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Anti-mitogenic Effect of Bitter Taste Receptor Agonists on Airway Smooth Muscle Cells

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Abstract

Airway remodeling is a hallmark feature of asthma and COPD. Clinical studies and animal models have demonstrated increased airway smooth muscle (ASM) mass, and ASM thickness is correlated with severity of the disease. Current medications control inflammation and reverse airway obstruction effectively yet have limited effect on remodeling. Recently we identified the expression of bitter taste receptors (TAS2R) on ASM cells, and activation with known TAS2R agonists resulted in ASM relaxation and bronchodilation. These studies suggest that TAS2R can be used as new therapeutic targets in the treatment of obstructive lung diseases. To further establish their effectiveness, in this study we aimed to determine the effects of TAS2R agonists on ASM growth and pro-mitogenic signaling. Pre-treatment of healthy and asthmatic human ASM cells with TAS2R agonists resulted in a dose-dependent inhibition of ASM proliferation. The anti-mitogenic effect of TAS2R ligands was not dependent upon activation of PKA, PKC, or high/intermediate conductance calcium activated K^+ channels. Immunoblot analyses revealed that TAS2R agonists inhibit growth factor-activated Akt phosphorylation without affecting the availability of phosphatidylinositol-3,4,5-trisphosphate, suggesting TAS2R agonists block signaling downstream of PI3K. Furthermore, the anti-mitogenic effect of TAS2R agonists involved inhibition of induced transcription factors (AP-1, STAT3, E2F, NFAT) and inhibition of expression of multiple cell cycle regulatory genes suggesting a direct inhibition of cell cycle progression. Collectively, these findings establish the anti-mitogenic effect of TAS2R agonists and identify a novel class of receptors and signaling pathways that can be targeted to reduce or prevent airway remodeling as well as bronchoconstriction in obstructive airway disease.

Keywords: Asthma, bitter taste receptor, airway remodeling, GPCR, TAS2R

Introduction

G protein-coupled receptor (GPCR) signaling plays a vital role in the regulation of airway smooth muscle (ASM) contraction, relaxation, and proliferation (4; 12). Exaggerated presentation of pro-contractile GPCR agonists in the airways during allergic inflammation contributes to bronchoconstriction in obstructive airway disease such as asthma. Another salient feature of inflammatory airway diseases is airway remodeling that is characterized by excessive proliferation and accumulation of resident cells including ASM cells. Animal models demonstrate ASM mass is increased by allergic airway inflammation, while human studies demonstrate a progressive increase in ASM mass in asthmatics that increases both dynamic and fixed airway resistance, limiting the effectiveness of current rescue bronchodilators (11; 21; 22; 26). Current anti-asthma therapies, including beta-agonists and corticosteroids, aim at alleviating bronchoconstriction and inflammation, respectively, but have a very limited effect on remodeling (21). Thus increase in ASM mass occurs unimpeded in asthmatics irrespective of how effectively asthma symptoms are managed. In addition to their lack of effect *in vivo*, both corticosteroids and beta-agonists have limited efficacy in inhibiting ASM proliferation by relevant mitogens in cell-based assays (5; 6; 10; 18; 21; 28; 30; 47; 55). Our previous studies have demonstrated that PKA plays a central role in mediating the functional effects of beta-agonists on ASM (37; 39; 55). However, beta-agonist-stimulated PKA activity in ASM appears constrained by beta-2-adrenoceptor (β_2AR) desensitization (4; 13), rendering beta-agonists relatively weak anti-mitogenic agents. Thus, the collective clinical and basic science findings to date support the need to identify new drugs that effect both ASM relaxation and inhibition of growth via novel and robust pathways.

Recently we identified expression of Type 2 taste receptors (TAS2Rs) known as bitter taste receptors ~~(BTRs)~~ on human ASM cells and demonstrated that stimulation of these receptors with known TAS2R agonists results in intracellular calcium elevation and, somewhat paradoxically, relaxation of ASM (14). Three independent laboratories have confirmed the airway relaxation effect of TAS2R agonists using mouse (51; 56), human (2; 14; 19) and guinea pig (44) airways. Aerosol exposure of airways to TAS2R agonists results in bronchodilation in normal and allergen- sensitized and challenged mice. In a recent study by Robinett et al. using asthmatic ASM cells and lung slices, TAS2R expression, signaling, and ASM relaxation and bronchodilatory effects were not altered under airway inflammatory conditions (1; 46). These studies suggest that agonists of TAS2Rs possess unique properties that can be exploited as a new class of anti-asthma drugs (16; 34). Studies to date of TAS2Rs in airways/ASM have focused on investigating acute effects of TAS2R agonists of airway resistance and ASM contraction. In the current study, we investigated the anti-mitogenic effects of chronic exposure of ASM cells to TAS2R agonists, and identify TAS2R agonists as potential novel therapeutics capable of modulating two important pathogenic mechanisms of asthma.

Experimental procedures

Materials

Antibodies against vasodilator-stimulated phosphoprotein (VASP) were from BD Biosciences (San Jose, CA, USA), and phospho-p42/p44, p38, p70S6K, cyclin D, and phospho-Akt antibodies were from Cell Signaling Technology (Beverly, MA, USA). IRDye 680 or 800 secondary antibodies were from Rockland (Gilbertsville, PA, USA). CyQUANT cell proliferation assay kit and propidium iodide were from Life Technologies (Grand Island, NY, USA). Papain dissociation kit, collagenase, and elastase were purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA). Quantitative PCR arrays and SYBR green reagents were purchased from RealTime Primers (Elkins Park, PA, USA) and Applied Biosystems (Grand Island, NY) respectively. Lentivirus expressing luciferase reporter was obtained from SABiosciences (Valencia, CA). Chloroquine, quinine, saccharine and other materials were obtained from Sigma (St. Louis, MO, USA) or from previously identified sources (11, 13).

Cell culture

Human ASM cultures were established from human tracheae or primary bronchi using an enzyme dissociation method (42). Human tracheae or bronchi were obtained from either National Disease Research Interchange or from human lung resection surgery and autopsy performed at Thomas Jefferson University under a protocol approved by the Thomas Jefferson University Institutional Review Board. Briefly, ~0.5 g of wet tissue was obtained from trachealis muscle under sterile conditions. The tissue was minced and resuspended in 5 ml of Earle's Balanced Salt Solution (EBSS) buffer containing papain and DNase, and incubated at

115 37°C for 45 min. Collagenase (5 mg), elastase (10 U/ml) and 125 mg BSA were added to the
116 tissue. Enzymatic dissociation of the tissue was performed for 45-60 min in a shaking water bath
117 at 37°C. The cell suspension was transferred to a new tube and cells separated by centrifugation.
118 The cell pellet was resuspended in EBSS containing ovomucoid inhibitor and the cell suspension
119 was slowly overlaid on the ovomucoid solution in a new tube. Cells were separated by
120 centrifugation and resuspended in Ham's F-12 complete medium containing 10% fetal bovine
121 serum (FBS; HyClone, Logan, UT) and 100 U/ml of penicillin, 0.1 mg/ml of streptomycin, and
122 amphotericin B.

123 ASM cells in subculture during the second through fifth cell passages were used. These
124 cells retain functional signaling pathways that are important in mediating ASM excitation and
125 contraction as determined by agonist-induced changes in cytosolic calcium (42). The cells were
126 maintained in F-12 medium with no serum and supplemented with 5.7 μg/ml insulin and 5 μg/ml
127 transferrin (arresting medium) for 48-72 h before the experiments. A select set of experiments
128 were carried out using human ASM cells isolated from severe asthmatics (obtained from the
129 laboratory of Dr. Reynold Panettieri, University of Pennsylvania, PA).

130 *Retroviral and lentiviral infection*

131 Stable expression of GFP, PKI-GFP, and RevAB-GFP was achieved by retroviral
132 infection, as described previously (15; 20; 29; 55). Briefly, retrovirus for the expression of each
133 was produced by cotransfecting GP2–293 cells with pVSV-G vector (encoding the pantropic
134 VSV-G envelope protein) and either pLNCX2-GFP, or pLNCX2-PKI-GFP, and viral particles
135 were harvested from supernatant. ASM cultures were infected with retroviral particles and
136 selected to homogeneity (typically >95% GFP-positive, as demonstrated in ref. (20)) with 250

μg/ml G418. Stable lines expressing GFP exhibited properties similar to those of uninfected naive cells with respect to mitogen-stimulated DNA synthesis and cell proliferation, as reported previously (20; 30).

Cignal Lenti luciferase reporter viral particles for different transcription factors were purchased from SA Biosciences and ASM cultures were infected with lentivirus as per manufacturer's recommendation. Stable lines were selected using puromycin and maintained in complete medium containing selection antibiotic.

Cell proliferation assay

Cells, naive or stably selected after retroviral infection as described above, were plated in either a 96-well plate (CyQUANT assay), or 6-well plate (cell count, flow cytometry) and maintained in complete Ham's F-12 medium supplemented with 10% FBS. After 24 h, cells were switched to arresting medium and treated with growth factors (10% FBS, 10 ng/ml PDGF or 10 nM EGF). 30 minutes before adding growth factors cells were pretreated with different bitter taste receptor agonists: chloroquine, quinine and saccharin, at concentrations noted in the Results section. After 72 h treatment with growth factors with vehicle or TAS2R agonists, media was changed to assay buffer containing CyQuant dye and fluorescence intensity measured as per manufacturer's instructions. In some experiments cells were pretreated with the PKC inhibitor Bis I (5 or 50 μM) or calcium-activated potassium channel inhibitors IbTx (10 or 100 nM) or TRAM-34 (10 or 100 nM) prior to treatment with growth factor +/- TAS2R agonists.

In an additional set of experiments, cells grown on 6-well plates treated as mentioned above were harvested by trypsinization and cell counts determined using a Coulter counter (Beckman Coulter, Fullerton, CA, USA).

Propidium iodide staining after treating cells with growth factor +/- TAS2R agonists for 24 hours was performed as per (45). Briefly, human ASM cells were grown in F12 complete medium supplemented with 10% FBS and antibiotics. Sub-confluent cells were serum starved for 48 h and incubated in fresh medium containing PDGF with or without TAS2R agonists (250 μM), cells harvested by trypsinization at 24 h and fixed in cold 70% ethanol. After counting, ~500,000 cells were treated with RNAse and stained with propidium iodide (BD Pharmingen, San Jose, CA) for cell-cycle analysis. The samples were analyzed by flow cytometry (FACScan, BD Pharmingen) and Flowjo commercial software.

Immunoblotting

Cells were grown to near confluence in 6-well plates and growth arrested for 72 h in serum-free Ham's F-12/IT medium as described above. The cells were then stimulated with indicated TAS2R agonists for 15 min followed by PDGF or EGF for 30 min. In a select set of experiments, cells were treated as described above for 12 or 24 h. Cells were then washed twice with ice-cold buffer (25 mM Tris and 150 mM NaCl, pH 8.0) then solubilized in a 25 mM Tris buffer (pH 8.0) containing 150 mM NaCl, 20 mM NaF, 5 mM EGTA, 1 mM EDTA, 10 mM sodium pyrophosphate, 10 mM p-nitrophenyl phosphate, 1 mM benzamidine, 0.1 M phenylmethylsulfonyl fluoride, and 1% (v/v) Nonidet P-40 (lysis buffer) for 30 min at 4°C. Following scraping, cell lysates were centrifuged at 13,200 g at 4°C for 10 min. Supernatants were collected, then electrophoresed on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and subsequently probed with the indicated primary antibodies and secondary antibodies conjugated with infrared fluorophores (15).

Luciferase (Luc) reporter assay

For luciferase assays, human ASM cells were stably transfected with different luciferase constructs using lentivirus as described above, then harvested and plated into 24-well plates. The following luciferase constructs were investigated: CRE, STAT3, E2F, C/EBP, SRE, Myc, NFκB, NFAT, Smad, and AP-1. Cells were treated with vehicle or PDGF with or without TAS2R agonists for 8, 12 or 24 h. Cells were subsequently harvested in lysis buffer, protein concentration determined and equal amount of total protein loaded directly in the well with a reaction mix containing firefly luciferase substrate (Bright-Glo Luciferase Assay System, Promega, Madison, WI, USA) as per manufacturer's instructions. Luminescence [relative light units (RLU)/well] was quantified by a microplate luminometer. RLU data was normalized using total protein loaded onto to each well.

RNA isolation, RT-PCR and Real-Time PCR array

Cells grown on 6-well plates were treated with PDGF or vehicle with or without pretreatment with TAS2R agonists for 24 h and total RNA harvested using Trizol method as described in our previous studies (36; 48). Total RNA (1 μg) was converted to cDNA by RT reaction and the reaction stopped by heating the samples at 94^{o} C for 5 min. Real-Time PCR array for cell cycle genes (catalog # HCC-1) was performed using SYBR green master mix as per the manufacturer's recommendation using Applied Biosystems real time PCR machine. Raw Ct values were obtained using software recommended threshold fluorescence intensity. RNA expression data was calculated as described previously using internal control gene β-actin (14; 48).

Cellular phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) lipid production

Phosphatidylinositides are cell membrane components and key molecules for growth factor activation and PI3K signaling. Human ASM cells plated on 15 cm plates were stimulated with PDGF with or without pretreatment with chloroquine and quinine for 30 min and phosphatidylinositides were extracted using chloroform/methanol (1:2, v/v), and PIP3 concentration determined by Cova-PIP ELISA (Echelon Biosciences Inc) as per manufacturers' instructions and as described previously (52).

Statistical analysis

Data are presented as mean ± SE values from n experiments, in which each experiment was performed using a different ASM culture derived from a unique donor. Individual data points from a single experiment were calculated as the mean value from 3 replicate observations for CyQuant assay, cell proliferation assay, flow cytometry, and luciferase assay. Data from ASM growth assays and luciferase assay were calculated and reported as fold change from basal or vehicle treated group. For immunoblot analyses, band intensities representing signals from secondary antibody blots conjugated with infrared fluorophores were visualized and quantified directly using the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE, USA). These values were normalized to values determined for β-actin or GAPDH and compared among stimuli and experimental groups. Statistically significant differences among groups were assessed by either analysis of variance (ANOVA) with Fisher's PLSD post hoc analysis using Prism Graphpad software (Graphpad, La Jolla, CA, USA), with values of $p < 0.05$ sufficient to reject the null hypothesis.

Results

TAS2R agonists inhibit airway smooth muscle growth

TAS2R *agonists inhibit airway smooth muscle growth* - In this study, we used three different mitogens (FBS, PDGF, and EGF) to induce ASM growth and determined the effect of three different TAS2R agonists (chloroquine (Chloro), quinine (Quin) and saccharin (Sacch)) on mitogen-induced ASM growth. ASM growth was determined using CyQuant assay. Pretreating human ASM cells with chloroquine or quinine significantly inhibited FBS- (66 and 74%, respectively) (Figure 1A), PDGF- (78 and 66%, respectively) (Figure 1B), or EGF (79 and 48%, respectively) (Figure 1C)-induced ASM growth in a dose-dependent manner (Figure 1). Saccharin was less effective in inhibiting ASM growth, yet significantly inhibited FBS, PDGF and EGF-induced ASM growth by 40, 60 and 33%, respectively, but only at the highest pretreatment concentration of 300 μM.

Because a recent study has demonstrated that TAS2R expression and signaling is not altered under inflammatory conditions in human airways (46), and ASM from asthmatics have been shown to proliferate at a higher rate than the healthy controls (43), we tested the growth inhibitory effect of TAS2R agonists on asthmatic ASM cells. PDGF-induced ASM growth ~~was higher~~ in asthmatic ASM cells (Figure 1 D) and TAS2R agonists significantly inhibited this induction.

To explore whether the anti-mitogenic actions of TAS2R agonists are mediated via their effect on cell hypertrophy or hyperplasia, we assessed regulation of mitogen-induced increases in ASM cell number by standard cell counting. PDGF treatment resulted in a significant increase in human ASM cell counts, and TAS2R agonists chloroquine, quinine and saccharin inhibited this

hyperplasia by 79 ± 3, 41 ± 9, and 37 ± 3%, respectively (Figure 2). Changes in cell size (hypertrophy) were determined by forward scatter analysis using flow cytometry. There was no significant effect of either PDGF or TAS2R agonists on ASM cell size as assessed by forward scatter analysis using flow cytometry (data not shown).

TAS2R agonist-mediated anti-mitogenic effect does not involve PKA or PKC

We have recently demonstrated that PKA mediates the anti-mitogenic effect of several agents on ASM proliferation (37; 39; 55). To assess the potential role of PKA in the growth inhibitory effect of TAS2R agonists, we stably expressed PKI, a PKA inhibitory peptide, in ASM cultures as described previously (36; 37; 55). PDGF-induced ASM growth was similarly inhibited by TAS2R agonists, chloroquine and quinine, in both GFP and GFP-PKI expressing ASM cultures (Figure 3A). Further, PKA activation was assessed by determining phosphorylation of VASP and luciferase assay using CRE-luc expressing cells. In GFP-expressing cells, stimulation of cells with isoproterenol, but not chloroquine, resulted in phosphorylation of VASP as indicated by the mobility shift from 46 to 50 kDa (Figure 3B). Isoproterenol-induced VASP phosphorylation was significantly attenuated in PKI-GFP expressing cells confirming our previous observations. Treatment of ASM cells stably expressing CRE-luc with TAS2R agonists for 12 h did not result in any change in the expression of CRE-induced luciferase (Figure 3C). As predicted, isoproterenol, prostaglandin E_2 and forskolin treatment induced expression of luciferase robustly (Figure 3C, right panel). These findings suggest that PKA does not play a role in the TAS2R agonist-mediated anti-mitogenic effect on ASM.

TAS2R signaling in ASM involves activation of PLC and release of calcium from intracellular stores. Diacylglycerol (DAG) produced by PLC in turn activates PKC. To assess the potential role of PKC in the TAS2R agonist-induced anti-mitogenic effect on ASM, cells were pretreated with 5 or 50 μM Bis I, a pan-PKC inhibitor. Both concentrations failed to reverse the TAS2R agonist-induced growth inhibitory effect (Figure 4A and B). These findings suggest that PKC does not play a role in mediating the anti-mitogenic effect of TAS2R agonists.

TAS2R agonists are known to induce membrane hyperpolarization potentially mediated via calcium-activated K^+ channels in ASM when stimulated acutely. We therefore examined whether a change in ~~electrical~~ the activity of intermediate/high-conductance calcium activated K^+ channels across the ASM plasma membrane plays a role in the regulation of ASM growth by TAS2R agonists. Pretreatment with IbTX, an inhibitor of large conductance calcium-activated potassium channels, did not affect the anti-mitogenic effect of chloroquine or quinine (Figure 4C, D). Pretreatment of cells with another potassium channel inhibitor (intermediate conductance), TRAM-34, similarly did not inhibit the anti-mitogenic effect of chloroquine and quinine (data not shown).

Effect of TAS2R agonists on mitogenic signaling in ASM

Because mitogenic signaling in ASM involves activation of MAP kinases (ERK and p38) (24; 27; 40), we assessed the regulatory effect of TAS2R agonists on MAP kinase activity in human ASM cells. Stimulation of human ASM cells with PDGF or EGF resulted in increased phosphorylation of p42/p44 and p38 MAP kinase, as reported previously (37; 55). TAS2R agonists did not inhibit PDGF or EGF-induced activation of p42/p44 and p38 MAP kinase

(Figure 5). ~~Similarly, growth factors activated p38 MAP kinase and TAS2R agonists had no effect on this activation (Figure 5).~~

Further, we assessed the effect of TAS2R agonists on the PI3K pathway by determining phosphorylation of (downstream) Akt. PDGF and EGF treatment resulted in an increased phosphorylation of Akt, and TAS2R agonists chloroquine and quinine significantly inhibited this phosphorylation (Figure 6). Previous studies have demonstrated that p70S6 kinase is a critical effector of mitogenic signaling mediated by receptor tyrosine kinases, GPCRs, and PI3K in ASM (3; 29; 32). As previously demonstrated, PDGF and EGF stimulation resulted in increased activation of p70S6 kinase. The TAS2R agonists chloroquine and quinine both significantly inhibited mitogen-induced activation of p70S6 kinase (~75% and 90% for PDGF, 73% and 69% for EGF, respectively; Figure 6). Saccharin was less effective in inhibiting phosphorylation of either Akt (28% for PDGF and 37%) or p70S6 kinase (48% for PDGF and 77% for EGF).

To test whether TAS2R agonists directly block phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), production, we measured cellular production of PIP_3 lipids after TAS2R agonists' chloroquine and quinine treatment. PDGF stimulation increased PIP_3 lipids significantly in human ASM cells. Yet, TAS2R agonists did not inhibit PIP_3 production (Figure 7), suggesting TAS2R agonists blocked Akt kinase phosphorylation at a point downstream of PI3K activation in ASM cells. Thus, the growth inhibitory effect of TAS2R agonists does not appear to be mediated by the regulation of phospholipid accumulation upstream of PI3K.

Collectively, these data suggest that the anti-mitogenic effect of TAS2R agonists in ASM involves inhibition of Akt kinase and S6 kinase, yet does not involve regulation of calcium-

activated potassium channel activity, PIP_3 accumulation, or PKA, PKC, p42/p44 or p38 pathways.

TAS2R agonists inhibit activation of transcription factors capable of stimulating cell growth

ASM growth is promoted by growth factors, chemokines and inflammatory cytokines, and involves activation of multiple intracellular signaling cascades that ultimately induce several key transcription factors involved in regulating cell proliferation (9). Using luciferase reporter assays, we investigated activation of ten (CRE, STAT3, E2F, C/EBP, SRE, Myc, NFκB, NFAT, Smad, and AP-1) different transcription factors to gain further insight into the mechanisms mediating the anti-mitogenic effects of TAS2R agonists. As shown in Figure 8, PDGF-induced activation of AP-1, E2F, STAT3, and NFAT, and the TAS2R agonists chloroquine and quinine significantly inhibited activation of each of these transcription factors (Figure 8). Activation of CRE, SRE, NFkB, and Smad were not affected by TAS2R agonists. STAT3 activation reflects induction of PI3K signaling by PDGF, and TAS2R agonists inhibit this response consistent with our results from immunoblot analyses. An AP-1 reporter was used to determine the effect of TAS2R agonists on MAP kinase signaling. Although TAS2R agonists did not inhibit acute p42/p44 or p38 activation by growth factors, the luciferase assay data suggest that TAS2R agonists inhibit MAP kinase signaling under chronic treatment conditions. Inhibition of E2F activation by TAS2R agonists indicate inhibition of cell cycle progression induced by growth factors. To further confirm the transcriptional activation, we carried out real-time PCR arrays using cell cycle gene arrays. Table 1 depicts a list of genes that were upregulated at least 2-fold by PDGF, and inhibited by TAS2R agonists chloroquine or quinine. Cell cycle genes such as cyclins, cyclin-dependent kinases, G2/S-phase expressed gene, and cell division cycle 2, and proliferation markers such as proliferating cell nuclear antigen (PCNA) and Ki-67 were the

notable genes induced by PDGF, with all inhibited by TAS2R agonists. These real-time PCR data were further confirmed by assessing regulation of cyclin D protein. PDGF induced expression of cyclin D protein in a time-dependent manner, and TAS2R agonists inhibited this induction (Figure 9). Collectively these data suggest that the anti-mitogenic effect of TAS2R agonists involves inhibition of cell cycle proteins in human ASM cells.

TAS2R agonists inhibit cell cycle progression

Findings from real-time PCR studies suggested that TAS2R agonists inhibit expression of cell cycle regulatory genes. We further analyzed the effect of TAS2R agonists on ASM cell cycle regulation using propidium iodide staining to assess the proportion of cells in G0 or G2/M/S phase. Pretreatment with chloroquine and quinine significantly decreased the proportion of cells in G0. PDGF treatment resulted in a modest but significant increase in the proportion of cells in S or G2/M phase suggesting mitosis (Figure 10). Pretreatment with TAS2R agonists chloroquine and quinine resulted in a higher proportion of cells in S and G2/M phases, suggesting that TAS2R agonists inhibit cell cycle progression.

Discussion

In this study we establish that TAS2R agonists inhibit human ASM proliferation induced by a wide range of mitogens, and do so through a mechanism distinct from other known ASM anti-mitogenic agents. The TAS2R agonists chloroquine and quinine both inhibited ASM proliferation induced by FBS, PDGF, or EGF, whereas saccharine, previously demonstrated to be a relatively weak TAS2R agonist (35), showed a modest anti-mitogenic effect. These anti-mitogenic effects were associated with a reduction in mitogen-induced PI3K and p70 S6 kinase activity yet had no effect on PKA or PKC activity, PIP3 accumulation, p42/p44 or p38 MAPK signaling. We further found that TAS2R agonists inhibited the induction of multiple pro-mitogenic transcription factors by PDGF, including AP-1, STAT3, NFAT and E2F, as well as the induction of specific genes involved in cell cycle regulation. STAT3 activation reflects induction of PI3K signaling by PDGF and TAS2R agonists inhibit this response, consistent with our finding our TAS2R agonist inhibition of Akt phosphorylation (Akt phosphorylation occurs downstream of PI3K activation). Regulation of AP-1 reporter activity was used to further assess the regulatory effect of TAS2R agonists on mitogenic pathways. Although TAS2R agonists did not inhibit acute p42/p44 or p38 activation by growth factors, inhibition of PDGF-induced AP-1 reporter activity suggests that TAS2R agonists inhibit MAP kinase signaling that occurs with chronic mitogen treatment. Growth factors mediating ASM growth activate both MAP kinase PI-3 kinase signaling and presumably regulate gene expression and cell growth via activation of multiple transcription factors. In fact, PI3K signaling is known to play a major role in the regulation of both nonasthmatic and asthmatic ASM cell growth (3; 7; 25; 31; 33; 38; 50; 57). Thus, a strong inhibitory effect of TAS2R agonists on PI3K signaling and inhibition of multiple transcription factor activation is predicted to mitigate inflammation-induced airway smooth

muscle remodeling in asthma. Inhibition of E2F activation by TAS2R agonists indicates inhibition of cell cycle progression induced by growth factors. This was further supported by strong inhibition of cell cycle genes induced by PDGF.

TAS2R agonists failed to induce PKA activity, evidenced by lack of cytosolic PKA substrate (VASP) phosphorylation or PKA-dependent transcriptional activity, and PKA inhibition had no effect on TAS2R inhibition of ASM proliferation. This is in agreement with the previous studies using human, guinea pig and murine airways that demonstrated a lack of PKA involvement in effecting ASM relaxation and bronchodilation. Beta agonists, mainstay asthma drugs, inhibit mitogen-stimulated increases in cell number or DNA synthesis in cultured ASM cells by only ~25%, whereas the more effective PKA activator PGE_2 is a much stronger (~75% inhibition) anti-mitogen (37; 55). Our previous studies have demonstrated that both ASM relaxation, as well as the modest anti-mitogenic effect of beta agonists, is primarily mediated via activation of PKA. TAS2R agonists on the contrary do not generate cAMP and do not activate PKA in mediating ASM relaxation, and our current findings similarly reveal the anti-mitogenic effect of TAS2R agonists to be PKA-independent. Therefore, TAS2Rs represent a novel class of asthma targets that mediate beneficial effects via a distinct mechanism.

Further, our findings establish that the TAS2R mediated anti-mitogenic effect does not involve activation of either PKC or membrane hyperpolarization. TAS2R signaling involves activation of phospholipase C resulting in accumulation of DAG and activation of PKC. Our previous studies have demonstrated that TAS2R agonist stimulation results in hyperpolarization of the ASM membrane (8; 14). Membrane potential is known to be involved in the regulation of cell proliferation; hyperpolarization is associated with a quiescent cell phenotype. However, our results indicate that ~~membrane potential~~ activation of intermediate/high conductance calcium

activated K^+ channel does not play a major role in mediating the anti-mitogenic effect of TAS2R agonists.

Bitter taste receptors are expressed on human, murine, and guinea pig ASM and at least 5-6 subtypes are expressed at a mid-high level (14; 19; 44; 51; 56). TAS2Rs are activated by a variety of structurally diverse chemical agents. Promiscuity of receptor activation by different ligands is evident in airways as well. High throughput screening of different bitter tastants using HEK293 cells expressing all the known human bitter taste receptors (also known as TAS2Rs) revealed that chloroquine and quinine bind to at least 3 subtypes of TAS2Rs expressed on human ASM cells and therefore may act as full agonists by eliciting a response via all the three subtypes. Saccharine on the other hand binds to only one subtype (35), which likely contributes to its relatively weak anti-mitogenic effect in human ASM. It is also possible that certain subtypes of TAS2Rs may activate different signaling mechanisms leading to a differential effect on ASM proliferation and anti-mitogenic signaling. Chloroquine and quinine demonstrated different level of inhibition of growth factor-induced gene expression in ASM cells presumably due to differences in the activation of signaling by different subtypes of TAS2Rs. We also recognize that the studies do not address signaling via any specific subtype of TAS2R due to a lack of sensitive tools to address receptor specificity. There are no well-characterized, commercially available antagonists of TAS2Rs. Additional medicinal chemistry and computational modeling studies are needed to develop novel antagonists of TAS2R. Furthermore, TAS2Rs have evolved as low affinity and low specificity receptors (41) and therefore, require μM concentrations of the agonists to activate these receptors. Similar ranges of concentrations are reported in studies using heterologous expression models as well (35).

414 Additional medicinal chemistry and computational modeling studies are needed to develop novel
415 high affinity agonists and antagonists of TAS2R.

416 In this study we focused primarily on investigating cell hyperplasia and hypertrophy as
417 potential cellular mechanisms by which ASM growth is regulated. However, ASM growth is also
418 regulated by additional mechanisms such as apoptosis and necrosis. ASM cells undergo
419 apoptosis under various conditions and a decreased rate of apoptosis has been reported to
420 contribute to excessive ASM mass in asthma (21). Recent studies demonstrated that statins
421 inhibit ASM growth by inducing apoptosis of ASM cells (17). Effects of TAS2R agonists on
422 ASM mass could also be due to cytotoxicity or necrosis. Future studies will address additional
423 cellular mechanisms involved in the anti-mitogenic effect of TAS2R agonists.

424 Airway remodeling continues to be a major clinical problem as none of the anti-asthma
425 medications used currently for clinical management of asthma symptoms effectively mitigate
426 features of airway structural changes (21; 22). The current findings demonstrate that TAS2R
427 agonists inhibit mitogen-induced growth both in normal and asthmatic ASM cells. Under in-vitro
428 conditions, beta-agonists modestly inhibit ASM growth (30; 55), and no clinical evidence exists
429 supporting an *in vivo* anti-mitogenic effect of β-agonists. Clinical studies using biopsy samples
430 obtained from asthmatics suggested no effect of long acting beta agonists on ASM mass (21).
431 One study has suggested leukotriene receptor antagonists possess growth inhibitory effects in
432 animal models (23), yet no human studies have provided evidence for an anti-remodeling effect
433 of leukotriene receptor antagonists (21; 28). Interestingly, a recent *in vitro* study by Trian et al.,
434 using human bronchial epithelial and smooth muscle cell (obtained from severe persistent
435 asthmatics) co-culture model demonstrated that epithelium-generated paracrine factors including
436 leukotrienes regulate ASM proliferation that could be inhibited by pre-treating cells with

leukotriene receptor antagonist, montelukast (53). Additional *in vivo* studies are needed to further ascertain the effect of leukotriene receptor antagonists on airway remodeling in asthmatics. Beta agonists are the drug of choice for managing acute exacerbations, but several problems associated with the use of beta agonists such as tachyphylaxis, individual variations in responsiveness, and safety concerns (54) have been noted. The recent discovery of taste receptor expression in ASM and the bronchodilatory effect of TAS2R ligands raise the possibility of a novel class of safe, effective anti-asthma medications.

Interestingly, TAS2Rs are also expressed on ciliary epithelium and activation of these receptors results in an increased ciliary beat frequency suggesting that TAS2R agonists are useful in clearing mucus during airway inflammation (49). The findings from the present study demonstrate, for the first time, the anti-mitogenic effect of TAS2R agonists. Future *in vivo* studies are needed to corroborate these *in vitro* findings. Collectively, the findings to date suggest TAS2R agonists represent an exciting new class of anti-asthma drugs, based on their capacity to address multiple features of asthma pathology, including bronchospasm, airway mucus accumulation, and airway remodeling.

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Conflict of interest

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Effect of bitter taste receptor (TAS2R) agonists on mitogen-induced ASM growth. Human ASM cells were pretreated with different concentrations of chloroquine (Chloro), quinine (Quin) or saccharine (Sacch) for 15 min and treated with FBS (A), PDGF (B) or EGF (C) for 72 h. ASM cells obtained from severe asthma patients were treated with PDGF with or without pre-treatment with TAS2R agonists (D). Total DNA content was determined by CyQuant assay and data presented as fold change in fluorescence from baseline. Note a significant (* $p<0.05$, n=6) inhibition of growth factor-induced ASM growth by TAS2R agonists. B-basal, F-FBS, E-EGF, P-PDGF.

Figure 2. Bitter taste receptor agonists inhibit PDGF-induced ASM hyperplasia. Human ASM cells were pretreated with 50 or 100 μM chloroquine (Chloro), quinine (Quin) or saccharine (Sacch) and PDGF-induced hyperplasia was determined by cell count. Note a significant (* $p<0.05$) decrease in the ASM cell number by bitter tastants (n=6). ~~Forward scatter analysis using flow cytometer revealed no effect of TAS2R agonists on ASM size (data not shown).~~

Figure 3. Role of PKA in TAS2R-induced anti-mitogenic effects on ASM. We used human ASM cells stably expressioning PKI-GFP chimera or GFP alone and assessed cell growth by CyQuant assay (A). Pre-treatment with 100 μM Chloroquine (Chloro) or Quinine (Quin), inhibited FBS (left) or PDGF (right)-induced ASM growth in both GFP and PKI-GFP expressing ASM cells. cAMP/PKA activation in ASM cells was further assessed by western blotting (B) and CRE-Luc assay (C). TAS2R agonists treatment of ASM cells did not activate PKA as determined by phosphorylation of VASP in GFP cells (B). Isoproterenol was used as a positive

control. Stimulation of ASM cells for 8 h with Chloro and Quin did not activate CRE-Luc (C). Isoproterenol, prostaglandin E2 and forskolin (FSK) robustly induced CRE-Luc activation. (NS: non-significant; n=3-5). Collectively, these data suggest that TAS2R agonists do not activate cAMP/PKA pathway in ASM cells.

Figure 4. Role of PKC and calcium-activated potassium channels in TAS2R-induced anti-mitogenic effect on ASM. Human ASM cells were pretreated with vehicle, PKC inhibitor Bis I (A, B), or calcium activated potassium channel inhibitor IbTx (C, D) for 15 min followed by treatment with 100 μM chloroquine (Chloro) or quinine (Quin), and PDGF- (A and C) and FBS- (B and D) induced ASM growth was determined using the CyQuant assay. Inhibition of PKC or calcium-activated potassium channel did not affect anti-mitogenic effect of TAS2R agonists (NS: non-significant; n=5).

Figure 5. Immunoblot analysis of effects of TAS2R agonists on mitogenic (MAPK) signaling in ASM. Human ASM cells were pretreated with chloroquine (Chloro), quinine (Quin) or saccharine (Sacch) for 15 min and stimulated with PDGF (left) or EGF (right) for 30 min, and lysates were harvested and subjected to immunoblot analysis for phospho-p42/44 (top), p38 (bottom) (A). GAPDH expression was used as internal control. Shown are the representative images (A). Densitometric analysis of western blot images suggests that TAS2R agonists do not inhibit PDGF or EGF induced activation of ERK or p38 MAP kinase in ASM cells (n=4) (B).

Figure 6. Effects of TAS2R agonists on PI3K and S6 kinase signaling in ASM. Human ASM cells were pretreated with chloroquine (Chloro), quinine (Quin) or saccharine (Sacch) and

stimulated with PDGF (left) or EGF (right), lysates were harvested and subjected to immunoblot analysis for phospho-Akt and phospho-p70S6K. Expression of β-actin was used as loading control. Shown are the representative western blot images (A). Densitometric analysis of western blot images from multiple experiments (n=5) suggests that TAS2R agonists significantly (* $p<0.05$) inhibit PDGF or EGF induced phosphorylation of Akt and p70S6K (B).

Figure 7. Effect of TAS2R agonists on the induction of phosphatidylinositol-3,4,5-trisphosphate (PIP3). Human ASM cells were stimulated with PDGF with or without pretreatment with 250 μM chloroquine (Chloro) and quinine (Quin) for 30 min and PIP3 concentration were determined by ELISA. PDGF stimulated PIP3 induction was unaffected by TAS2R agonists (* $p<0.05$, n=4).

Figure 8. Inhibition of multiple transcription factors by TAS2R agonists. Human ASM cells stably expressing luciferase under the control of STAT3 (A), E2F (B), NFAT (C) and AP-1 (D) were treated with PDGF with or without pretreatment with chloroquine (Chloro), quinine (Quin) or saccharine (Sacch), and luciferase activity assessed after 24 h by a luminometer. Note a significant inhibition of PDGF-induced transcriptional activation by TAS2R agonists Chloro and Quin (* $p<0.05$, n=3-5).

Figure 9. TAS2R agonists inhibit expression of cyclin D1 in ASM cells. PDGF treatment resulted in an increased expression of cyclin D1 at 12 h (data not shown) or 24 h, and Chloroquine (Chloro) and quinine (Quin) inhibited this response, (* $p<0.05$, n=3-5). Top: representative western blot image, Bottom: densitometric analysis.

710 **Figure 10.** Human ASM cell cycle analysis. Using flow cytometry and propidium iodide
711 staining we determined the proportion of cells in G0, S and G2/M phase of cell cycle after
712 treating cells with PDGF +/- TAS2R agonists. Chloroquine (Chloro) and quinine (Quin)
713 pretreatment decreased proportion of G0 cells (A) and increased cells in S (B) and G2/M (C)
714 phases of cell cycle (* $p<0.05$, n=4).
715

Genes inhibited by choroquine and quinine treatment				
Gene name	**PDGF (P)**	**P+Chloroquine**	**P+Quinine**	**P+Saccharin**
Baculoviral IAP repeat-containing 5 (survivin)	8.64 ± 2.29	0.22 ± 0.09	3.82 ± 1.19	9.44 ± 2.17
Breast cancer 2, early onset	4.92 ± 1.66	0.21 ± 0.07	2.36 ± 0.92	7.25 ± 4.97
Cyclin A2	6.37 ± 2.7	0.18 ± 0.06	3.08 ± 1.1	8.52 ± 4.3
Cyclin B1	4.72 ± 2.15	0.64 ± 0.14	2.77 ± 0.95	5.86 ± 3.5
Cyclin B2	6.02 ± 1.7	0.22 ± 0.1	3.42 ± 1.24	7.91 ± 2.6
Cyclin D1	10.71 ± 6.38	2.36 ± 1.25	3.62 ± 1.9	13.05 ± 9.9
Cyclin D2	1.39 ± 0.25	0.22 ± 0.07	1.60 ± 0.53	0.88 ± 0.16
Cyclin E2	11.43 ± 3.35	0.97 ± 0.28	5.62 ± 0.8	11.05 ± 5.7
Cell division cycle 2 G1 to S and G2 to M	8.48 ± 2.93	0.09 ± 0.03	3.93 ± 1.7	14.25 ± 7.28
Cycle division cycle 20 homolog (S. cerevisiae)	11.79 ±7.9	0.95 ± 0.26	3.52 ± 1.05	10.64 ± 3.7
Cyclin-dependent kinase 2	2.59 ± 0.81	0.46 ± 0.11	2.31 ± 0.76	1.78 ± 0.37
CDK inhibitor 3 (CDK2-associated dual specificity phosphatase)	5.05 ± 1.6	0.30 ± 0.07	3.23 ± 1.71	3.78 ± 1.3
CDC28 protein kinase regulatory subunit 1B	2.34 ± 0.72	0.44 ± 0.09	1.78 ± 0.56	1.73 ± 0.36
DEAD/H box polypeptide 11 (CHL1-like helicase homolog , S. cerev	2.64 ± 1.12	0.29 ± 0.12	2.19 ± 0.61	3.08 ± 0.63
Kinetochore associated 1	3.44 ± 1.1	0.25 ± 0.18	2.44 ± 0.7	2.65 ± 0.63
Karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	2.84 ± 0.8	0.78 ± 0.19	2.67 ± 0.87	1.99 ± 0.6
MAD2 mitotic arrest deficient-like 1 (yeast)	4.65 ± 1.46	0.45 ± 0.13	2.83 ± 0.94	4.05 ± 0.81
MCM2 minichromosome maintenance deficient 4 (S. cerevisiae)	3.92 ± 1.3	0.32 ± 0.10	2.89 ± 0.93	2.65 ± 0.44
MCM2 minichromosome maintenance deficient 5, cell division cycle	5.93 ± 2.2	0.06 ± 0.02	3.56 ± 0.88	3.43 ± 0.68
Antigen identified by monoclonal antibody Ki-67	10.17 ± 2.91	0.14 ± 0.09	4.56 ± 1.7	10.72 ± 3.49
Proliferating cell nuclear antigen	2.34 ± 0.8	0.23 ± 0.07	1.64 ± 0.49	1.53 ± 0.4
RAD51 homolog (RecA homolog E. coli) (S. cerevisiae)	6.62 ± 1.8	0.66 ± 0.5	4.62 ± 1.22	5.71 ± 1.4
Retinoblastoma-like 1 (p107)	3.14 ± 0.8	0.18 ± 0.14	2.68 ± 0.95	2.22 ± 0.67
Genes not sensitive to either chloroquine or quinine treatment				
Cyclin A1	4.98 ± 1.64	4.98 ± 1.93	5.09 ± 1.58	4.61 ± 1.67
Cyclin E1	2.01 ± 0.48	1.03 ± 0.14	2.31 ± 0.88	1.19 ± 0.22
Cyclin F	2.61 ± 0.76	0.20 ± 0.10	2.18 ± 0.5	1.89 ± 0.36
Cycle division cycle 34 homolog (S. cerevisiae)	1.38 ±0.39	0.56 ± 0.03	2.51 ± 1.07	0.53 ± 0.08
CDKinhibitor 2B (p15, inhibits CDK4)	2.34 ± 1.25	1.38 ± 1.02	2.92 ± 1.31	1.39 ± 0.69
MCM2 minichromosome maintenance deficient 2, mitotin (S. cerevis	3.54 ± 0.92	0.41 ± 0.28	3.36 ± 1.02	2.56 ± 0.52
MCM2 minichromosome maintenance deficient 3 (S. cerevisiae)	2.77 ± 0.7	0.27 ± 0.10	2.38 ± 0.69	1.90 ± 0.30
Retinoblastoma binding protein 8	2.26 ± 0.4	0.82 ± 0.18	2.31 ± 0.44	1.24 ± 0.09

Table 1: Effect of TAS2R agonists on genes up-regulated by PDGF in human ASM cells. Human ASM cells were treated with PDGF +/- chloroquine (Chloro) or quinine (Quin) and total RNA harvested after 24 h. Gene expression was assessed by real-time PCR using cell cycle real-time PCR gene arrays. Shown in the table are the genes up-regulated ≥2 folds above the basal by PDGF. Note several cell cycle regulatory genes were induced by PDGF and TAS2R agonists inhibited the expression of these genes (n=5).

Figure 1

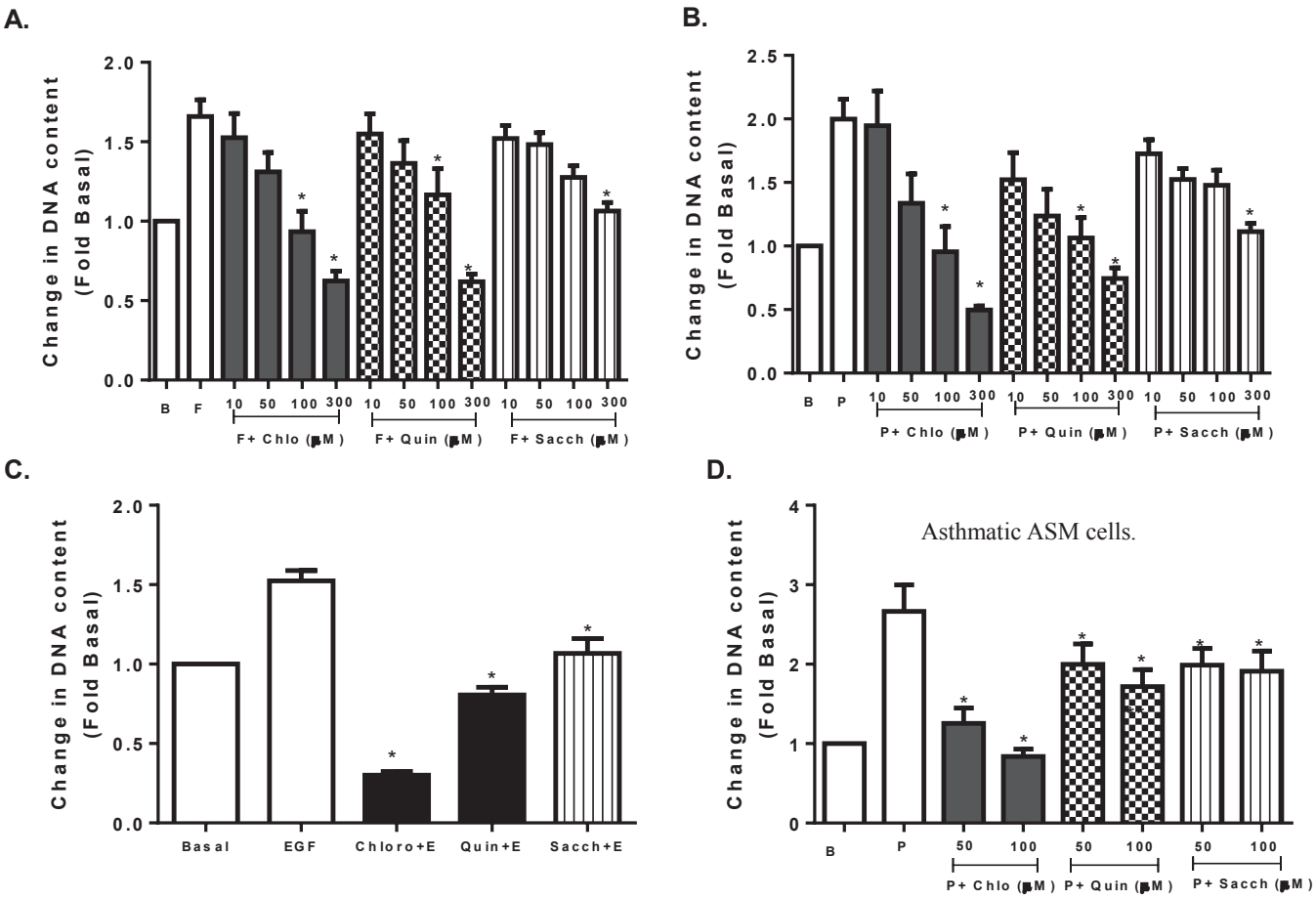

A.
Change in DNA content (Fold Basal)
2.0
1.5
1.0
0.5
0.0
B F 10 50 100 300 10 50 100 300 10 50 100 300
F+ Chlo (μM) F+ Quin (μM) F+ Sacch (μM)
B.
Change in DNA content (Fold Basal)
2.5
2.0
1.5
1.0
0.5
0.0
B P 10 50 100 300 10 50 100 300 10 50 100 300
P+ Chlo (μM) P+ Quin (μM) P+ Sacch (μM)
C.
Change in DNA content (Fold Basal)
2.0
1.5
1.0
0.5
0.0
Basal EGF Chloro+E Quin+E Sacch+E
D.
Asthmatic ASM cells.
Change in DNA content (Fold Basal)
4
3
2
1
0
B P 50 100 50 100 50 100
P+ Chlo (μM) P+ Quin (μM) P+ Sacch (μM)

Figure 2

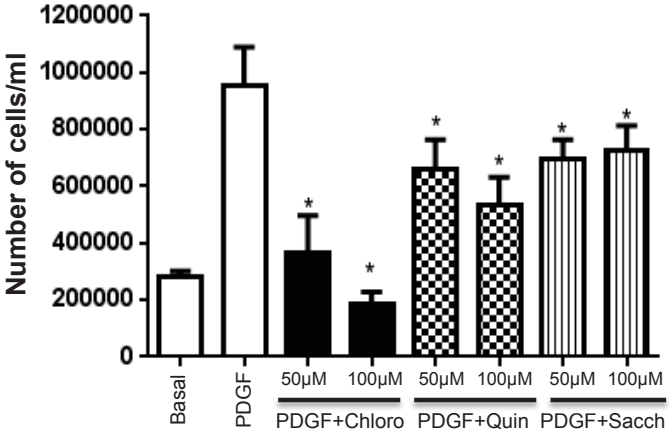

Number of cells/ml
1200000
1000000
800000
600000
400000
200000
0
Basal
PDGF
50μM
100μM
50μM
100μM
50μM
100μM
PDGF+Chloro
PDGF+Quin
PDGF+Sacch

Figure 3

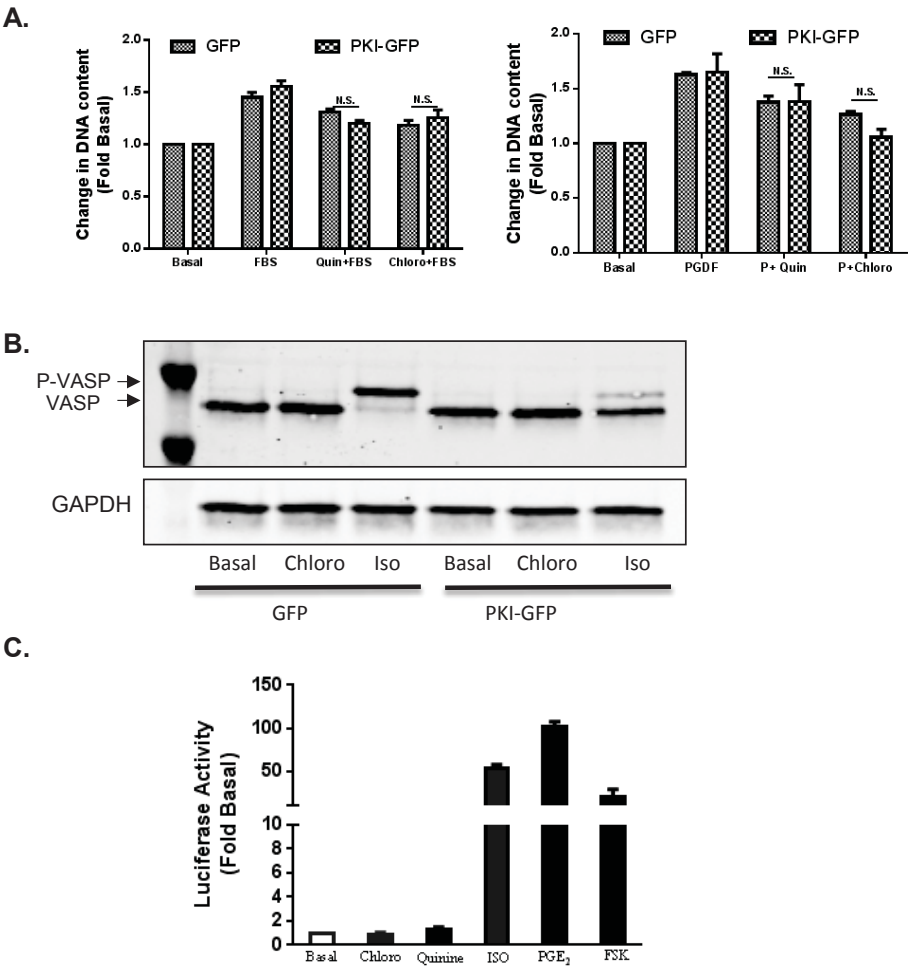

A.
GFP
PKI-GFP
Change in DNA content (Fold Basal)
2.0
1.5
1.0
0.5
0.0
N.S.
N.S.
Basal
FBS
Quin+FBS
Chloro+FBS
GFP
PKI-GFP
Change in DNA content (Fold Basal)
N.S.
N.S.
Basal
PGDF
P+ Quin
P+Chloro
B.
P-VASP
VASP
GAPDH
Basal
Chloro
Iso
Basal
Chloro
Iso
GFP
PKI-GFP
C.
Luciferase Activity (Fold Basal)
150
100
50
10
8
6
4
2
0
Basal
Chloro
Quinine
ISO
PGE2
FSK

Figure 4

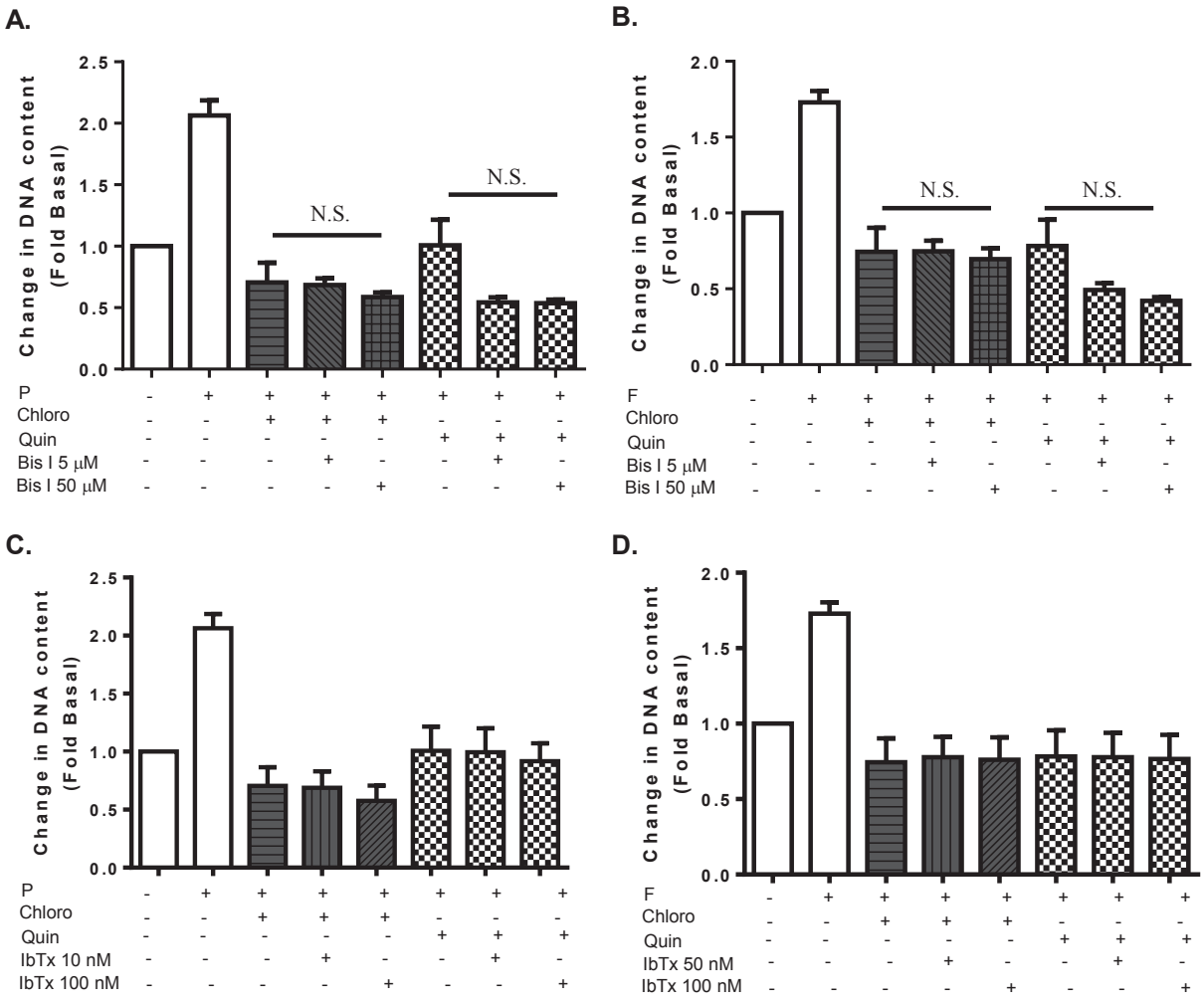

A.
Change in DNA content (Fold Basal)
2.5
2.0
1.5
1.0
0.5
0.0
N.S.
N.S.
P - + + + + + + +
Chloro - - + + + - - -
Quin - - - - - + + +
Bis I 5 μM - - - + - - + -
Bis I 50 μM - - - - + - - +
B.
Change in DNA content (Fold Basal)
2.0
1.5
1.0
0.5
0.0
N.S.
N.S.
F - + + + + + + +
Chloro - - + + + - - -
Quin - - - - - + + +
Bis I 5 μM - - - + - - + -
Bis I 50 μM - - - - + - - +
C.
Change in DNA content (Fold Basal)
2.5
2.0
1.5
1.0
0.5
0.0
P - + + + + + + +
Chloro - - + + + - - -
Quin - - - - - + + +
IbTx 10 nM - - - + - - + -
IbTx 100 nM - - - - + - - +
D.
Change in DNA content (Fold Basal)
2.0
1.5
1.0
0.5
0.0
F - + + + + + + +
Chloro - - + + + - - -
Quin - - - - - + + +
IbTx 50 nM - - - + - - + -
IbTx 100 nM - - - - + - - +

Figure 5

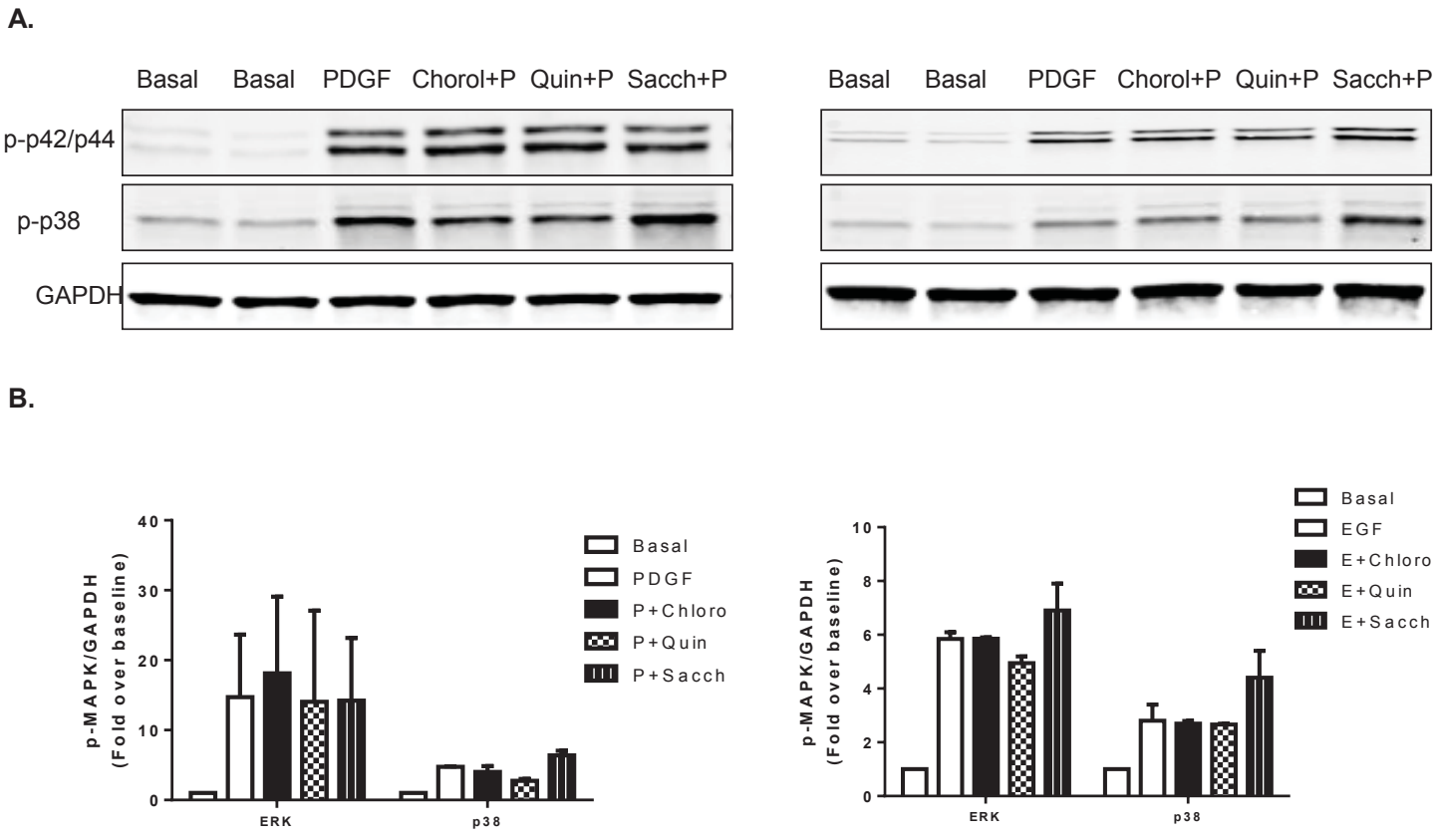
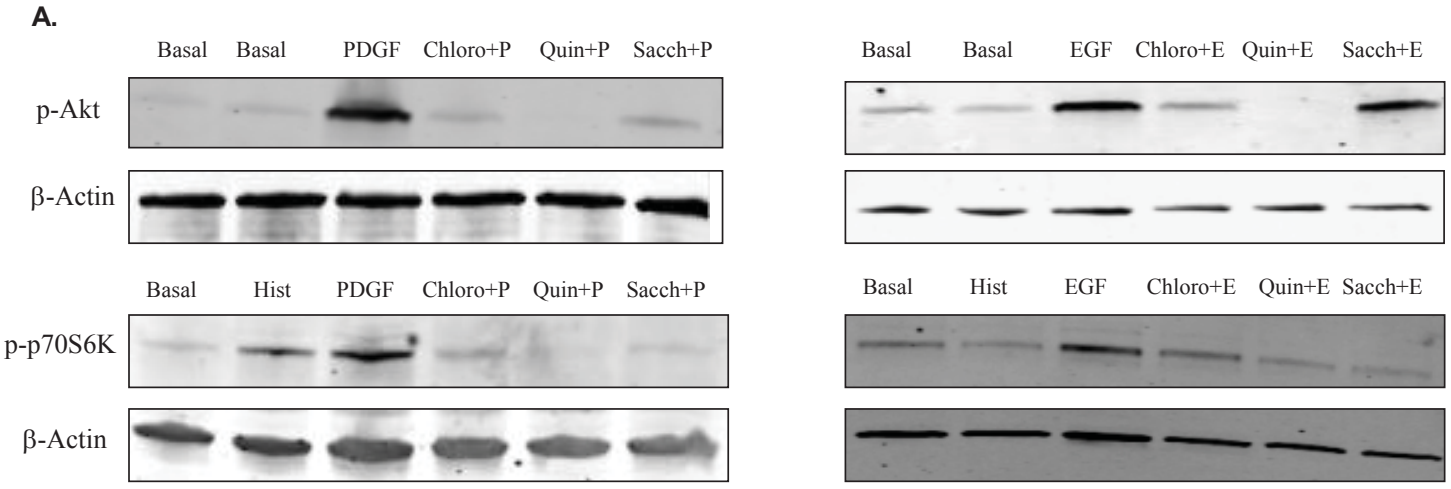

A.
Basal
Basal
PDGF
Chorol+P
Quin+P
Sacch+P
p-p42/p44
p-p38
GAPDH
Basal
Basal
PDGF
Chorol+P
Quin+P
Sacch+P
B.
p-MAPK/GAPDH
(Fold over baseline)
40
30
20
10
0
ERK
p38
Basal
PDGF
P+Chloro
P+Quin
P+Sacch
p-MAPK/GAPDH
(Fold over baseline)
10
8
6
4
2
0
ERK
p38
Basal
EGF
E+Chloro
E+Quin
E+Sacch

Figure 6


A.
Basal
Basal
PDGF
Chloro+P
Quin+P
Sacch+P
p-Akt
β-Actin
Basal
Basal
EGF
Chloro+E
Quin+E
Sacch+E
Basal
Hist
PDGF
Chloro+P
Quin+P
Sacch+P
p-p70S6K
β-Actin
Basal
Hist
EGF
Chloro+E
Quin+E
Sacch+E

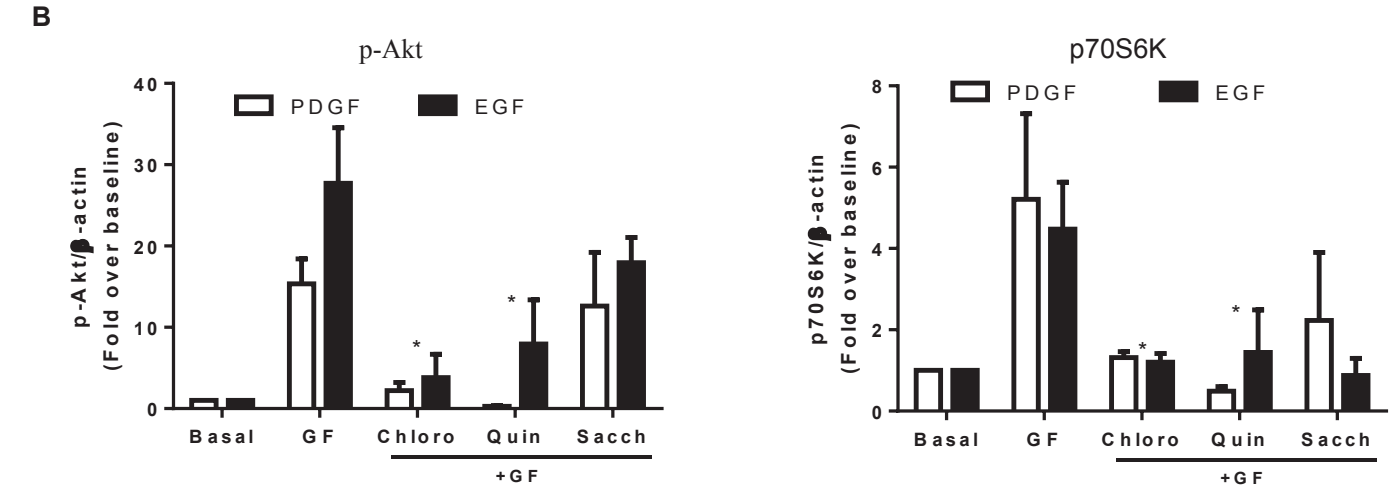

B
p-Akt
PDGF
EGF
p-Akt/β-actin
(Fold over baseline)
40
30
20
10
0
Basal
GF
Chloro
Quin
Sacch
+GF
p70S6K
PDGF
EGF
p70S6K/β-actin
(Fold over baseline)
8
6
4
2
0
Basal
GF
Chloro
Quin
Sacch
+GF

Figure 7

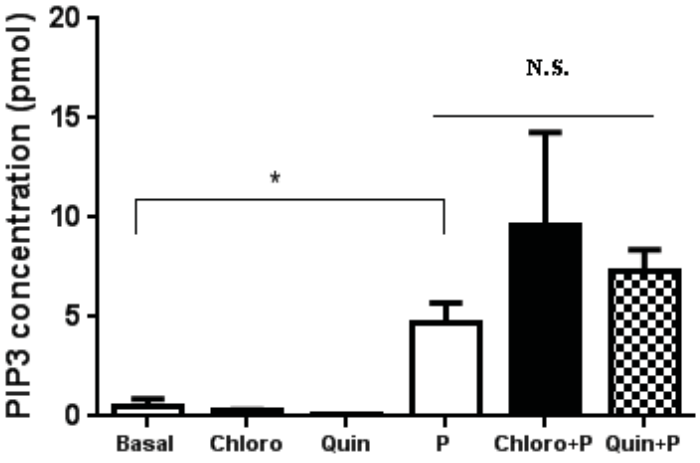

PIP3 concentration (pmol)
20
15
10
5
0
*
N.S.
Basal
Chloro
Quin
P
Chloro+P
Quin+P

Figure 8

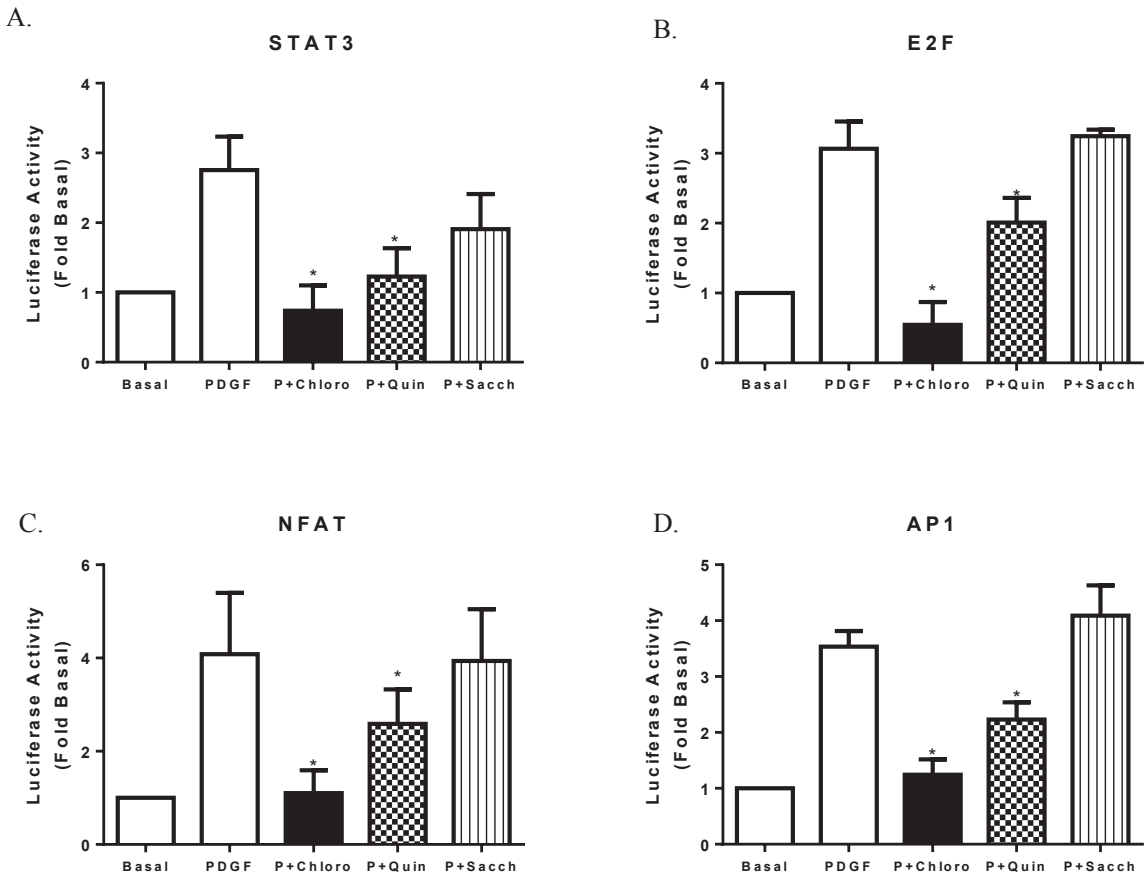

A.
STAT3
Luciferase Activity (Fold Basal)
0
1
2
3
4
Basal
PDGF
P+Chloro
P+Quin
P+Sacch
*
B.
E2F
Luciferase Activity (Fold Basal)
0
1
2
3
4
Basal
PDGF
P+Chloro
P+Quin
P+Sacch
*
C.
NFAT
Luciferase Activity (Fold Basal)
0
2
4
6
Basal
PDGF
P+Chloro
P+Quin
P+Sacch
*
D.
AP1
Luciferase Activity (Fold Basal)
0
1
2
3
4
5
Basal
PDGF
P+Chloro
P+Quin
P+Sacch
*

Figure 9

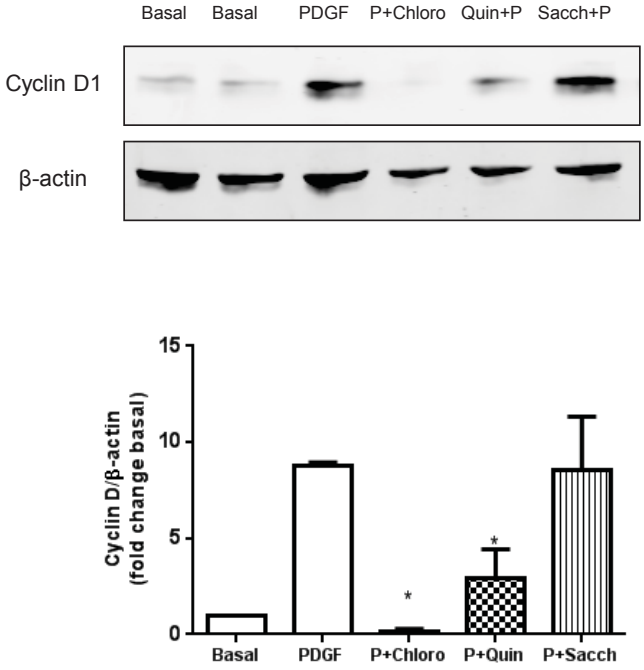
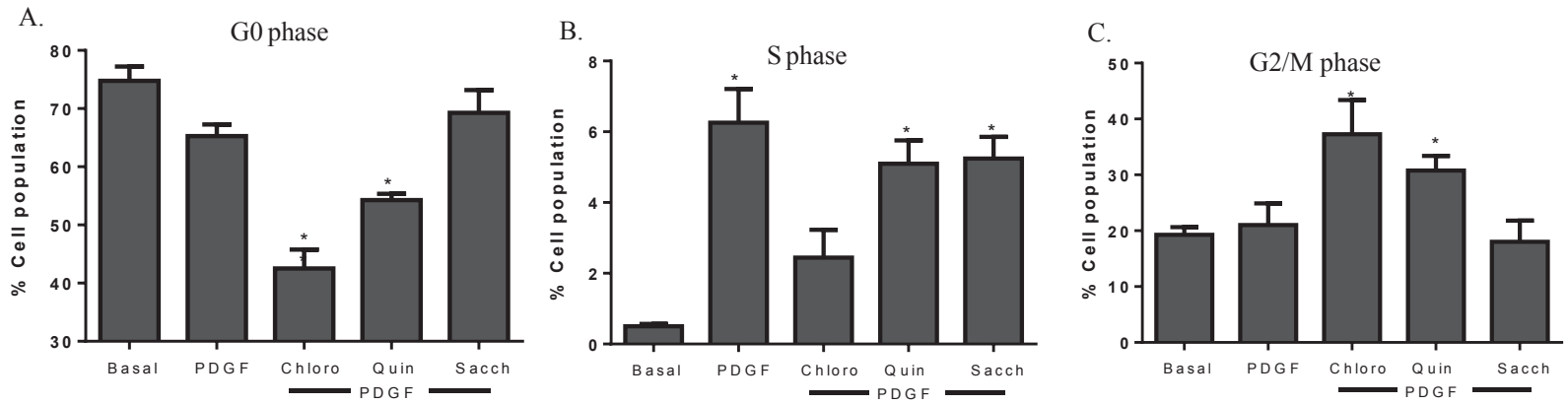

Basal
Basal
PDGF
P+Chloro
Quin+P
Sacch+P
Cyclin D1
β-actin
Cyclin D/β-actin (fold change basal)
15
10
5
0
*
*
Basal
PDGF
P+Chloro
P+Quin
P+Sacch

Figure 10


A.
G0 phase
% Cell population
80
70
60
50
40
30
Basal
PDGF
Chloro
Quin
Sacch
PDGF
B.
S phase
% Cell population
8
6
4
2
0
Basal
PDGF
Chloro
Quin
Sacch
PDGF
C.
G2/M phase
% Cell population
50
40
30
20
10
0
Basal
PDGF
Chloro
Quin
Sacch
PDGF