molecular docking of RalA with quinine was shown in Figure 10 C, D. The ligand interaction map shows Arginine 79 (R79) as the possible interacting residue with quinine. Quinine has quinoline and quinuclidine ring. The RalA-GDP-Quinine complex

structure shows that quinine is anchored by H-bond interaction by distance measurement of 2.2 Aº. The R79 residue is located on the flexible switch II loop region.

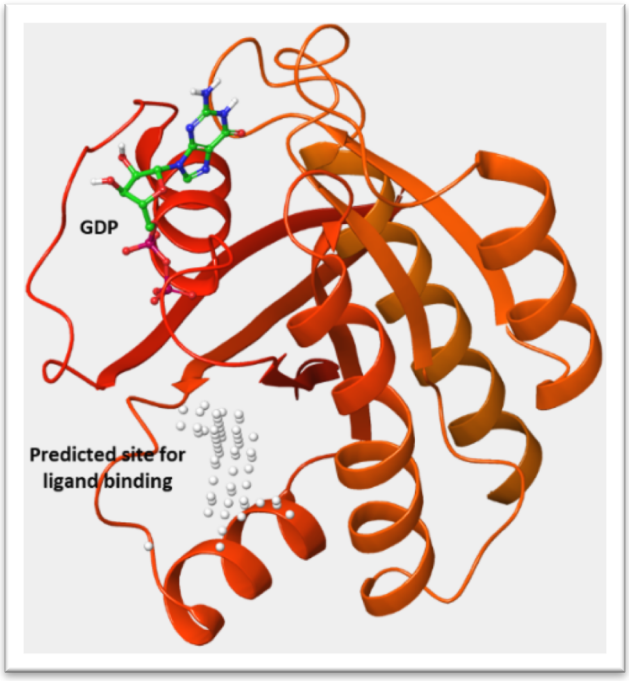

Figure 14. Predicted binding sites using SiteMap module. The Schrödinger's SiteMap module was used to determine the binding site of the compound on the prepared structure. The white sphere/surfaces indicated the putative binding area in the binding cavity.

6. Discussion

Ras p21 proteins as small GTPases are involved in transducing the extracellular signals to the effector pathways inside the cells. The combination of Ras p21 proteins with their GAPs and GEFs provide molecular switches cycling between ‘on’ and ‘off’ states as a result of binding to GTP or GDP, respectively (Bos et al. 2007).

Ras p21 pathway is reported as one of the most prevalently de-regulated pathways in cancer patients. Approximately 30% of all human tumors are caused by mutations in *RAS* genes (Maertens & Cichowski, 2014). However, direct targeting of the oncogenic Ras through various approaches has not been successful so far. Therefore, alternative approaches are required by targeting alternative Ras p21 downstream pathways such as RalA, which is involved in cell proliferation, cell survival and metastasis of various cancers in human (Yan et al. 2014).

Pivotal pieces of evidence have demonstrated that Ral GTPases also play an essential role in cancer which is both dependent and independent of Ras p21 (Gildea et al. 2002; Guin et al. 2013; Lim et al. 2006; Male et al. 2012; Rybko et al. 2011; Saito et al. 2013; Singhal et al. 2006; Smith et al. 2006; Spiczka and Yeaman 2008). The current study was designed to investigate the role of bitter taste agonist, quinine, in Ras-Ral pathway and analyze the regulation of the activity of Ras p21-Ral pathway.

As already mentioned, bitter taste receptors belong to the G protein-coupled receptors family. They have recently been reported to be expressed in various extra oral tissues and act as the mediator of signal transduction apart from the canonical bitter taste signaling pathway

(Gilca and Dragos 2017). Several bitter compounds have been shown to activate GPCRs; and quinine which we used in this study is reported as one of the ligands that can activate multiple GPCRs (Slack et al. 2010; Pydi et al. 2012; Singh et al. 2020).

The results of our study showed that quinine causes the activation of Ras p21 in MCF10A cells and MCF7 cells leading to the activation of the MAPK pathway, a downstream target of the Ras p21 pathway (Delire and Stärkel 2015).

As mentioned earlier, inhibiting Raf-MEK-ERK and PI3K/AKT-mTOR, which are the downstream effector pathways of Ras p21, has been the most effective strategy to target Ras signaling (Roberts and Der 2007; Yap et al. 2008). However, RalGDS-Ral signaling pathway has been reported as the third downstream effector of Ras signaling cascade in the last decade (Gentry et al. 2015). This has brought attention to developing new tools to manipulate Ral signaling, which is potentially considered as a therapeutic strategy to target Ras-related cancers in humans. RalGDS is a common intermediate between Ras p21 and Ral small GTPases and activation of Ras p21 can result in RalA activation via RalGDS.

Our results using MCF10A cells showed that RalA activity was inhibited in response to quinine stimulation even though Ras p21 is activated in these cells in response to quinine challenge. In MCF7 cells, quinine did not cause any change in RalA activity even though Ras p21 is activated. The results suggested that the Ras p21-RalGDS-RalA pathway may not be linked in these cells. This was despite the fact that our results confirmed the presence of RalGDS in MCF10A cells. There was very weak signal for RalGDS in MCF7 cells.

Our results suggest an alternative mechanism for the observed effects of quinine on RalA. One possible mechanism by which quinine might cause its effects is through direct

effect on RalA protein itself that is independent of bitter taste receptor activation. In 2017, Sidhu et al., demonstrated that quinine has a cell permeant nature and can cause direct effect on G protein activation (Sidhu et al. 2017). The other possibility is that quinine uncouples the RalGDS link between Ras p21 and RalA. This would disrupt Ras p21 activation from causing a corresponding RalA activation. It is also possible that quinine enhances the GAP activity of RalA which would explain the inhibition of RalA activity in MCF10A cells. The effect of quinine on RalA in MCF7 cells were different from those observed with MCF10A cells. Quinine did not cause any decrease in RalA activity in MCF7 cells. This could be due to the reason that MCF7 cells are metabolically active due to their oncogenic status and G proteins would be expected to be in an activated state. More studies are required to elucidate these mechanisms.

Our results pointed to a direct action of quinine on RalA. Thus, studies were carried out to investigate if RalA has the ability to physically interact with quinine. The modeling results showed that there is a possible interaction between quinine and RalA at Arginine 79 (R79). R79 is located in the flexible switch II loop region (residues 69-81) which causes conformational change during the GDP/GTP exchange cycle (Martin and Der 2012). We anticipate that effect of quinine on this conformational change may cause inhibition of RalA activation whether RalGDS is present in the cell. Site-directed mutagenesis studies may help elucidate the role of this residue in RalA-quinine interaction and its role in RalA inhibition.

On the other hand, it has been demonstrated that Calmodulin can cause RalA activation both in a calcium-dependent and -independent manner (Clough et al. 2002; Sidhu 2016). Considering the fact that quinine has structural similarity (Barry and Bernal 1993) to W.7.HCL (N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide), which is a calmodulin

antagonist, the possibility exists that quinine acts as an antagonist for calmodulin and results in RalA inhibition in MCF10A cells. Further studies needed to elucidate more these mechanisms.

7. Conclusion

Quinine cause the activation of Ras p21 and ERK1/2 in both MCF10A and MCF7 cells. However, it inhibited RalA activity on MCF10A cells and had no effect of MCF7 cells. These effects were not mediated through RalGDS, suggesting that quinine may have a direct effect on RalA activity in these cell lines.

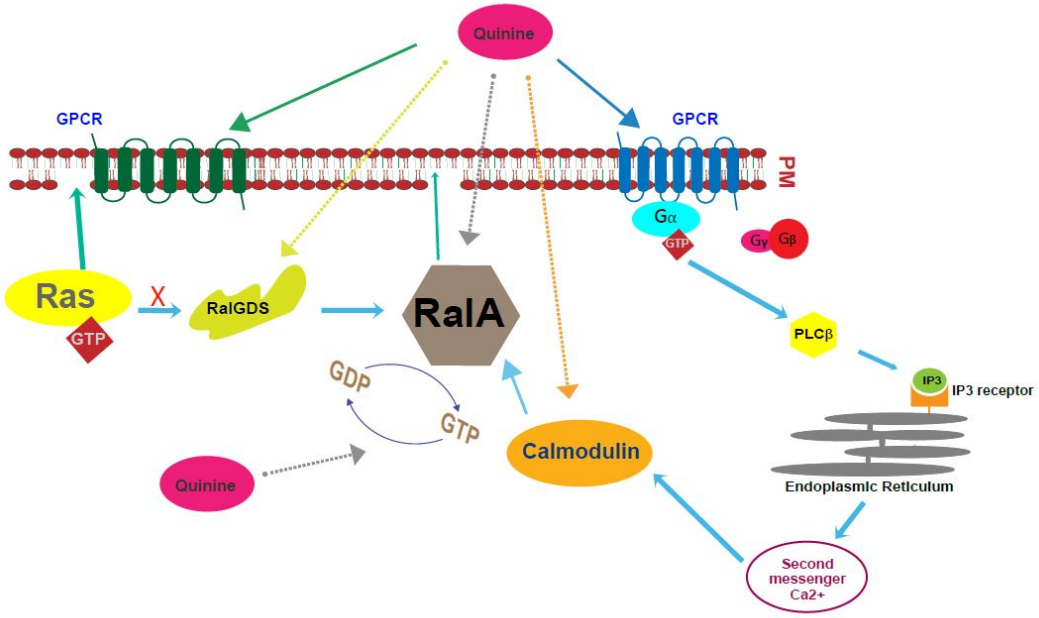

Figure 15. Proposed model for RalA regulation through GPCR-dependent and -independent pathways. The model shows that in MCF10A cells, the inhibition of RalA in the presence of quinine may be due to its effect on enhancing the intrinsic GTPase activity of RalA or activating a RalGAP. It is also possible that RalA activity is inhibited due to the action of quinine as an antagonist for calmodulin, a calcium binding protein implicated in RalA activation. An alternative mechanism can be a direct action of quinine on RalA that results in RalA inhibition in MCF10A cells. Quinine has no significant effect on RalA activity in MCF7 cells.

8. Future directions

8.1 Investigate the receptor-mediated activation of Ras p21 by quinine

Site directed mutagenesis and/or bitter taste receptor inhibitors can be used to clarify whether quinine causes Ras p21 activation through GPCRs or whether it acts directly due to its membrane-permeability properties.

8.2 Investigate the effect of quinine on regulation of RalGDS in mammary epithelial cells

More studies are needed to determine the effect of quinine on RalGDS and to investigate whether quinine disrupts the Ras p21/RalGDS pathway and inhibits the corresponding RalA activation.

8.3 Evaluate the receptor independent effects of quinine on RalA regulation

In order to determine the possibility of physical interaction between RalA and quinine, site directed mutagenesis assays are needed to elucidate whether quinine regulates RalA activation directly. *In vitro* studies can be carried out to demonstrate direct interaction between RalA and quinine.

8.4 Investigate if Ras p21/RalGDS/Ral pathway is active in mammary epithelial cells

In order to determine if the Ras p21/RalGDS/Ral is active in MCF10A and MCF7 cells, Ras p21 activating agonist such as EGF can be used to treat the cells and analyze the effect on this pathway.

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