The effect of polyphenols on the angiotensin II receptor type 1 activation

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Abstract

Hypertension, an increase in blood pressure, can lead to many serious complications, one of which is heart failure. Angiotensin (Ang) II is associated with hypertension; specifically, the Ang II receptor type 1 (AGTR1) is known to cause vasoconstriction. Currently, medication is available to lower blood pressure, but it presents limitations. For this reason, naturally occurring substances are being examined in combination with antihypertensive medication. Two polyphenols that have been shown to lower blood pressure are resveratrol and pterostilbene. Although they present a possible ability to lower blood pressure, they both require a more intensive understanding of how the compound works to reduce blood pressure. The purpose of the study was to examine the effect of resveratrol and pterostilbene at the receptor level for the first time using parallel receptor-ome expression and screening via transcriptional output-transcriptional activation following arrestin translocation (PRESTO-TANGO). This novel method will allow AGTR1 activation to be measured through luminescence. This study demonstrated that resveratrol alone at concentrations of 50 μM, 100 μM, and 200 μM activates AGTR1 and also when combined with Ang II. Ang II with 50 μM and 100 μM resveratrol had a greater activation than Ang II alone. It was also found that 20 μM pterostilbene combined with Ang II activated AGTR1 and had a greater activation than Ang II alone. Although the results were not statistically significant, the trends suggested that resveratrol and pterostilbene do promote the activation of AGTR1. Since this is the first time that the effect of resveratrol and pterostilbene on AGTR1 has been examined, more studies will need to be conducted at the receptor level to understand the compound's ability to regulate receptor activity.

Lay Summary

The mission statement of the Lakehead University Department of Biology states, "Faculty and students in the Department of Biology are bound together by a common interest in explaining the diversity of life, the fit between form and function, and the distribution and abundance of organisms." This research project was part of the human sciences, specifically in regard to the study of hypertension. It aimed to investigate two naturally occurring polyphenols, resveratrol and pterostilbene, at the receptor level using the PRESTO-TANGO method. By analyzing the angiotensin II receptor type 1 (AGTR1) activation, it was concluded that the increase in AGTR1 receptor activation by angiotensin II was not attenuated by resveratrol and pterostilbene . Future studies are directed towards investigating the role of these polyphenols at receptor level.

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Abbreviations

Ang – angiotensin

ACE – angiotensin-converting enzyme

AGTR1 – angiotensin II receptor type 1 (human)

AGTR2 – angiotensin II receptor type 2 (human)

agtr1 – angiotensin II receptor type 1 (rodent)

agtr1a – angiotensin II receptor type 1 subunit a (rodent)

agtr1b – angiotensin II receptor type 1 subunit b (rodent)

agtr2 – angiotensin II receptor type 2 (rodent)

ARB – angiotensin receptor blockers

BrdU – bromodeoxyuridine

DMEM – Dulbecco's Modified Eagles Medium

GPCR – G-protein-coupled receptor

FBS – fetal bovine serum

LB – Lysogeny Broth

PBS – phosphate buffered saline

PRESTO-TANGO – parallel receptor-ome expression and screening via transcriptional output- transcriptional activation following arrestin translocation

RAAS – renin-angiotensin-aldosterone system

TANGO – transcriptional activation following arrestin translocation

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Chapter 1: Introduction

1.1 Hypertension

Cardiovascular disease is one of the leading causes of death worldwide [1]. Cardiovascular disease can include coronary artery disease, heart failure, cardiac arrest, ischaemic stroke, and ventricular arrhythmias [1]. Hypertension has been defined as having a high blood pressure, with a systolic blood pressure ≥ 140 mmHg and/or a diastolic pressure ≥ 90 mmHg [2, 4]. It is a chronic condition that can lead to serious complications and is a major risk factor of cardiovascular disease [1, 2-4]. Blood pressure is dependent on cardiac output and systematic vascular resistance [5]. For example, the narrowing of blood vessels can cause the heart to work harder to pump blood, resulting in an increased blood pressure.

Hypertension can develop in one of two ways, namely, primary hypertension and secondary hypertension. Primary hypertension, also known as essential hypertension, has no known cause, meaning that hypertension develops over time [4, 6]. It is believed to be a combination of genetic, environmental, and behavioral factors [4]. Along with aging, lifestyle factors such as decreased exercise and increased stress are thought to contribute to increase blood pressure [7]. Secondary hypertension occurs when disease is the result of an identifiable cause, such as through thyroid and renal disease [6].

To manage hypertension and prevent future complications associated with high blood pressure, standard treatment options are available. Treatment of hypertension involves changes in lifestyle and the use of blood pressure-reducing medication [5, 8]. By changing lifestyle habits, such as reducing sodium intake and alcohol consumption, and by increasing physically activity, blood pressure can be reduced in some instances [8].

Lifestyle changes, in combination with blood pressure-reducing medications, are also commonly used.

Multiple pharmacologically produced medications such as α and β-blockers, angiotensin (Ang)II receptor blockers (ARBs), diuretics, calcium channel blockers, Ang-converting enzyme (ACE) inhibitors and direct vasodilators can help to decrease blood pressure [5, 9]. Two major classes of antihypertensive medications that target the renin-angiotensin-aldosterone system (RAAS) include ACE inhibitors and ARBs [10]. ACE inhibitors such as benazepril and captopril inhibit the ACE, which then inhibits the formation of Ang II [10]. ARBs such as losartan and candesartan block the binding of Ang II to Ang II receptors [10].

The combination of ACE inhibitors and ARBs have been shown in a meta-analysis conducted in various populations to reduce blood pressure, but no long-term effects were concluded from this study [10]. It has been advised that treatment of hypertension should not include ARBs/ACE inhibitors in combination [10]. Although medications are available, some drugs might not fully block binding, and medications can present adverse side effects [5, 10, 11]. Therefore, naturally occurring compounds are being studied as an alternative.

1.2 Angiotensin II

Ang II is a octapeptide hormone that plays an important role in regulating blood pressure and in the cardiovascular system because of its ability to cause the vasoconstriction of arteries and veins [12, 13]. It is part of the RAAS, which is controlled through the actions of the cardiovascular system; the central nervous system; and the kidneys, which allow blood pressure homeostasis to be controlled [12, 14, 15].

The RAAS pathway, depicted in Figure 1, starts with the formation of angiotensinogen, the precursor to Ang I [12]. Angiotensinogen, a Serpin A8 protein, is produced mainly in the liver [12,15]. When it combines with renin, which is produced largely in the kidneys, it is converted into Ang I [15]. The renin allows for the 10 amino acid cleavage from the N-terminus of the angiotensinogen [12, 15]. It should be noted that Ang II levels are dependent on renin levels because renin is a rate-limiting step in RAAS [14]. Ang II is converted from Ang I by the removal of two amino acids from the C-terminus by ACE [12, 13, 15]. Ang II is the major biological product of RAAS, although Ang II can be converted further into Ang 1-7 [15]. It works through two specific G-protein-coupled receptors (GPCRs) known as angiotensin II receptor type 1 (AGTR1) and angiotensin II receptor type 2 (AGTR2) [15, 16]. Ang II is known to mediate cardiac contractility and be involved with various aspects of cardiac remodeling [13].

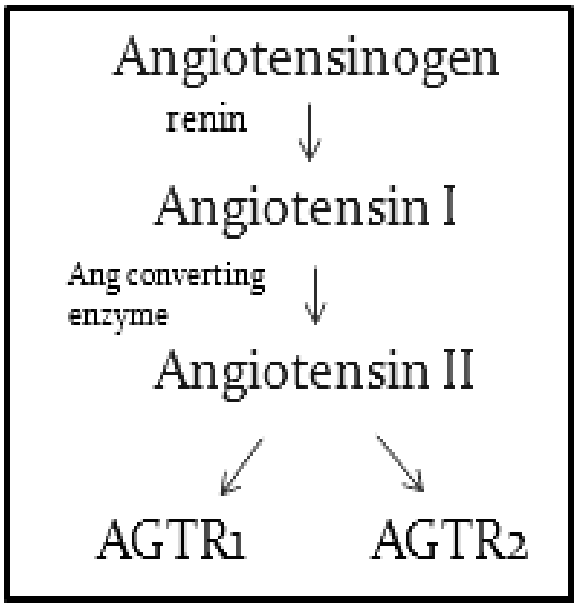


Figure 1. Renin-angiotensin-aldosterone pathway depicting the formation of Ang II. Adapted from M. Sparks et al. *Comprehensive Physiology* (2014). Cleavage of angiotensinogen by renin forms Ang I. The ACE then converts Ang I into Ang II. The biologically active compound then works through the two specific GPCRs, AGTR1 and AGTR2.

1.3 G-Protein-Coupled Receptors of Angiotensin II

GPCRs also are known as a seven-transmembrane domain receptors that are capable of turning an extracellular signal into an internal signal inside the cell [17- 20]. GPCRs are involved with facilitating many physiological processes, specifically processes caused by hormones, neurotransmitters, and the environment [18]. For this reason, they are considered important drug targets [18, 19]. The main purpose of GPCRs is to bind to their agonist to activate the G-protein, which results in activating downstream signaling pathways [19]. It has been estimated that there are at least 800 human GPCRs, but not all functions are known because of limited structural detection methods [19, 20]. Specifically, GPCRs can have different signalling and transduction

pathways, making it difficult to determine the actual effect of just ligand and GPCR binding without system interference [18].

Ang II, the major active product of the RAAS, is known to mediate blood pressure [12, 21]. Ang II can be found in different tissues types, including cardiac tissue and is involved in cardiac remodeling [21, 22]. Ang II works through two specific GPCRs, known as AGTR1 and AGTR2 [22]. Other proposed receptors include Ang II receptor type 3 (AGTR3) and Ang II receptor type 4 (AGTR4) [22]. However, AGTR1 and AGTR2 are the major receptors because they meet the criteria of being operational, having transduction and structural compatibility [22]. The effects of Ang II are mediated through AGTR1 and opposed by AGTR2 [23]. The reported physiological outcomes of AGTR1 activaition by Ang II include being vasoconstrictive, hypertrophic, antinatriuretic, antidiuretic (via increased antidiuretic hormone release), proinflammatory, prooxidative stress, profibrotic, and prothrombotic [24]. The reported physiological outcomes of AGTR2 activaition by Ang II include being vasodilative, antiproliferative, proapoptotic [24]. Chronic exposure to Ang II downregulates its receptors [21]. It should be noted that AGTR1nomeclature is different in animals and humans. AGTR1 in rodents is denoted in literature as Agtr1 and contains two subunits, Agtr1a and Agtr1b (Figure 2) [21]. This is important to note as the systems are not identical between humans and all animals.

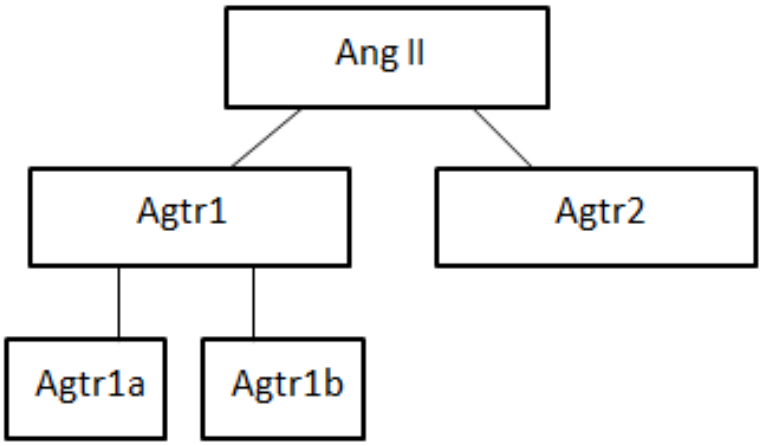


Figure 2. Ang II major receptors in rodents. Agtr1a and Agtr1b are the subunits of Agtr1 and Agtr2 in rodents [21, 22].

In humans, AGTR1 is located on chromosome 3 [21, 22]. It is found in a variety of tissues throughout the body, such as the brain, lung, heart and blood vessels [21]. The approximate molecular mass of AGTR1 is 40 kDa [21, 22]. AGTR1 binds with both agonists and antagonists [25]. Binding to a receptor is determined by specific residues that are located either on the extracellular region or on the transmembrane domain [21]. The effect of AGTR1 when activated though binding by an agonist such as Ang II promotes vasoconstriction. AGTR1 also can be upregulated and downregulated by agonists [21]. When Ang II is increased for a short time, AGTR1 is increased [21]. However, Ang II is considered a downregulator of AGTR1 when exposed chronically, possibly due to desensitaization [21]. Blocking agents can be used to prevent an agonist from binding to AGTR1 [22]. For example, losartan, an Ang II receptor blocker, can be used to block the agonist [22].

In rodents, Agtr1a and Agtr1b are located on chromosomes 17 and 2 [21]. The approximate size of Agtr1a is 84kb, and the approximate size of Agtr1b is 15kb [22].

Agtr1a is expressed throughout the body, including the heart [22]. Studies have suggested that the heart contains only Agtr1a , with Agtr1b being confined to the areas of the adrenal gland, brain, and testis [22]. There is a 95% similarity between their amino acid sequences, making them almost indistinguishable [21]. However, it has been shown that Agtr1a plays a larger role in the regulation of blood pressure, although research has suggested that they are functionally the same [21].

When AGTR1 binds to Ang II, it induces multiple signalling pathways that include both G protein- and non-G protein-related signalling [21]. The major pathways that are activated to produce the functions associated with AGTR1 are through phospholipase C, phosphoinositide hydrolysis and Ca^{2+} signalling [22]. This binding also signals cross-talk between tyrosine kinases [21]. Reactive oxygen species produced by activation of NAD(P)H oxidases are associated with the effects produced by Ang II binding to AGTR1 [21]. Serine/Threonine kinases (MAPK pathways) and the Jak/STAT pathway also are activated [21, 22].

In humans, the approximate molecular mass of AGTR2 is 41kDa [21]. The AGTR2 encoding gene is located on chromosome X in humans and in rodents [21, 22]. It is approximately 34% similar to AGTR1 but displays different functions than AGTR1 [21]. Ang II, when bound to AGTR2, is known to be a vasodilator [24]. It also is capable of producing antiproliferative and proapoptotic effects [21, 24]. It has been thought that AGTR2 plays a large role in fetal development because it is ubiquitously expressed at birth and then declines in some tissues [21, 22]. AGTR2 has been shown in the heart to remain at consistent levels [22]. However, it has been thought that AGTR2 can be induced later in life under certain conditions, such as chronic heart failure [21]. It has

been shown that when Ang II binds to AGTR2, it activates tyrosine and serine/threonine phosphates which inhibits some of the AGTR1 pathways [21]. It also has been found that AGTR2 can cause hypertrophy in cardiomyocytes independent of Ang II [21]. The role of AGTR2 must be examined more closely because its functions are not fully understood at this time [21].

1.4 PRESTO-TANGO System

The human genome produces approximately 350 nonolfactory GPCRs, of which just over one third are orphan receptors, meaning that their ligands are unknown [26]. Unknown GPCRs are a major concern because GPCRs are important in the treatment of various diseases since they are a key target for current medications in the market, with 30% to 40% targeting nonolfactory GPCRs [26- 28]. GPCRs are the primary target of many candidate drugs, but GPCRs can also be non-specifically targeted [28]. This poses a problem when designing medications specifically meant to target a specific GPCR because this might produce unwanted side effects [28]. Therefore, detecting and understanding GPCRs and ligand interaction are essential to prescribing current and future medications. However, the detection of GPCRs has been limited due to deficient detection methods.

Detection methods have involved chemical and physical approaches, but both have presented limitations [28]. Chemical detection of GPCRs has involved looking at interactions between small molecule and GPCRs, as well as larger scale detection methods such as chemical databases [28]. Using the information available in chemical databases about GPCRs allows predictions about targets to be made [28]. However, these methods can be time-consuming efforts that rely on accurate data to be entered [28].

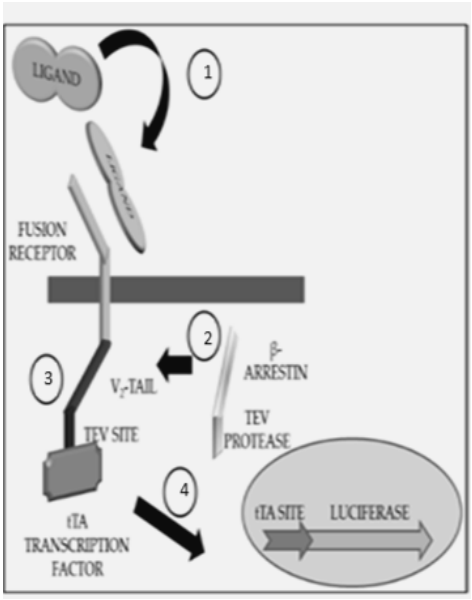
Physical detection has historically relied on radioligand detection, but this method has been limited by the number of labelled ligands [27, 28]. Other methods have involved G-protein-dependent functional assays such as cAMP assay, IP_3/IP_1 and Ca^{2+} assay, GTPγS binding assay, and reporter assays [27].

G-protein-dependent functional assays also can involve G-protein-mediated events [27]. Therefore, detection methods independent of the classical GPCR pathway are termed G-protein-independent functional assays [27]. The ability of most GPCRs to recruit β-arrestin is important because β-arrestin signalling and G-protein signalling are independent of classical G-protein signalling [28]. Although other assays use β-arrestin, the transcriptional activation following the arrestin translocation (TANGO) method has shown promising results [26, 28]. Therefore, it was expanded upon to create a parallel receptor-ome expression and screening via transcriptional output-transcriptional activation following arrestin translocation (PRESTO-TANGO) [26, 28].

PRESTO-TANGO is a new method developed by Dr. Bryan Roth and colleagues that expanded on the TANGO method, which is an efficient chemical integration method that permits the scanning of one target with one receptor at a time [26, 28]. This system allows the ligand and GPCR to be scanned without interference from other G-protein-mediated factors [27, 28]. The PRESTO-TANGO method expands upon this system and allows multiple compounds to be scanned against the human genome at once [26]. The PRESTO-TANGO method, a cell culture-based system, facilitates the determination of agonists of GPCRs. The cells that are used in this model are a HTLA cell line, a derivative of the HEK293 cell line, which is capable of expressing tetracycline transactivator (tTA) luciferase -β-arrestin-2-Tobacco Etch Virus (TEV) fusion genome

[26]. For each GPCR, a TANGO construct was created, resulting in 315 constructs. However, not all constructs could be validated by agonists, even if they were nonorphans [26].

In general, the system works by a ligand binding to the GPCR, which has been transfected into the cell, and upon binding, the β-arrestin -2-TEV fusion protein is recruited [26]. The fusion protein is then cleaved, which facilitates the release of tTA [26]. The tTA then binds to the nucleus and activates the luciferase reporter gene [26]. The luminescence produced can then be measured (Figure 3) [26]. This system, through the activation of a receptor (by increased luminescence), allows for the determination of agonists of GPCRs (Figure 4). This is a revolutionary system because of its ability to scan multiple GPCRs simultaneously with ligands [26].

a) 

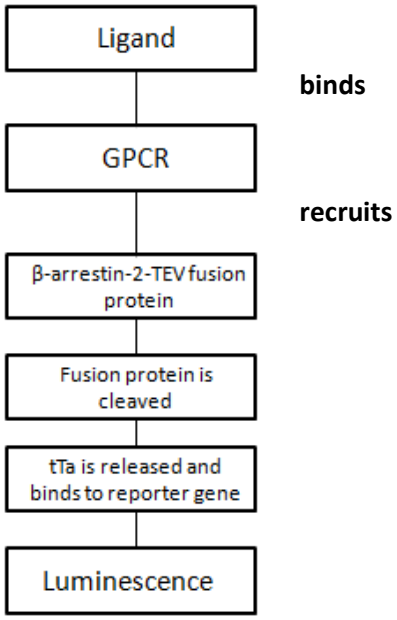
b) 

Figure 3. PRESTO-TANGO system. Adapted from W. Kroeze et al. *Nature Structural & Molecular Biology* (2015) [26]. a) 1. Ligand binds to GPCR, 2. β-arrestin -2-TEV fusion protein is recruited to receptor, 3. fusion protein in then cleaved and tTA is released and 4. tTA bind to nucleus producing luminescence. b) PRESTO-TANGO steps.

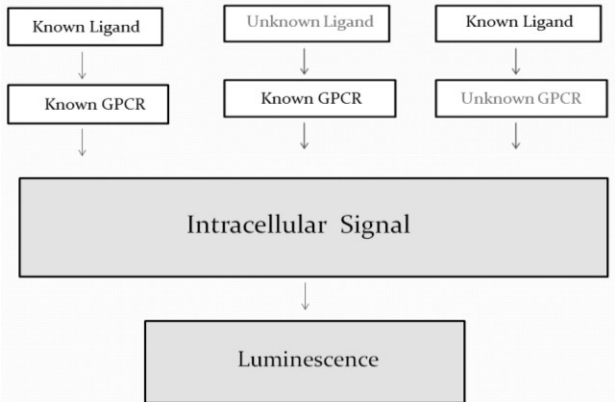
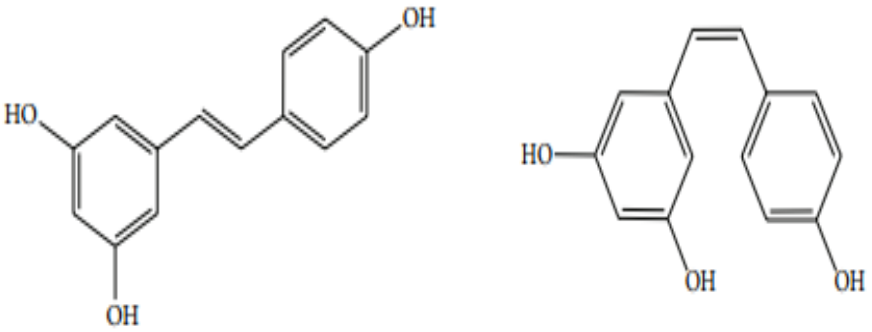


Figure 4. Mechanism of PRESTO-TANGO system. Luminescence signal can be produced as long as either the ligand or GPCR is known.

1.5 Resveratrol

Resveratrol (3,5,4′-trihydroxy-trans-stilbene), a polyphenol, is a naturally occurring stilbene compound [29- 31]. It has a stilbene structure that consists of two

phenolic rings attached by a double bond (Figure 5) [31]. Resveratrol is present in two isomer formations, the *cis*- and *trans*- formation, with the trans-formation being the more stable of the two [31].



a) b)

Figure 5. Molecular structure of resveratrol. Image from J. Gambini et al. *Oxidative Medicine and Cellular Longevity* (2015) [31] a) *trans*-resveratrol and b) *cis*-resveratrol [31].

Resveratrol is found in a variety of plants and many different foods [30, 31]. Specifically, some plants produce resveratrol in response to stress [30]. Common foods in which resveratrol is found include, grapes, blueberries, blackberries, and peanuts [29, 30, 31]. However, based on the Mediterranean diet, resveratrol is consumed mostly through red wine [31]. The resveratrol present in wine comes from the grapes, with the skin, seeds, petioles, and woody parts being the most concentrated source [31]. A high concentration has been reported in the grape types of Vitis vinifera, labrusca, and muscadine at a concentration of 50 to 100 μg/g in skin and seeds [32]. The amount of resveratrol found in plants varies because of different conditions, such as environmental factors [31].

Resveratrol has been studied for approximately 70 years but more so in the last decade. It has been reported to have many beneficial effects, including antioxidant, anticancer, and anti-inflammatory properties [31]. Resveratrol offers a great range of potential health benefits, but it has been difficult to suggest a certain dosage for treatment or supplement [33]. Many studies have examined the benefits of resveratrol *in vitro* and *in vivo* using animal models, and even though research has been performed in human studies, research still has been lacking in clinical trials [31]. This is mainly due to the lacking information about the absorption and metabolism of resveratrol [33].

Resveratrol's efficiency is dependent on the way it is consumed [31]. Low bioavailability and the ways in which resveratrol's metabolites are absorbed can affect its efficiency [31]. It has been suggested that metabolites such as glucuronides and sulfates, rather than free resveratrol, might be responsible for some of the benefits because they usually are detected in urine up to 9 hours after ingestion [33, 34]. The potential side effects of long-term use also are unknown at this time [33]. The reason this is important is because resveratrol can accumulate in tissue [33]. One study has estimated that on average, men consume 1629 μg/day and women consume 235 μg/day of resveratrol [33]. Concentrations at these amounts have shown no adverse side effects [33].

The bioavailability of resveratrol varies greatly, depending on the method of distribution (i.e., orally, infusion) [31]. Bioavailability of resveratrol using oral absorption has been reported at 75% [35]. However, a concern with oral absorption of resveratrol has been its low plasma concentration levels [31]. It also has been reported that the use of resveratrol might be limited because of poor absorption [31].

In animal studies, resveratrol has been found effective in reducing blood pressure in hypertensive rats and also working in combination with antihypertensive medication [36]. Resveratrol has been reported as lowering systolic blood pressure at a concentration of ≥ 150 mg/day [37]. However, in a meta-analysis combining six studies with 247 participants, the compiled previously reported data indicated that resveratrol had no significant effect on systolic or diastolic blood pressure [37]. There have been limited studies regarding the antihypertensive role of resveratrol treatment in clinical trials.

1.6 Pterostilbene

Pterostilbene (trans-3,5-dimethoxy-4-hydroxystilbene) is a derivative, a dimethylated form, of the polyphenol resveratrol [31, 38]. Although very similar in structure, it is believed that pterostilbene has better bioavailability because of the two methoxy groups [38]. The addition of the methoxy groups improves its lipophilic nature and oral absorption (Figure 6) [38].

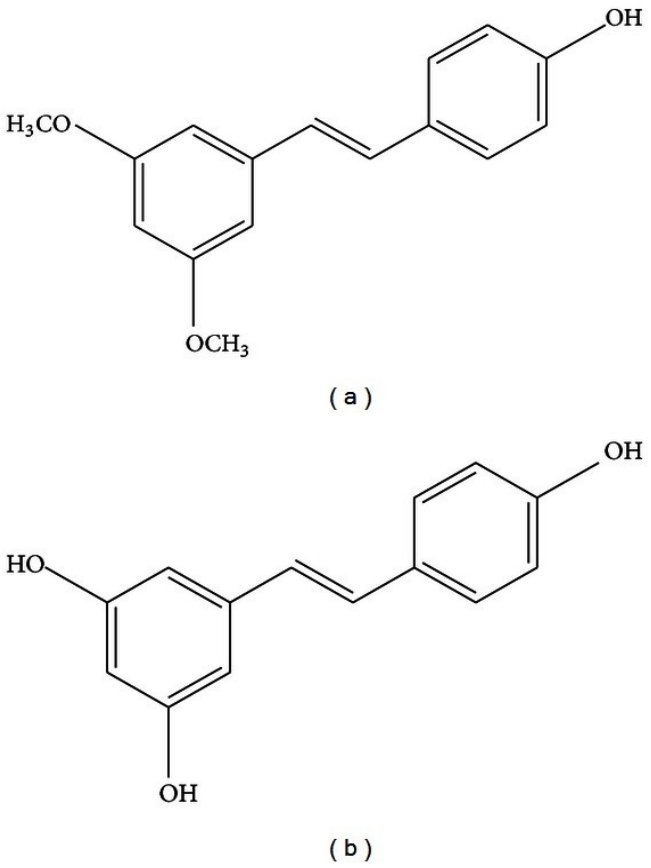


Figure 6. Molecular structure of pterostilbene and resveratrol. Image from D. McCormack & D. McFadden. *Oxidative Medicine and Cellular Longevity* (2013) [38]. Image (a) pterostilbene and (b) resveratrol [38].

Pterostilbene sources are found in foods such as fruits, nuts, and plants [31, 38]. The most well-known source is blueberries, with an average of between 99 and 520 ng/gram per berry [38]. Although it is considered concentrated in grapes, grapes contain smaller amounts of pterostilbene in comparison to resveratrol [39, 40]. Recent studies have reported pterostilbene posses antioxidant capability and has the ability to reduce oxidative stress [38, 39, 40]. Along with pterostilbene showing antioxidant capability, it has been shown to possess antiproliferative and anti-inflammatory abilities [38, 39]. It also has been shown that pterostilbene can inhibit apoptosis and autophagy

[38]. It has been found that pterostilbene has greater bioavailability in oral administration compared to resveratrol [38]. One animal study reported that pterostilbene had an 80% bioavailability compared to resveratrol at 20% [38, 41]. Again, more studies need to be conducted regarding resveratrol and pterostilbene pharmacokinetics.

Pterostilbene is an understudied compound that needs to be examined more closely in a clinical setting, especially in regard to hypertension and associated cardiovascular complications. In particular, there has been limited research on this compound's effect on blood pressure regulation. Evidence has shown that pterostilbene does lower blood pressure in adults with hypercholesterolemia [42]. The study, which was conducted in 2014, concluded that a higher dosage (250 mg/day) was effective in lowering blood preassure [42]. The safety of 250 mg/day also was studied, and it was reported to be generally safe [43]. However, more research is needed to investigate the role of pterostilbene in hypertensive patients.

1.7. Rationale

Ang II is associated with hypertension, and it is known to increase blood pressure when bound to AGTR1. Ang II will activate AGTR1 in the PRESTO-TANGO system because it is an agonist. Although resveratrol and pterostilbene in some cases have been reported to lower blood pressure, their mechanisms of action are not well understood. This study sought to determine the effect of resveratrol and pterostilbene on AGTR1 activation. Targeting AGTR1 will help to clarify the role of these ligands in hypertension.

However, it must be noted that before resveratrol and pterostilbene effects can be determined on AGTR1 activation the PRESTO-TANGO system must be optimized for the study. The PRESTO-TANGO system has not been used to study the effects of these

compounds on AGTR1 activation before. Therefore many aspects of this system must be optimized such as AGTR1 concentration, the effect of time on the system, treatment concentrations and cell densities. Until the system is optimized the effect of resveratrol and pterostilbene on AGTR1 activation cannot be determined.

1.8 Hypothesis

In the presence of Ang II, resveratrol and/or pterostilbene will prevent Ang II induced AGTR1 activation.

1.9 Specific Aims

1. Optimize the PRESTO-TANGO method with AGTR1 receptor.
2. Determine the effect of resveratrol on AGTR1 using the optimized PRESTO-TANGO method.
3. Determine the effect of pterostilbene on AGTR1 using the optimized PRESTO-TANGO method.

Chapter 2: Methods

2.1 Transformation of Competent Cells

Escherichia coli D5H-α competent cells (Fisher, NH, USA) were transformed using the plasmids AGTR1-Tango (Addgene, MA, USA cat. #66222) or pGL4.54 [luc2/TK] Vector (Promega, WI, USA cat. #E5061). This was done by mixing 1μL of plasmid with 50 μL of competent cells. The mixture was then incubated on ice for 30 minutes and then heat shocked for 1 minute at 43° C. To the plasmid and competent cell mixture, 400 μL of sterile LB Broth (Fisher, NH, USA) was added and then placed in a 37° C shaking incubator for 1 hour. The mixture was then spread at a volume of 50 μL on to LB agar plates containing 100 μg/mL of ampicillin because both vectors contained the ampicillin resistant gene. After allowing the plates to dry, they were placed in an incubator at 37° C, 5% CO_2, and 100% humidity overnight.

The following day, colony growth was verified by examining a negative control containing only competent cells and no plasmid. A single colony was then added to a flask containing 100 mL of sterile LB Broth and 100 μg/mL of ampicillin. This protocol was always done in duplicates. Furthermore an extra mL was made of bacterial culture for the future storage of plasmids. The flasks were then placed in a 37° C shaking incubator and left overnight.

The following day, the extra 1 mL of bacterial culture was combined with 1 mL of 50% glycerol and stored at -80° C . Maxi preps were made with the remaining bacterial culture following the ZymoPure™ Plasmid Maxiprep Kit (ZYMO RESEARCH, CA, USA cat.# D4202) manufacturing protocol. The eluted plasmids from the maxi preps

were stored at -20° C, and concentrations were read using a Take3™ microplate reader with Gen5 program (BioTek, VT, USA).

2.2 Cell Culture Methods

HTLA cells, a derivative of the HEK293 cell line, are capable of expressing tetracycline transactivator (tTA) luciferase -β-arrestin -2-Tobacco Etch Virus (TEV) fusion genome. The cells were obtained from the laboratory of Dr. Gilad Barnea. These cells were grown and cultured in 100 x 20 mm vacuum-glass plasma treatment tissue culture dishes (Corning, NY, USA). The cells were split at approximately 90% confluency and were seeded at 1,440,000 cells per plate. Cells were grown in a supplemented medium (10 mL per 100 x 20 mm) that consisted of Dulbecco's Modified Eagles Medium (DMEM; Sigma-Aldrich, MO, USA); 10% fetal bovine serum (Hyclone, PA, USA); 1% antibiotic-antimycotic (Gibcco, CA, USA); 1% gentamicin (Gibcco, CA, USA); 0.2% hygromycin B (Fisher, NH, USA); and 0.05% puromycin (Gibcco, CA, USA). Cells were then incubated at 37° C, 5% CO_2, and 100% humidity for all experiments, unless otherwise stated.

2.3 Transfection

When cells were at approximately 60% confluency, the transfection mixture (300 µL) was added to the cells. The transfection mixture consisted of DMEM; FuGENE 6 (Promega, WI, USA); and the receptor plasmid AGTR1 (Addgene, MA, USA) at 0.1µg. FuGENE 6 to AGTR1 was added at a 60:1 ratio in DMEM. Control cells received 300 µl of DMEM only. All other wells received AGTR1 transfection mixture and pGL4.54 had its own transfection mixture substituted for AGTR1 using the same ratio. Cells were then incubated for 24 hours.

2.4 Treatment

After 23.5 hours of being transfected, the cells were split into six-well plates (Corning, NY, USA) with 400,000 cells/mL. Cells were grown in supplemented media at 1 mL/well, along with the addition of 1 mL of corresponding treatment. Control cells and AGTR1 control cells not receiving specific treatment had 1 mL of supplemented media added. Cells were then incubated for 24 hours.

2.4.1 Resveratrol Treatment

A 5-mM stock concentration of resveratrol was prepared by dissolving 0.028 g of resveratrol (Sigma-Aldrich, MO, USA) into 20 mL of distilled water. The concentrations of 50 μM, 100 μM, and 200 μM were made from resveratrol stock using supplemented media, and then 1 mL of resveratrol treatment was added to each well. Treated cells were then incubated for 24 hours.

2.4.2 Ang II Treatment

A 32-mM stock concentration of Ang II was prepared by dissolving 0.005 g of Ang II (Sigma-Aldrich, MO, USA) into 15 mL of distilled water. A 43-nM concentration was made up from Ang II stock concentration using supplemented media, and then 1 mL of Ang II treatment was added to each well. Treated cells were then incubated for 24 hours.

2.4.3 Losartan Treatment

A 10-mM stock concentration of losartan was prepared by dissolving 0.072 g of losartan (Sigma-Aldrich, MO, USA) into 15 mL of distilled water. A 1333-nM concentration was made up from losartan stock concentration using supplemented media,

and then 1 mL of losartan treatment was added to each well. Treated cells were then incubated for 24 hours.

2.4.4 Ang II + Losartan Treatment

A cotreatment mixture of 43 nM of Ang II and 1333 nM of losartan was prepared together using supplemented media, and then 1 mL of cotreatment was added to each well. Treated cells were then incubated for 24 hours.

2.4.5 Ang II + Resveratrol Treatment

Cotreatment mixtures of 43 nM of Ang II and 50 μM, 100 μM, and 200 μM of resveratrol was prepared together using supplemented media, and then 1 mL of cotreatment was added to each well. Treated cells were then incubated for 24 hours.

2.4.6 Pterostilbene Treatment

A 5-mM stock concentration of pterostilbene was prepared by dissolving 0.001 g of pterostilbene (Sigma-Aldrich, MO, USA) into 15 mL of dimethyl sulfoxide (DMSO). A 20-μM concentration was made up from the pterostilbene stock concentration using supplemented media, and then 1 mL of pterostilbene treatment was added to each well. Treated cells were then incubated for 24 hours.

2.4.7 Ang II + Pterostilbene

A cotreatment mixture of 43 nM of Ang II and 20 μM of pterostilbene was prepared together using supplemented media, and then 1 mL of cotreatment was added to each well. Treated cells were then incubated for 24 hours.

2.5 Cell and Viability Test

Cell counts were performed when cells reached approximately 80% confluency (22 hours later). The media was then removed from the wells, and the cells were washed with 1 mL of phosphate buffered saline (PBS), and 500 µL of trypsin was added. Cells were then incubated for 2 minutes at 37˚ C, 5% CO_2, and 100% humidity. Once the cells were tryspinized, a 1:1 ration of Trypan Blue 0.4% (GE Healthcare, ON, Canada) was used. Cell counts were done using hemocytometer and 10x Nikon phase microscope in which live and dead cells were counted. Trypan Blue enters only nonviable cells, thus facilitating the detection of dead cells via the blue stained cells.

*2.6 Reading Luminescence (*Bright-Glo™ Luciferase Assay System*)*

Luminescence readings were taken 24 hours after treatment was added. Media were removed from the wells, and then 100 µL of Bright-Glo (Promega, WI, USA) was added to each well in the six-well plate. The six-well plate was then incubated for 5 minutes on Belly Dancer (Stovall, NC, USA) in the dark. After 5 minutes, the 100 µL in each well was transferred to a corresponding well on a white half area 96-well plate (Corning NY, USA). Using the FLUOstar OPTIMA (BMG LABTECH, Germany), luminescence readings were taken using the setting of 1 cycle with an interval measurement time of 4.06 seconds and a cycle time of 10 seconds, along with a wait time of 5 minutes prior to reading.

2.7 BrdU Fixing and Reading

When the HTLA cells were at approximately 70% confluency, the cells were treated with 1mM Bromodeoxyuridine (BrdU) and were incubated for 30 minutes. Media were removed, and the cells were washed with PBS (10 mL) and trypsinized (2

mL). Cells were then incubated for 5 minutes. Supplemented media (4 mL) were then added, and cell suspension was transferred to centrifuge tubes. The tubes were then spun at 500 x g for 5 minutes at 22° C. Supernatant was then removed, and the pellet was suspended in 200 μL of PBS. Cells were then fixed in chilled 70% ethanol, with ethanol being added dropwise while the centrifuge tube was being vortexed. Fixed cells were then stored in 4° C up to a week.

Cells were spun at 200 xg for 15 min at 22° C in centrifuge (Beckman Coulter, CA, USA). Pre-coating of microcentrifuge tubes was completed with a 0.1% bovine serum albumin-phosphate buffered saline (BSA-PBS). BSA-PBS at 1 mL was added to each microcentrifuge tube. Supernatant of BrdU was discarded, as was the BSA-PBS solution. To the precoated tube with pellet 670 μL of distilled water and 330 μL of 6M, hydrochloric acid was then added. Microcentrifuge tubes were then vortexed and incubated for 30 minutes at room temperature. Microcentrifuge tubes had 0.1M Borate Buffer added. Cells were spun at 200 x g for 15 minutes at 22° C in centrifuge. Supernatant was then discarded. Microcentrifuge tubes had 0.1M Borate Buffer B added. Cells were then spun at 200 x g for 5 minutes at 22° C in centrifuges. Supernatant was then discarded. Microcentrifuge tubes had 1 mL of BSA-PBS added. Cells were then spun at 200 x g for 5 minutes at 22° C in centrifuge. A anti-BrdU solution was made up with a 5 μL/100 μL of 0.1% BSA-PBS. Supernatant was then discarded. Each sample then had 50 μL of anti-BrdU/BSA-PBS added to it and was resuspended until no particle matter was visible. Microcentrifuge tubes were then incubated for 30 minutes in the dark; 400 μL of PBS were then added to each microcentrifuge tube, and the entire solution was

2.8 Statistics

The optimized data presented corresponds to four (n = 4) independent experiments, and all statistical analyses were performed using GraphPad Prism 5 software. Data was presented as mean ± standard error of the mean (SEM). All data used one-way ANOVA with post hoc Tukey's test with a $p \leq 0.05$ indicating significance.

Chapter 3: Results

3.1. Results of Optimization

Before the effect of resveratrol and pterostilbene on AGTR1 activation could be determined, the system had to be optimized. The PRESTO-TANGO system presents great potential for understanding the GPCR-ligand interaction, but because it is a new method, limited information is available. This study was the first time that AGTR1 had been examined with the compounds resveratrol and pterostilbene. As there has been no literature on this current method with these compounds, many steps in the procedure had to be optimized. This included AGTR1 itself, especially in regard to luminescence readings, time, treatments, and cell densities. Figure 7 displays the optimization steps that were taken to optimize the system. Section 3.1 includes a detailed discussion of these areas of optimization.

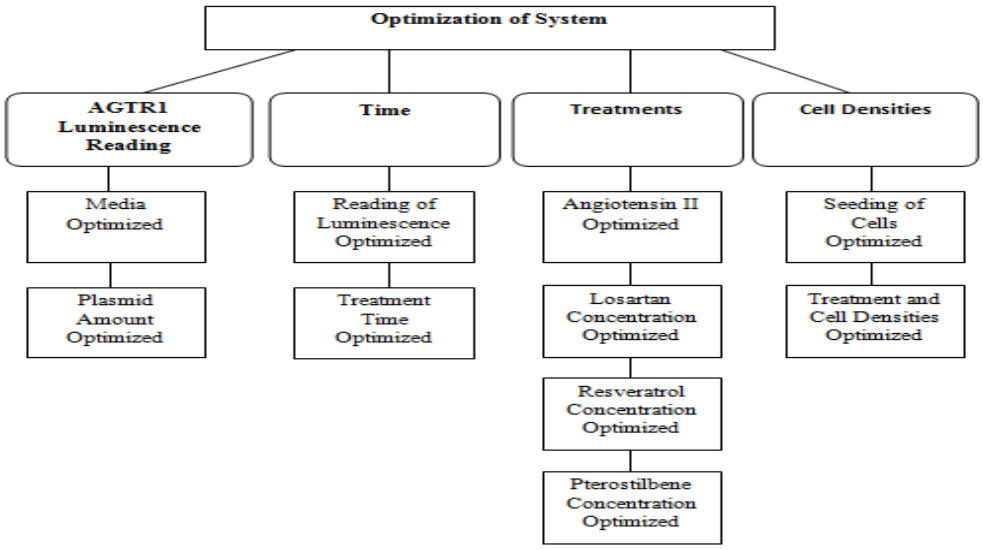


Figure 7. Optimization steps. Chart displays the major areas of AGTR1 luminescence reading, time, treatment and cell densities that needed to be optimized and the steps that lead to their optimization.

AGTR1 displays an increased activation in the PREST0-TANGO system

To test the system, HTLA cells were grown to approximately 90% confluency and then split into six-well plates. Cells were plated at a volume of 200 μL into 2 mL of supplemented media (Day 0). After 24 hours of cells being incubated (Day 1), they were transfected with a 6:1 ratio of FuGENE 6 and AGTR1 plasmid, with the exception of the control. After being incubated for another 24 hours (Day 2), the media was removed, and cells were treated with resveratrol at the concentrations of 50 μM, 100 μM, 200 μM, and 400μM or supplemented media. After being incubated for another 24 hours (Day 3), luminescence readings were taken. Luminescence reading are reported in relative light units (RLU). The average readings indicated that AGTR1 activation was approximately 40 times higher than the control (nontransfected cells) at a luminescence reading of 404,653.5 to 14,872 (Figure 8). Bright-Glo reagent and supplemented media alone had luminescence readings similar to the average blank (empty well; Figure 8). Average resveratrol treated cells showed an increase in luminescence reading with 50 μM at 440,473, 100 μM at 460,092 and 200 μM at 470,929 , except 400 μM of resveratrol caused a decrease in luminescence at a value of 196,763 (Figure 8). Based on the luminescence readings, these results showed that AGTR1 was being activated compared to the control. If AGTR1 was being activated, it was difficult to state the effect of resveratrol on the receptor.

To test the system, another trial was performed, except on Day 2, when cells were treated with the AGTR1 antagonist losartan (1333 nM). Average luminescence readings indicated that AGTR1 was significantly larger than the control alone at a reading of 999,013.5 to 21,966.5, but losartan reduced the luminescence reading (Figure 9). This

system reports the agonist's affect, this trial showed that losartan did not have an agonist effect on AGTR1 because it had a lower reading than that of AGTR1 alone. However, AGTR1 was still high compared to the control, and this experiment indicated that the system was working correctly with the antagonist. This finding suggests that AGTR1 was being activated by a possible ingredient in the media.

Reduced percentage of FBS lowers AGTR1 activation but reduces cell density.

Looking at the media alone, it was thought that testing different amounts of FBS might have lowered AGTR1 luminescence readings because FBS can contain unknown ingredients. The experiment was carried out as previously stated, except the cells were grown in supplemented media with decreasing amounts of FBS (10%, 5%, 2.5%, and 0%). Control had an average luminescence reading of 21,439 (Figure 10). As the percentage of FBS in supplemented media decreased, the average luminescence readings showed a decrease in AGTR1 activation with values of 1,846,472 for 10% FBS, 410,442 for 5% FBS, 247,177 for 2.5% FBS, and 46,939 for 0% FBS (Figure 10).

Another dilution FBS trial was performed using 10%, 5%, and 2.5% FBS in the supplemented media under the same conditions, and nontransfected cells also were assessed with the same dilutions of FBS. Reducing the amount of FBS in the supplemented media also showed a decrease in luminescence of cells transfected with AGTR1 because 10% FBS had a 3,975,003 average luminescence and 5% FBS had a 453,011 average luminescence (Figure 11). The exception was 2.5%, which had a 586,425 average luminescence (Figure 11). Control cells with different amounts of FBS in supplemented media showed similar average luminescence's (Figure 11). Based on this information it was obvious that reducing the % of FBS in media reduced the

AGTR1 activation, but it was unclear if the reduced nutrients were the reason for limiting cell growth.

Reducing the FBS might also have been limiting cell growth; therefore, another trial was performed under the same conditions, except 10% FBS was filter sterilized. To filter out small compounds from 10% FBS, it was thought that AGTR1 levels could be reduced without effecting the nutrient concentrations greatly. Control, 10% FBS, 10% filter sterilized FBS and 0% FBS conditions were examined under previous conditions. The control had a luminescence reading of 9,902 (Figure 12). AGTR1 transfected cells grown in 10% FBS-supplemented media had a luminescence reading of 730,836, 10% filter sterilized FBS-supplemented media had an increased luminescence reading of 857,875 and 0% FBS supplemented media had a decreased luminescence reading of 32,603 in regards to AGTR1 10% FBS (Figure 12). These results indicated that filter sterilizing FBS had no effect on AGTR1 luminescence readings.

To confirm the observation that reducing FBS caused a decrease in cells by Day 3, cell counts and viability tests were performed on Day 3 under the same conditions. As shown in Table 1, cell counts indicated a decrease in cell density with decreasing amounts of FBS in supplemented media, and cell viability remained consistent. This result confirmed that lowering the amounts of FBS reduced the number of cells, which might have affected the results of the treatment on the receptor activation.

Another trial was performed under the same conditions, except dialyzed 10% FBS in the supplemented media was examined by removing activating compounds from the FBS and maintaining nutrients. Control and AGTR1 were performed under the same conditions, except on Day 0, 250,000 cells/well at a volume of 2 mL were seeded, as

opposed to the volume of 200 µL into the six-well plates. Control had an average luminescence reading of 28,053.5, and AGTR1-transfected cells grown in 10% dialyzed FBS-supplemented media had an average luminescence reading of 2,453,582 (Figure 13). Indicating again AGTR1 was still being activated when compared to the control.

AGTR1 plasmid amount reduction reduces AGTR1 activation

Previous trials had used 1 µg of AGTR1 in transfection. To determine if the amount of AGTR1 had an effect on the increased luminescence values compared to the control, the AGTR1 concentrations of 0.03 µg, 0.05 µg, 0.07 µg, 0.1 µg, and 0.12 µg were examined. HTLA cells were grown to approximately a 90% confluency and then split into six-well plates (Day 0). Cells were plated at a volume of 200 µL into 2 mL of supplemented media. After 24 hours of cells being incubated, they were transfected with an increasing ratio of FuGENE 6 to AGTR1 plasmid, with the exception of the control. After being incubated for 24 hours (Day 2), media were removed, and cells were treated with supplemented media. After being incubated for another 24 hours (Day 3), luminescence readings were taken, with a wait time of 15 minutes before reading. Luminescence readings showed an increase in AGTR1 activation when the amount of AGTR1 and FuGENE 6 increased, except for 0.05 and 0.07 µg (Figure 14). AGTR1 0.03 µg had a luminescence reading of 91,372 , 0.05 µg had a luminescence reading of 63,957, 0.07 µg had a luminescence reading of 90,297, 0.1 µg had a luminescence reading of 128,747, and 0.12 µg had a luminescence reading of 160,564 (Figure 14). From the data obtained, 0.1 µg was chosen for future experiments.

Time does not significantly alter luminescence readings

To examine the effect of time on luminescence readings, the trial examining the effect of AGTR1 plasmid reduction (Figure 14) also was run over a course of 10 minutes. Luminescence values were read using the FLUOstar OPTIMA, for 20 cycles with cycle time of 30 seconds and a measurement interval time of 1 second. Figure 15a displays all 20 readings over the course of 10 minutes for each sample. Control showed a decrease over 10 minutes, as did all AGTR1 plasmid amounts. Blanks remained consistent over the 10 minutes. The average luminescence values over the 10 minutes were graphed for each sample (Figure 15b). These results indicated that a snapshot reading, which is a 1-point reading, is still an accurate form of measurement as the values over 10 minutes gradually decrease.

The other factor that could have been influenced the reading was the duration of the cell treatment. To examine the effect of time on the PRESTO-TANGO system with respect to treatment time, the effect of the agonist Ang II was examined. Following the information obtained thus far and until stated otherwise, HTLA cells were grown to approximately 90% confluency and then split into six-well plates (Day 0). Cells were plated at 250,000 cells/well at a volume of 2 mL of supplemented media. After 24 hours of cells being incubated, they were transfected with a 60:1 ratio of FuGENE 6 to AGTR1 plasmid (0.1 μg), with the exception of the control. After being incubated for 24 hours (Day 2), the media were removed, and cells were treated with supplemented media and Ang II. After being incubated for another 24 hours (Day 3). Luminescence readings showed a minor increase in readings, with a trend for increasing signal with increasing duration of AngII exposure, with the exception of Ang II at 36 hours (Figure 16). This

showed that the agonist worked because it was almost double that of AGTR1, indicating activation of receptor. It also demonstrated that time did not have a significant influence on luminescence readings.

The effect of Ang II and losartan on AGRTR1 and cell viability

The effect of the concentrations of 43 nM Ang II and 1333 nM losartan on cell viability and AGTR1 activation was examined. Methods were followed as previously stated, except on Day 2, cells were treated with supplemented media, Ang II, losartan, and Ang II + losartan (all treatments treated with AGTR1). After being incubated for another 24 hours, luminescence readings were taken, with a wait time of 15 minutes before readings. Average luminescence readings indicated that AGTR1 was slightly increased above control, Ang II caused an increased luminescence, losartan had a decreased luminescence, and Ang II + losartan had a decreased average luminescence with respect to AGTR1 (Figure 17). Cell density and viability of cells were calculated for Ang II and losartan. As shown in Table 2, Ang II and losartan both had a high cell viability. As these concentrations produced the expected agonist and antagonist effect (Ang II has an increased signal, and losartan has a decreased signal) and had high viability, these concentrations were selected for the remainder of the experiments.

The effect of resveratrol on cell viability

The effect of the concentrations 50 μM, 100 μM, 200 μM, and 400 μM of resveratrol on cell viability and AGTR1 activation was examined. Methods were followed as previously stated, except on Day 2, cells were treated with supplemented media, Ang II, resveratrol 50 μM, 100 μM, 200 μM and 400 μM (all treatments in the presence of AGTR1). Average luminescence readings indicated that AGTR1 was higher

than control. Ang II had a large increase with respect to AGTR1. Resveratrol 50 μM had a decreased average luminescence compared to AGTR1; resveratrol 100 μM and 200 μM had an increase (Figure 18). All of the resveratrol concentrations plus Ang II had an increased average luminescence over AGTR1, with resveratrol 50 μM having the largest increase and then descending with increasing concentration (Figure 18). Cell density and viability of cells were calculated for all controls and treatments, as shown Table 3. Although viability was high for all conditions, confluency was decreasing with treatment conditions compared to control and AGTR1.

Efect of pterostilbene on AGTR1 activation and cell viability

The effect of the concentrations 20 μM, 40 μM, 60 μM and 100 μM pterostilbene on cell viability and AGTR1 activation was examined. Methods were followed as previously stated, except on Day 2, cells were treated with supplemented media and pterostilbene (all treatments treated with AGTR1). Average luminescence readings indicated that AGTR1 had a higher luminescence then the control (Figure 19). Pterostilbene had an increased average luminescence reading when increasing concentrations, except for 100 μM pterostilbene, which had a reading close to control (Figure 19), although 20 μM, 40 μM, 60 μM had a similar reading to AGTR1. Cell density and viability of cells were calculated for all controls and treatments, as shown in Table 4. Although the viability was higher than 87% for all groups' cell densities were decreasing with higher concentrations of treatment conditions compared to control and AGTR1.

Another trial was performed to look at the effect of the concentration of 20 μM in the PRESTO-TANGO system. Pterostilbene 20 μM was chosen because it had the

highest cell density (Table 4). Following the above conditions, the experiment was repeated with control, AGTR1, Ang II, losartan , pterostilbene, and pterostilbene + Ang II. Control had an average luminescence of 46,419; AGTR1 had a similar average luminescence of 59,165 (Figure 20). Ang II had an increased average luminescence of 161,140 (Figure 20). Pterostilbene had an increased average luminescence over control and AGTR1 at 97,248 (Figure 20). Pterostilbene + Ang II had the highest increased average luminescence of 392,321 (Figure 20). Cell density and viability also were calculated, as shown in Table 5. Although the viability was greater than 95% for all conditions , treatment groups had a decreased cell density compared to that of control and AGTR1.

Optimization of cell densities with treatments

To investigate the decreasing cell densities of treatment groups further cell proliferation also was measured using the BrdU assay (Appendix). These results showed that the treatment groups had a higher proliferation rate than that of the control. For this reason, to determine if cell counts would match proliferation rates on Day 3, the experiment was changed to match BrdU methods. HTLA cells were grown to approximately 90% confluency and then split into 100 x20 mm tissue culture dishes (Day 0). Cells were plated at a volume of 1,440,000 cells/plate into a volume of supplemented media. After 24 hours of cells being incubated (Day 1), they were transfected with a 60:1 ratio of FuGENE 6 to AGTR1 plasmid (0.5 μg). After being incubated for 24 hours (Day 2), cells were split and plated into six-well plates at a volume of 250,000 cells/well in supplemented media and various treatments. Cells were split between Day 2 and Day 3 so that the cells would reach 70% confluency by Day 3 instead of 90% to match BrdU

results. After being incubated for another 24 hours (Day 3), luminescence readings were taken. However, results could not be obtained because not enough cells grew to run in the machine (error occurred).

To obtain a large enough confluency of cells by Day 3, a variety of cell seedings were performed to determine what volume of cells allowed for an 80% confluency in a six-well plate. The number of 400,000 cells/well showed an 80% confluency after 24 hours later. This experiment was performed again plating a volume of 400,000 cells/well between Day 2 and Day 3 (Figure 21). To examine this method with an 80% confluency control, AGTR1, Ang II, resveratrol 100 μM, resveratrol 100 μM + Ang II, and losartan groups were examined. Average luminescence for control had a value of 13,002; AGTR1 had an increased value of 57,624 (Figure 21). Ang II had an increased average luminescence reading as did resveratrol 100 μM (Figure 21). The highest increased average luminescence was resveratrol + Ang II (Figure 21). Losartan showed a decreased average luminescence to AGTR1 (Figure 21). As shown in Table 6, all cell densities were in close proximity to each other, and viability of the cells was above 97%.

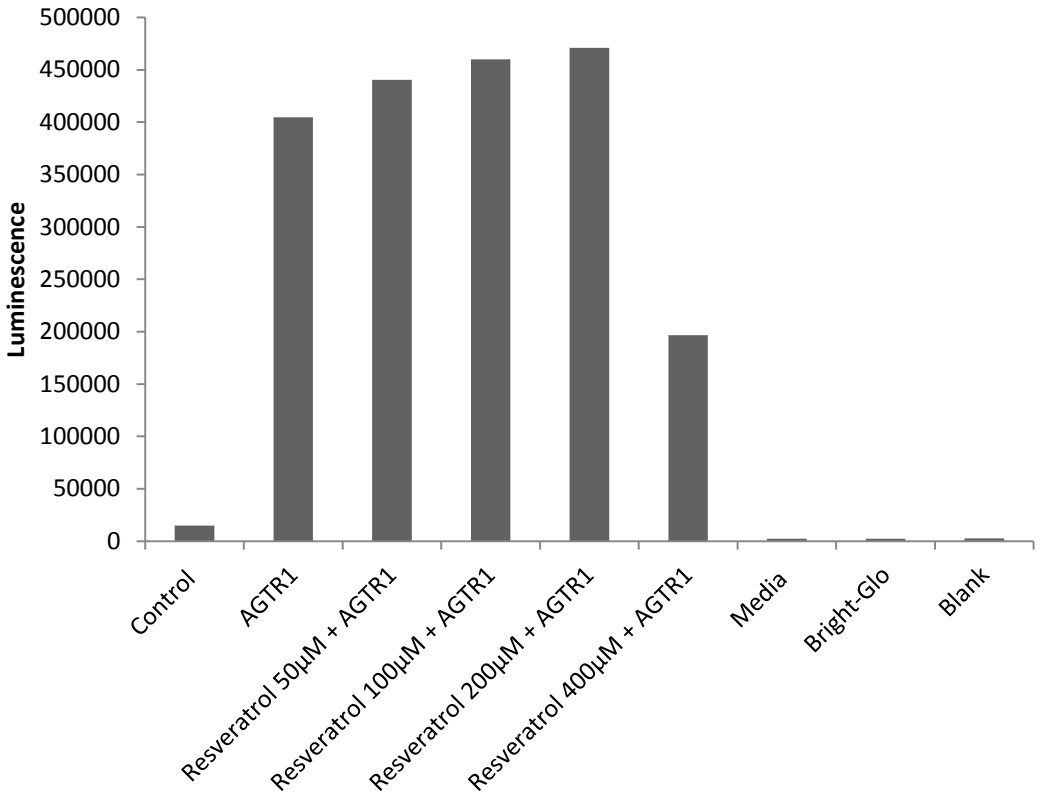


Figure 8. Effect of resveratrol on AGTR1 activation. Luminescence readings of control, 1μg of AGTR1, 50 μM, 100 μM, 200 μM, and 400 μM of resveratrol treatments with AGTR1, supplemented media, Bright-Glo, and Blank. Cells were seeded at a volume of 200 μL/well in six-well plates. Luminescence values were read using the FLUOstar OPTIMA one cycle with cycle time of 1 sec and a measurement interval time of 4.06 sec. n = 1.

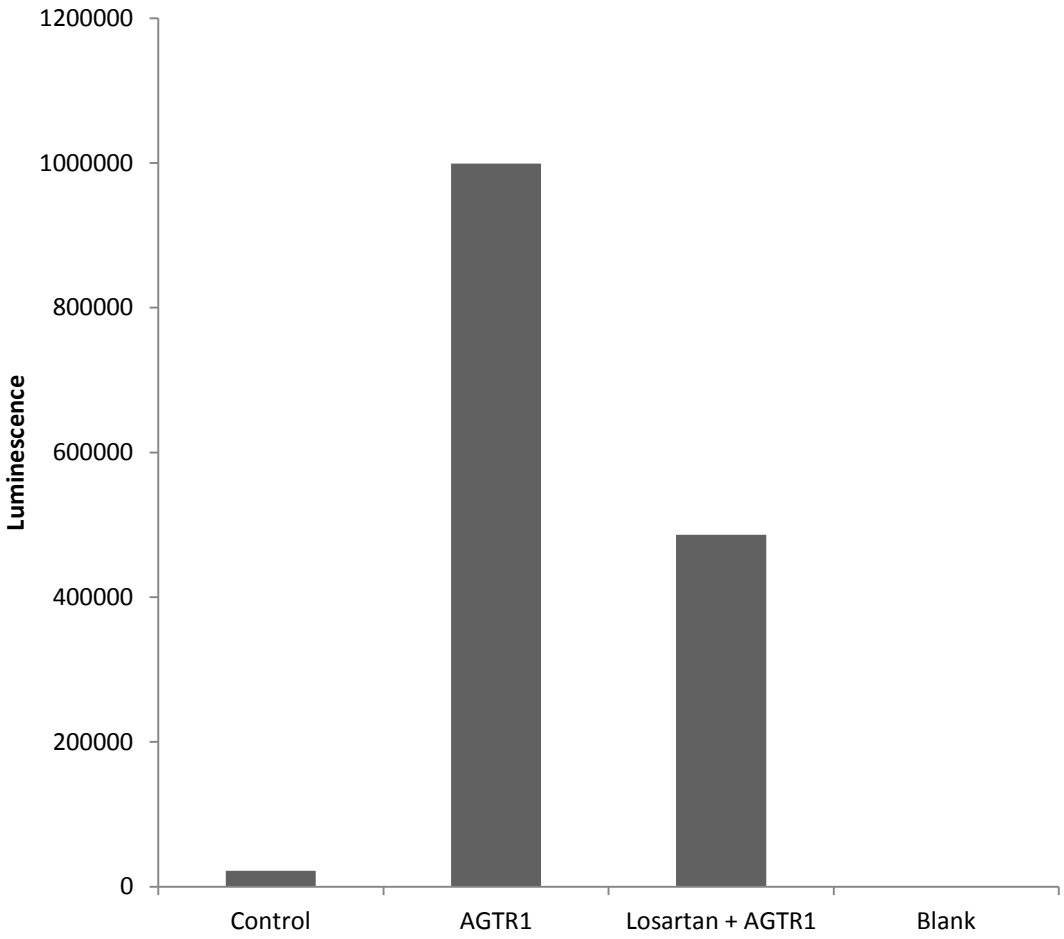


Figure 9. Effect of losartan on AGTR1 activation. Average luminescence readings of control, 1 μg of AGTR1, losartan (1333 nM) with AGTR1, and blank. Cells were seeded at a volume of 200 μL/well in six-well plates. Luminescence values were read using the FLUOstar OPTIMA one cycle with cycle time of 1 sec and a measurement interval time of 4.06 sec. n = 1.

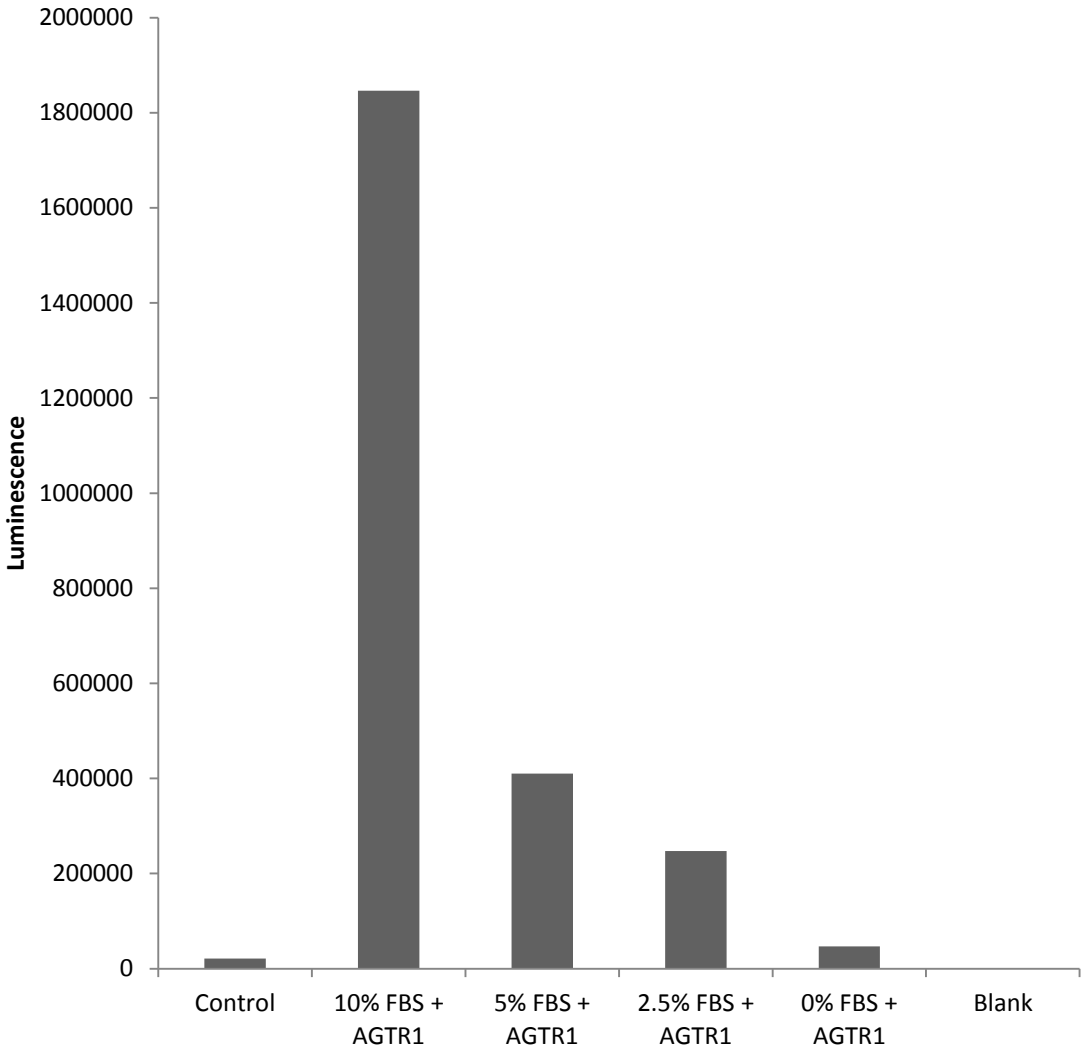


Figure 10. Effect of FBS on AGTR1 activation. Average luminescence readings of control and 1 μg of AGTR1 with FBS dilutions in supplemented media and blank. Cells were seeded at a volume of 200 μL/well in six-well plates. Luminescence values were read using the FLUOstar OPTIMA one cycle with cycle time of 1 sec and a measurement interval time of 4.06 sec. n = 1.

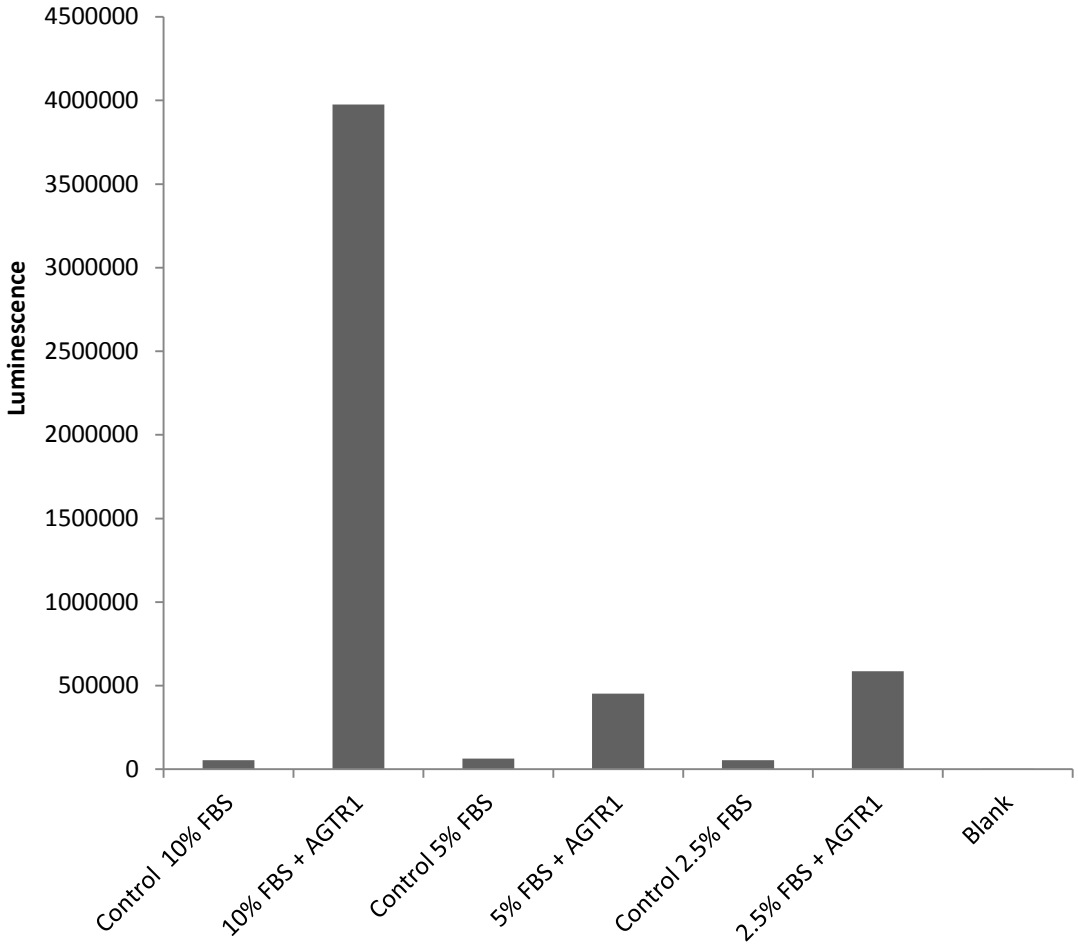


Figure 11. Effect of FBS on AGTR1 activation and control cells. Average luminescence readings of controls with 10%, 5%, and 2.5% FBS in supplemented media, 1 µg AGTR1 with FBS dilutions of 10%, 5%, and 2.5% in supplemented media. Cells were seeded at a volume of 200 µL/well in six-well plates. Luminescence values were read using the FLUOstar OPTIMA one cycle with cycle time of 1 sec and a measurement interval time of 4.06 sec. n = 1.

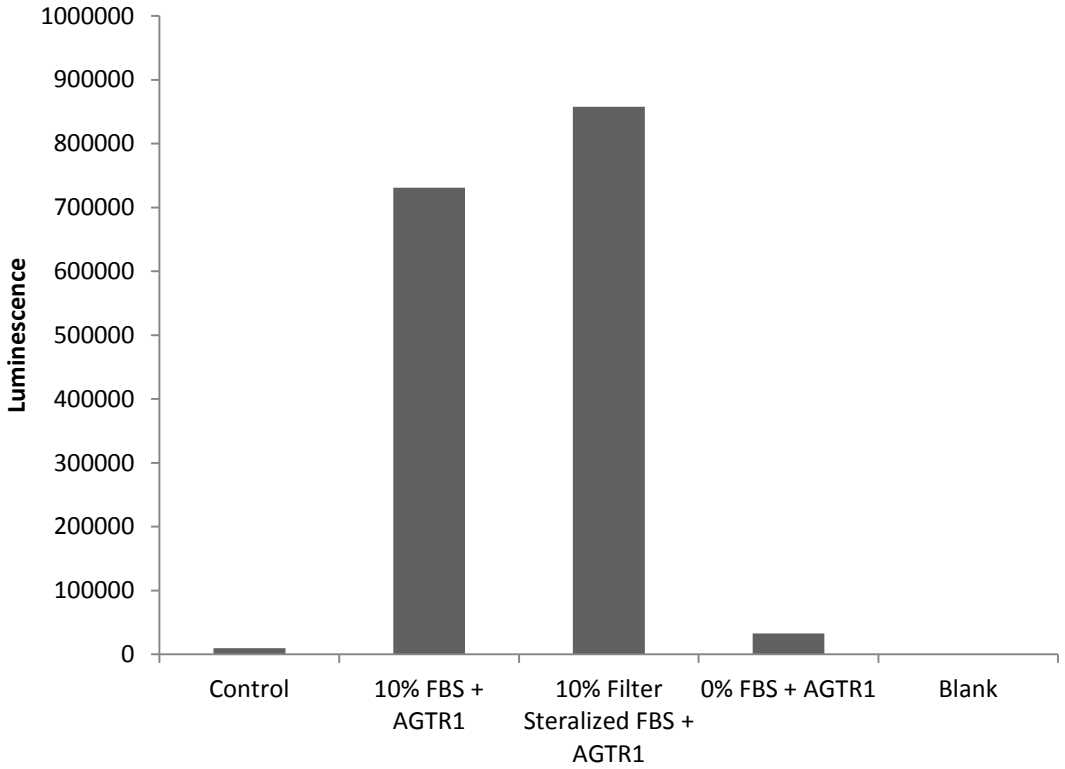


Figure 12. Effect of FBS, filter sterilized FBS, and no FBS on AGTR1 activation. Luminescence readings of control, 1 µg AGTR1 with FBS dilutions of 10%, filtered 10% and 0% supplemented media, and blank. Cells were seeded at a volume of 200 µL/well in six- well plates. Luminescence values were read using the FLUOstar OPTIMA one cycle with cycle time of 1 sec and a measurement interval time of 4.06 sec. n = 1.

Table 1. Cell Density and Viability 1. Number (#) of average viable, nonviable, and combined cells (average # of cells) per square for 10% FBS with AGTR1, 10% FBS filter sterilized with AGTR1, 5% FBS with AGTR1, 2.5% FBS with AGTR1, and 0% FBS with AGTR1. Calculated cell density and cell viability included. See appendix for formulas.

	Average # of Viable Cells (per square)	Average # of Non-Viable Cells (per square)	Average # of Cells (per square)	Cell Density (cells/ml)	Cell Viability %
10% FBS + AGTR1	48.89	1.27	50.17	1,003,300	97.4%
10% FBS Filter Sterilized + AGTR1	58.44	1.00	59.44	1,188,800	98.3%
5% FBS + AGTR1	47.39	1.50	48.89	977,778	97.0%
2.5% FBS + AGTR1	40.38	1.22	41.61	832,222	96.9%
0% FBS + AGTR1	13.33	0.94	14.28	285,600	93.3%

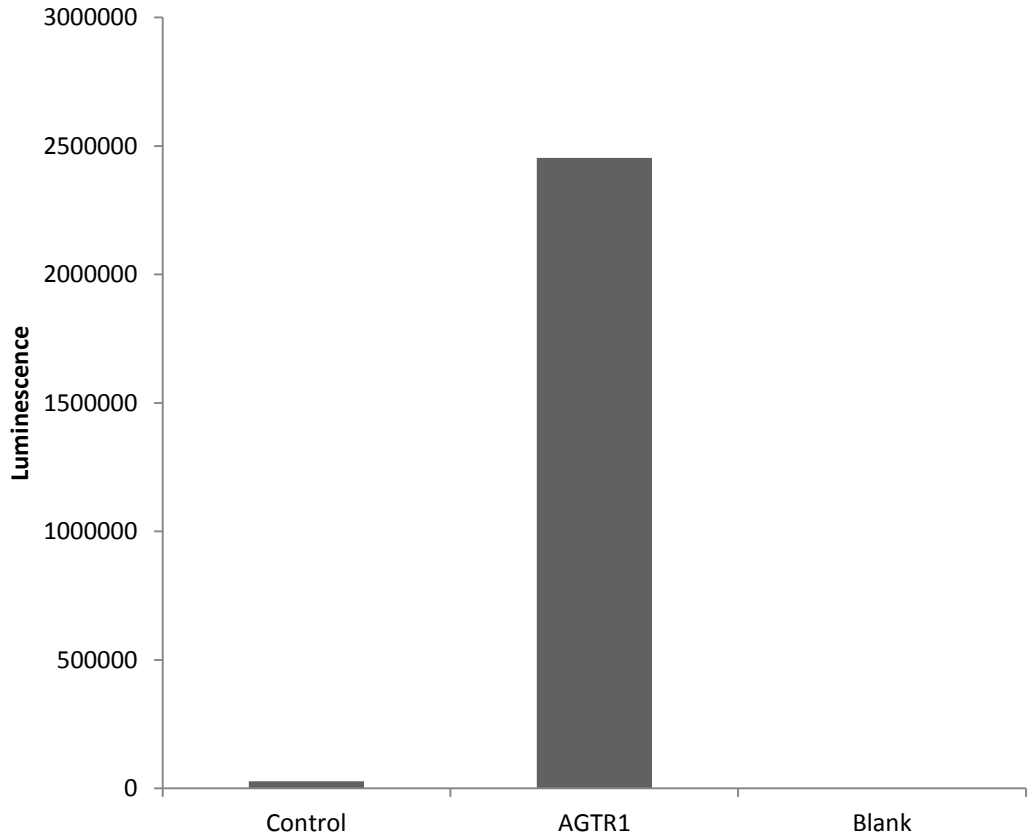


Figure 13. Effect of dialyzed FBS on AGTR1 activation. Average luminescence reading of control, AGTR1, and blank made with 10% dialyzed FBS in supplemented media. Cells were seeded at 250,000 cells/well in six-well plates. Luminescence values were read using the FLUOstar OPTIMA, one cycle with cycle time of 1 sec and a measurement interval time of 4.06 sec.n=1.

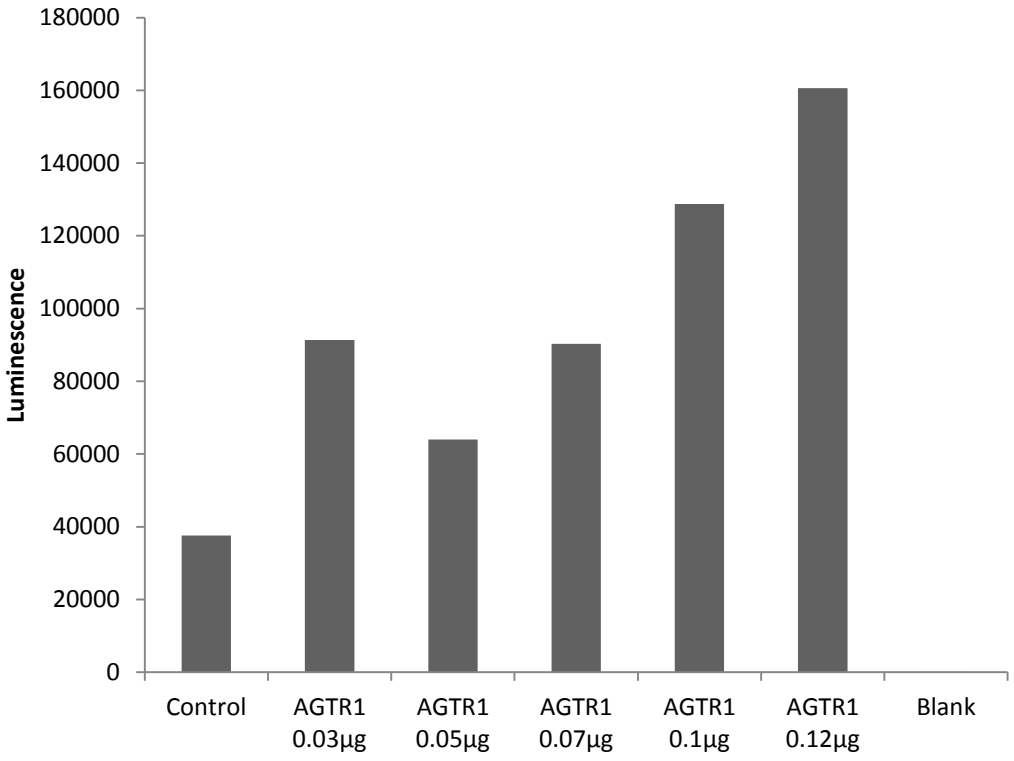
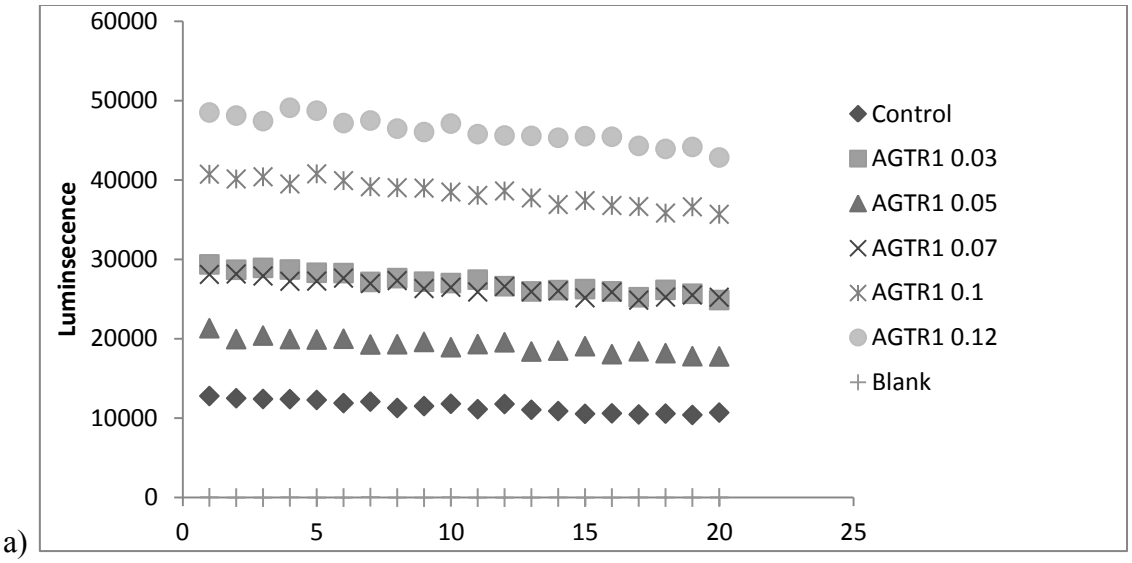
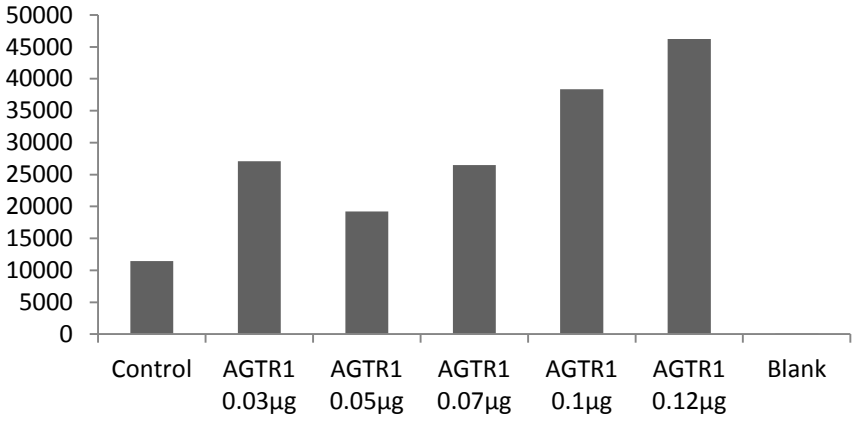


Figure 14. Effect of varying concentrations of AGTR1 plasmids. Luminescence readings of control, AGTR1 concentrations of 0.3 µg, 0.5 µg, 0.07 µg, 0.1 µg, 0.12 µg, and blanks. Cells were seeded at a volume of 200 µL/well in six-well plates. Luminescence values were read using the FLUOstar OPTIMA, for 1 cycle with cycle time of 1 sec and a measurement interval time of 4.06 sec. n = 1.



a)



b)

Figure 15. AGTR1 luminescence over time. Luminescence reading of control, AGTR1 concentrations of 0.3 µg, 0.5 µg, 0.07 µg, 0.1 µg, 0.12µg, and blank. Cells were seeded at a volume of 200 µL/well in six-well plates. When cells were at ~60% confluency, they were transfected with FuGENE 6 for 24 hr in incubator. Supplemented media were changed, and plate was incubated for another 24 hr. Luminescence values were read using the FLUOstar OPTIMA for 20 cycles with cycle time of 30 sec and a measurement interval time of 1 sec. n = 1. a) luminescence over 20 cycles b) average luminescence over 20 cycles.

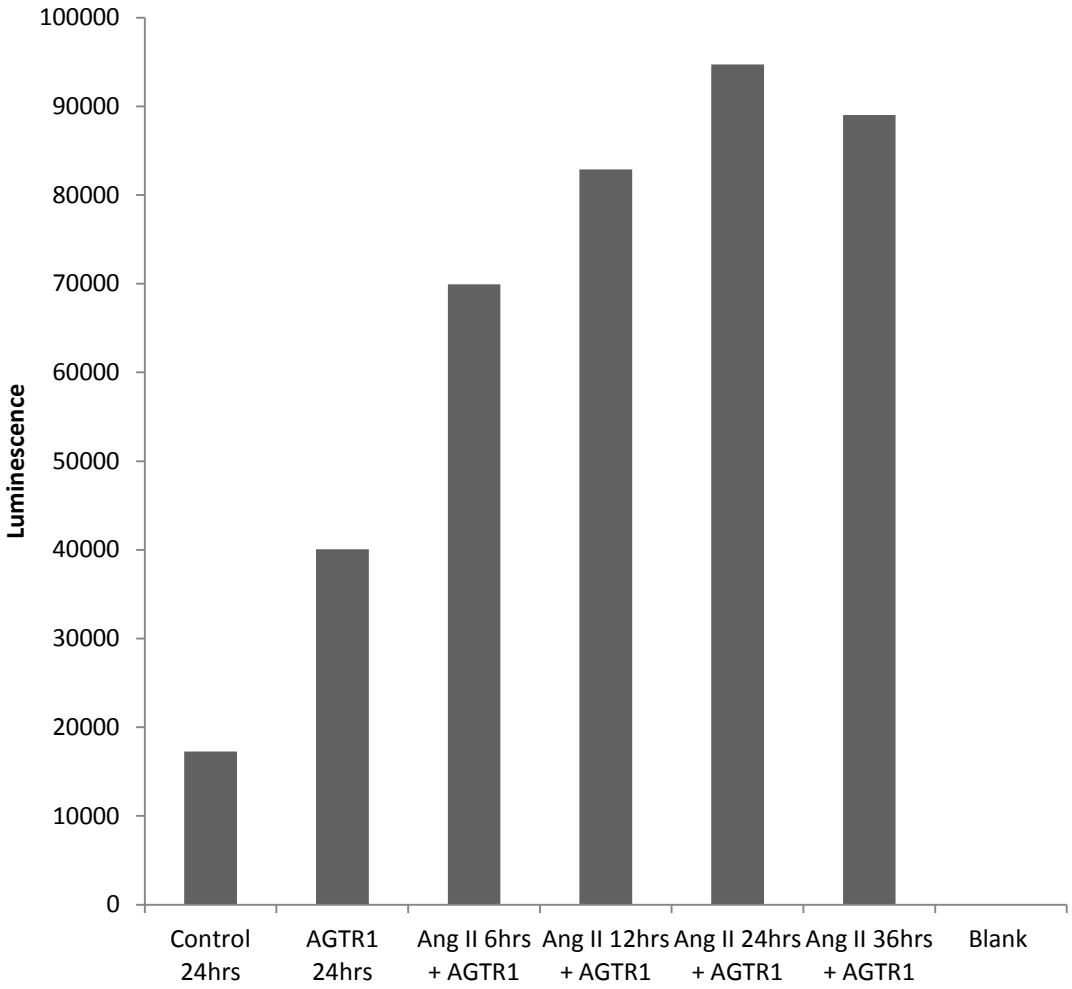


Figure 16. Ang II luminescence over time. Average luminescence reading of control, 0.1 µg of AGTR1, Ang II 6-, 12-, 24-, and 36-hr treatment periods with AGTR1 and blank. Cells were seeded with 250,000 cells/well in six-well plates. Luminescence values were read using the FLUOstar OPTIMA, for 1 cycle with cycle time of 1 sec and a measurement interval time of 4.06 sec. n = 1.

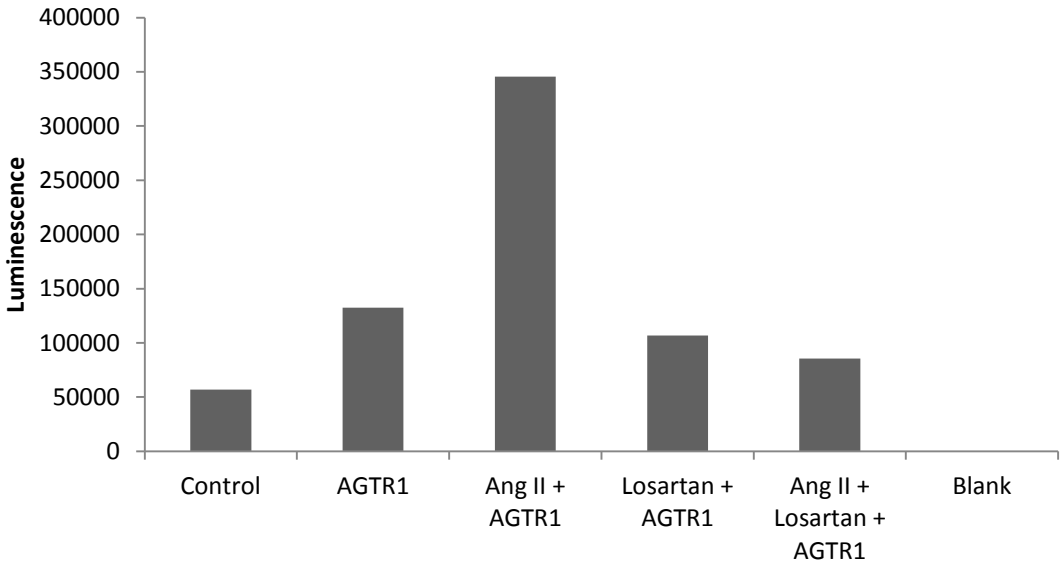


Figure 17. Effect of losartan in the presence of Ang II on AGTR1 activation. Average luminescence of control, 0.1 μg of AGTR1, Ang II (43 nM) with AGTR1, losartan (1333 nM) with AGTR1, Ang II + losartan with AGTR1, and blank. Cells were seeded with 250,000 cells/well in six-well plates. Luminescence values were read using the FLUOstar OPTIMA, for 1 cycle with cycle time of 1 sec and a measurement interval time of 4.06 sec. n = 1.

Table 2. Cell Density and Viability 2. Number (#) of average viable, nonviable, and combined cells (average # of cells) per square for Ang II with AGTR1 and losartan with AGTR1. Calculated cell density and cell viability included. See appendix for formulas.

	Average # of Viable Cells (per square)	Average # of Non-Viable Cells (per square)	Average # of Cells (per square)	Cell Density (cells/ml)	Cell Viability %
Ang II + AGTR1	98.30	1.17	99.5	1,990,000	98.8%
Losartan + AGTR1	87.00	2.11	89.11	1,178,222	97.6%

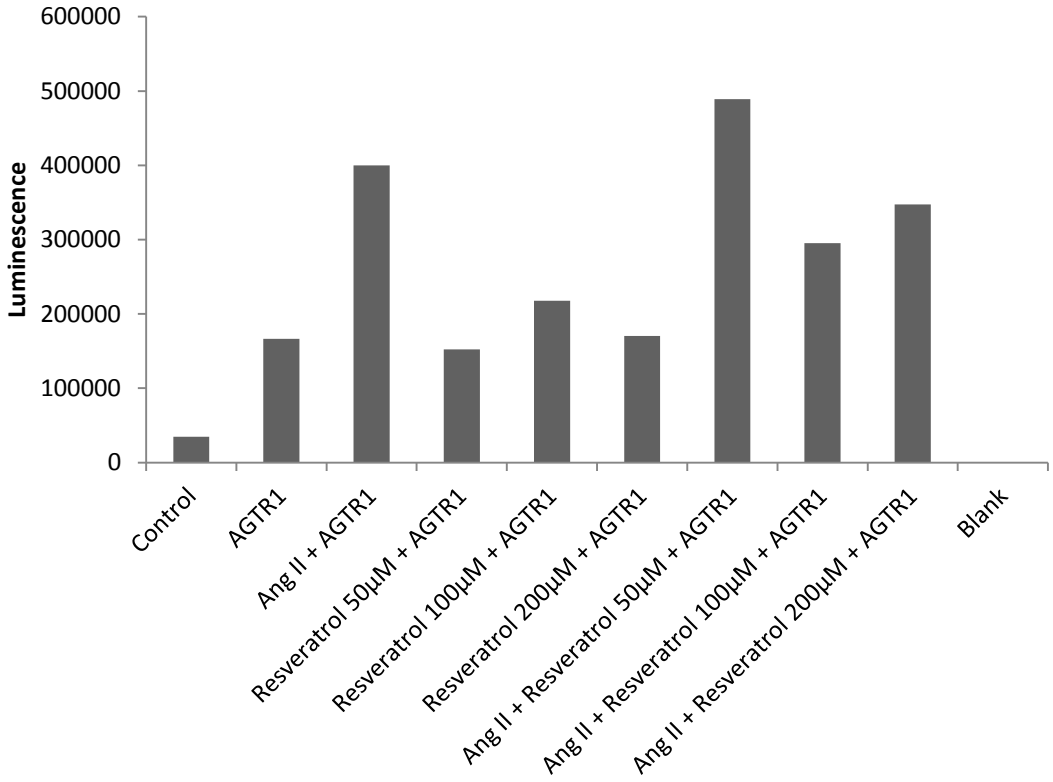


Figure 18. Effect of resveratrol in the presence of Ang II on AGTR1 activation. Cells were seeded with 250,000 cells/well in six-well plates Average luminescence reading of control, 0.1 μg of AGTR1, Ang II (43 nM) with AGTR1, 50 μM, 100 μM, and 200 μM resveratrol treatments with AGTR1, 50 μM, 100 μM, 200 μM resveratrol treatments + Ang II. Luminescence values were read using the FLUOstar OPTIMA 1 cycle with cycle time of 1 sec and a measurement interval time of 4.06 sec.
n = 1.

Table 3. Cell Density and Viability 3. Number (#) of average viable, non-viable and combined cells (average # of cells) per square for control, AGTR1, Ang II with AGTR1, resveratrol 50 μM,100 μM, and 200 μM with AGTR1. Calculated cell density and cell viability included. See appendix for formulas.

	Average # of Viable Cells (per square)	Average # of Non-Viable Cells (per square)	Average # of Cells (per square)	Cell Density (cells/ml)	Cell Viability %
Control	79.89	1.44	81.33	1,626,667	98.2%
AGTR1	71.28	2.28	73.56	1,471,200	96.9%
Ang II + AGTR1	59.33	2.50	61.82	1,236,667	98.8%
Resveratrol 50μM + AGTR1	52.17	2.50	54.67	1,093,333	95.4%
Resveratrol 100μM + AGTR1	49.38	2.05	51.44	1,028,889	95.9%
Resveratrol 200μM + AGTR1	50.00	2.72	52.72	1,054,444	94.8%
Resveratrol 50μM + Ang II + AGTR1	44.33	1.22	45.56	911,111	97.3%
Resveratrol 100μM + Ang II + AGTR1	41.11	1.00	42.11	842,222	97.9%
Resveratrol 200μM + Ang II + AGTR1	37.78	1.00	38.78	775,556	97.4%

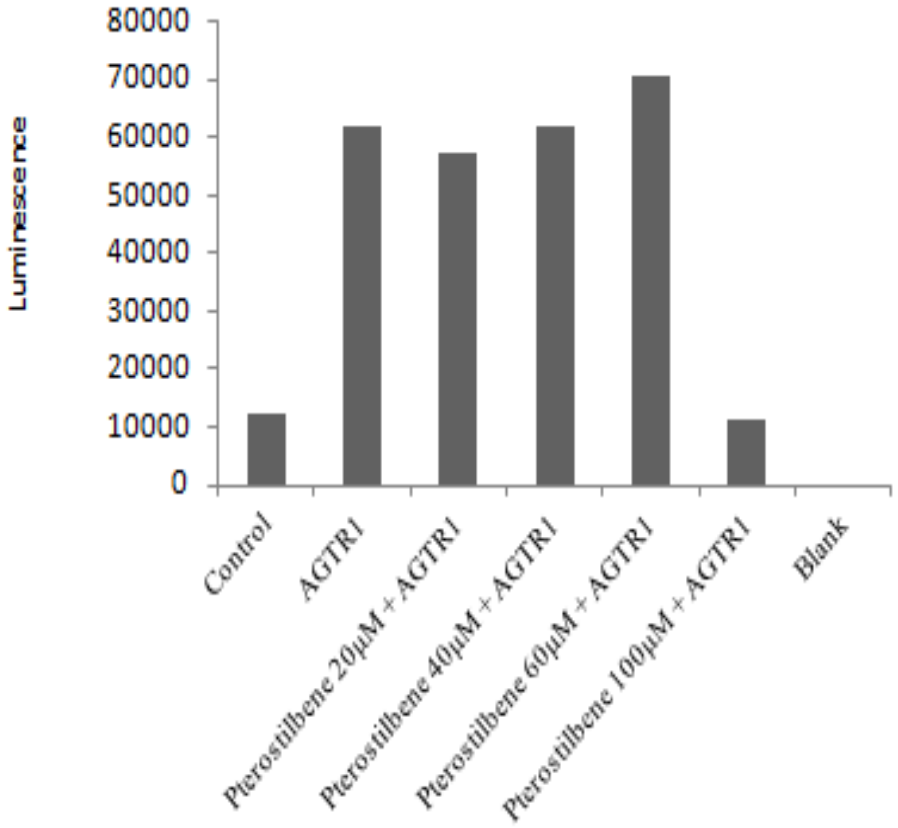


Figure 19. Effect of varying concentrations of pterostilbene on AGTR1 activation. Average luminescence reading of control, 0.1 µg of AGTR1, 20 µM, 40 µM, 60 µM, and 100 µM pterostilbene treatments with AGTR1, and blank. Cells were seeded with 250,000 cells/well in six-well plates. Luminescence values were read using the FLUOstar OPTIMA, for one cycle with cycle time of 1 sec and a measurement interval time of 4.06 sec. n = 1.

Table 4. Cell Density and Viability 4. Number (#) of average viable, nonviable,and combined cells (average # of cells) per square for AGTR1, Ang II with AGTR1, pterostilbene 20 µM, 40 µM, 60 µM, and 100 µM with AGTR1. Calculated cell density and cell viability included. See appendix for formulas.

	Average # of Viable Cells (per square)	Average # of Non-Viable Cells (per square)	Average # of Cells (per square)	Cell Density (cells/ml)	Cell Viability %
Pterostilbene 20µM + AGTR1	29.72	1.44	31.17	623,333	95.0%
Pterostilbene 40µM + AGTR1	18.55	1.44	20.00	400,000	92.7%
Pterostilbene 60µM + AGTR1	12.72	1.83	14.56	291,200	87.4%
Pterostilbene 100µM + AGTR1	3.11	0.66	3.78	75,600	82.3%
Control	49.05	1.39	50.06	1,001,120	98.9%
AGTR1	43.44	0.77	44.22	884,444	97.2%

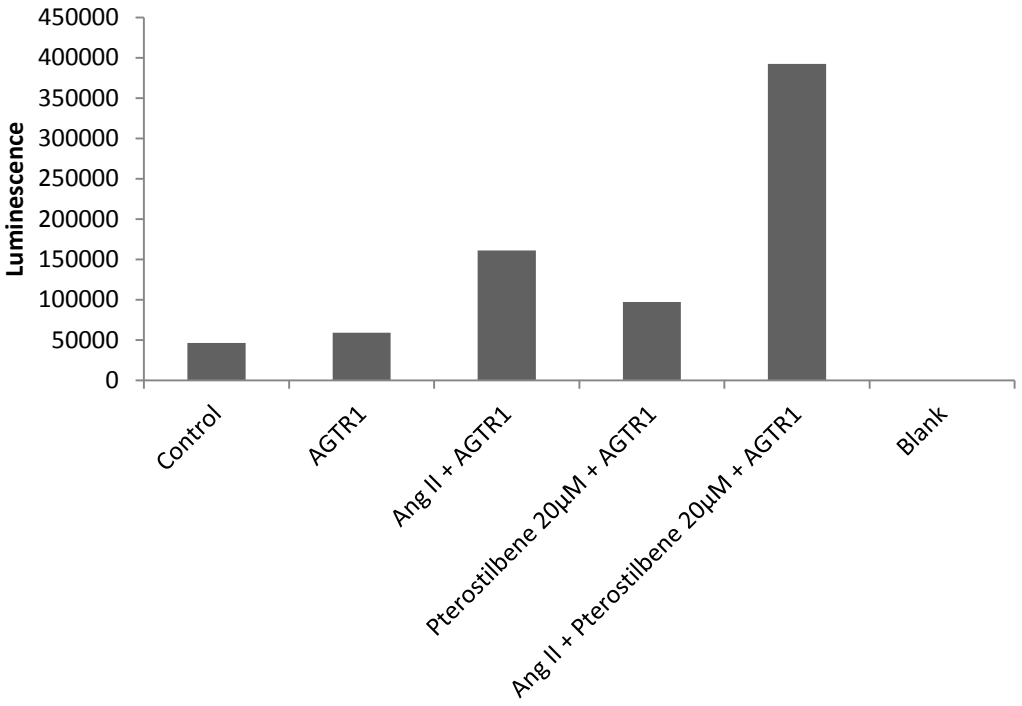


Figure 20. Effect of 20 μM pterostilbene in the presence of Ang II on AGTR1 activation. Average luminescence reading of control, 0.1 μg of AGTR1, Ang II (43 nM) with AGTR1, 20 μM pterostilbene treatment with AGTR1, 20 μM pterostilbene treatment + Ang II with AGTR1, and Blank. Cells were seeded with 250,000 cells/well in six-well plates. Luminescence values were read using the FLUOstar OPTIMA, for one cycle with cycle time of 1 sec and a measurement interval time of 4.06 sec. n = 1.

Table 5. Cell Density and Viability 5. Number (#) of average viable, nonviable, and combined cells (average # of cells) per square for control, AGTR1, Ang II (43 nM) with AGTR1, pterostilbene 20 µM and 100 µM with AGTR1. Calculated cell density and cell viability included. See appendix for formulas.

	Average # of Viable Cells (per square)	Average # of Non-Viable Cells (per square)	Average # of Cells (per square)	Cell Density (cells/ml)	Cell Viability %
Control	86.06	0.14	86.33	1,726,667	99.7%
AGTR1	73.61	1.00	74.61	1,492,200	98.6%
Ang II + AGTR1	46.38	4.80	47.61	952,200	97.4$
Pterostilbene 20µM + AGTR1	23.22	1.00	24.22	484,444	95.0%
Pterostilbene 20µM + Ang II + AGTR1	23.28	0.556	23.83	476,600	97.7%

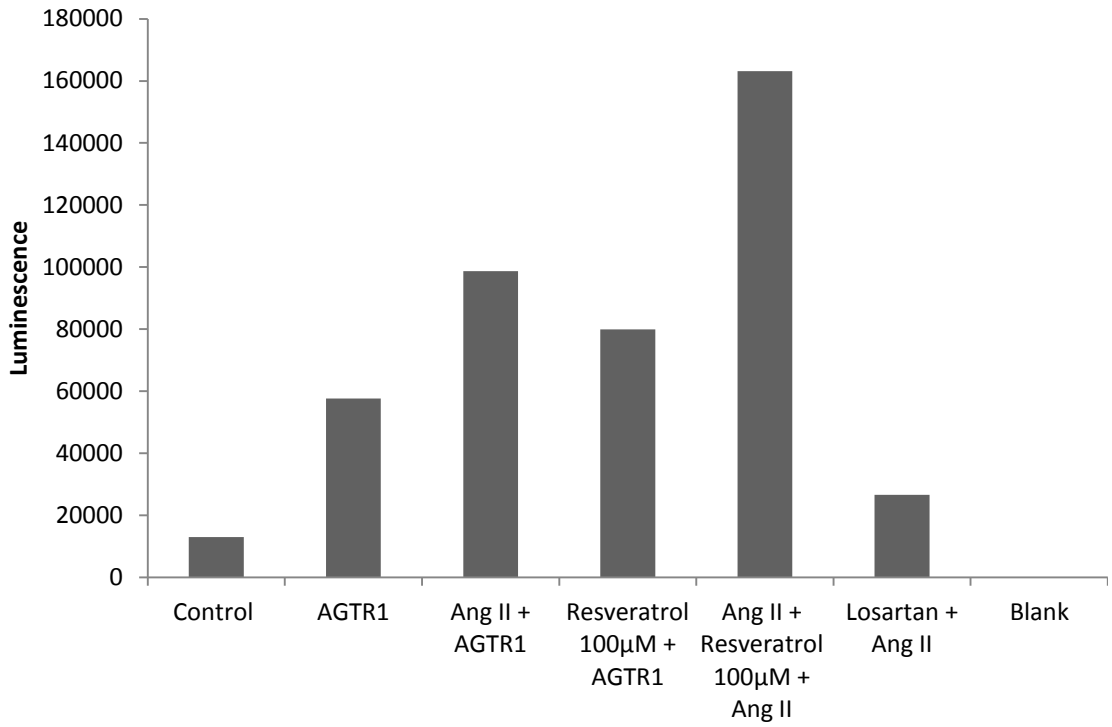


Figure 21. Effect of resveratrol 100 µM in the presence of Ang II on AGTR1 activation. Cells were seeded with 400,000 cells/well in six-well plates Average luminescence reading of control, 0.1 µg of AGTR1, Ang II (43 nM) with AGTR1, 100 µM resveratrol with AGTR1, 100 µM resveratrol + Ang II with AGTR1, and blank. Luminescence values were read using the FLUOstar, for one cycle with cycle time of 1 sec and a measurement interval time of 4.06 sec. n = 1.

Table 6. Cell Density and Viability 6. Number (#) of average viable, nonviable, and combined cells (average # of cells) per square for control, AGTR1, Ang II (43 nM) with AGTR1, resveratrol 100 µM with AGTR1, Ang II + 100 µM with AGTR1 and losartan (1333 nM) with AGTR1. Calculated cell density and cell viability included. See appendix for formulas.

	Average # of Viable Cells (per square)	Average # of Non-Viable Cells (per square)	Average # of Cells (per square)	Cell Density (cells/ml)	Cell Viability %
Control	30.00	0.67	30.67	613,333	97.8%
AGTR1	31.72	0.83	32.56	651,111	97.4%
Ang II + AGTR1	33.83	0.78	34.61	692,222	97.4%
Resveratrol 100µM + AGTR1	33.33	0.56	33.89	677,777	98.3%
Resveratrol 100µM + Ang II + AGTR1	30.33	0.78	31.11	622,222	97.4%
Losartan + AGTR1	32.72	0.61	33.33	666,667	98.2%

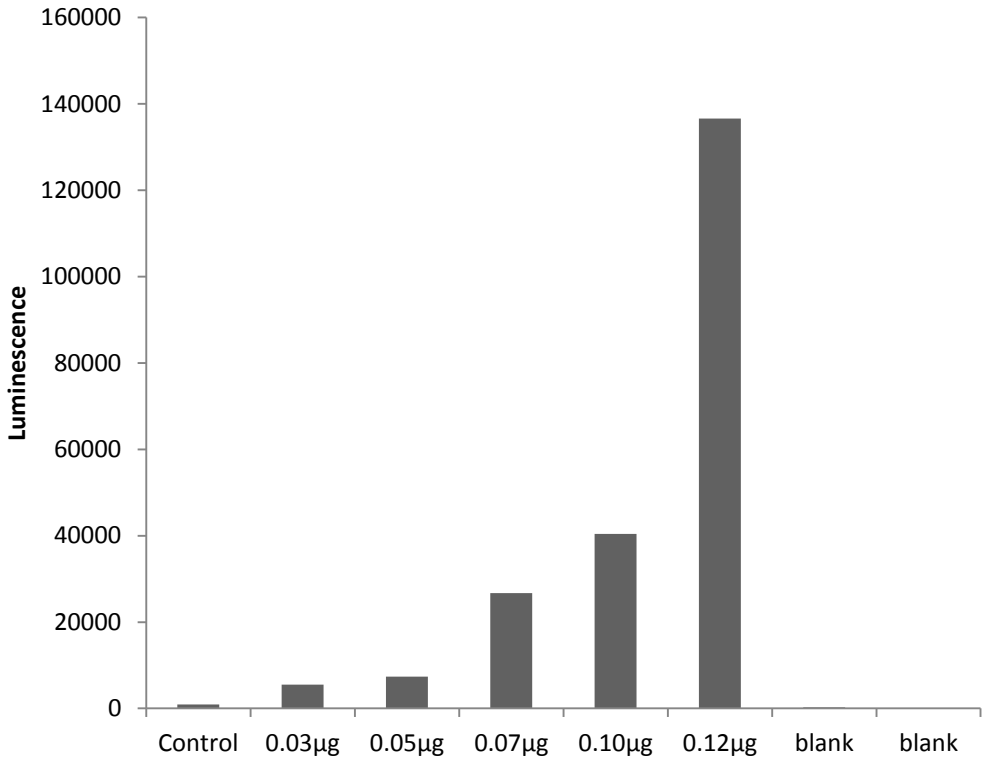


Figure 22. Effect of varying concentrations of pGL4.54 on AGTR1activation. Luminescence reading of control, pGL4.54 concentrations of 0.3 µg, 0.5 µg, 0.07 µg, 0.1 µg, 0.12 µg, and blanks. Cells were seeded at a volume of 200 µL in six-well plates. Luminescence values were read using the FLUOstar OPTIMA, for one cycle with cycle time of 1 sec and a measurement interval time of 4.06 sec. n = 1.

3.2. Optimized Results

The effect of resveratrol and pterostilbene on AGTR1 activation

In the previous optimization steps, treatment groups had similar cell densities as control and AGTR1 groups. Therefore, optimized steps, as indicated in the Methods section, included using 10% FBS containing media, using a AGTR1 plasmid concentration of 0.1 μg, splitting cells between Day 2 and Day 3 for optimal confluency, and taking a snapshot luminescence reading. Trials were performed using the groups that included control, AGTR1, Ang II, losartan, resveratrol 50 μM, resveratrol 100 μM, resveratrol 200 μM, resveratrol 50 μM + Ang II, resveratrol 100 μM + Ang II, resveratrol 200 μM + Ang II, pterostilbene 20 μM and pterostilbene 20 μM. In addition to treatment groups, pGL4.54, a control plasmid that was constantly expressed using Bright-Glo, was included. Before running the optimized trials, pGL4.54 was examined in the system with the different amount of 0.03 μg, 0.05 μg, 0.07 μg, 0.1 μg, and 0.12 μg. Control had a luminescence of 939 (Figure 22). The luminescence increased with an increase in pGL4.54 concentration as based on the following luminescence values of 0.03 μg at 5,513, 0.05 μg at 7,393, 0.07 μg at 26,695, 0.1 μg at 40,450, and 0.12 μg at 263 (Figure 22). Based on the numbers provided and the average luminescence of AGTR1, 0.1 μg of pGL4.54 was selected for the following optimized trials. Due to the ability of pGL4.54 to be constantly expressed, it acted as a parallel control by allowing luminescence readings to be standardizied against it.

The optimized trials were performed as described in the Methods section. The trials were performed four times , with each condition in a trial being performed in triplicate. Cell counts were taken 2 hours prior to the luminescence reading. As showing

in Tables 7, 8, 9 and 10, cell counts were all in close proximity to each other. However, the results were not statistically significant. As shown in Figure 23, the trends indicated that there was an increase luminescence in AGTR1 (0.73) from control (0.07). The treatment groups, in comparison to AGTR1, showed an increase with Ang II (1.09), a decrease with losartan (0.23), and a decrease with Ang II + losartan (0.21). Resveratrol groups of 50 μM (0.92), 100 μM (0.94), and 200 μM (1.2) showed an increase above AGTR1, with 200 μM being slightly higher than others. Resveratrol 50 μM (1.27), 100 μM (1.36) and 200 μM (1.05) + Ang II groups showed an increase above AGTR1 and showed a higher average luminescence than that of resveratrol groups, with the exception of resveratrol 200 μM + Ang II. Pterostilbene 20 μM (0.75) had remained similar to AGTR1. With the addition of Ang II, it had the large increase above AGTR1 (1.28). Ang + resveratrol 50 μM , 100 μM and pterostilbene 20 μM had an increase above Ang II alone.

Table 7. Cell Density and Viability Trial 1. Number (#) of average viable, non,viable, and combined cells (average # of cells) per square for control, AGTR1, pGL4.54, Ang II (43 nM) with AGTR1, losartan (1333 nM) with AGTR1, Ang II + losartan with AGTR1, resveratrol 50 μM, 100 μM, 200 μM with AGTR1, Ang II + resveratrol 50 μM,100 μM, 200 μM with AGTR1, pterostilbene 20 μM with AGTR1 and Ang II + pterostilbene 20 μM with AGTR1. Calculated cell density and cell viability included. See appendix for formulas.

	Average # of Viable Cells (per square)	Average # of Non-Viable Cells (per square)	Average # of Cells (per square)	Cell Density (cells/ml)	Cell Viability %
Control	32.17	0.72	32.89	657,777.8	97.8%
AGTR1	32.22	0.33	32.56	651,111.1	99.0%
pGL4.54	30.44	0.83	31.28	625,555.6	97.3%
Ang II + AGTR1	33.44	0.50	33.94	678,888.9	98.5%
Losartan + AGTR1	30.56	0.50	31.06	621,111.1	98.4%
Ang II + Losartan + AGTR1	34.67	1.06	35.72	714,444.4	97.1%
Resveratrol 50μM + AGTR1	30.44	0.16	30.61	612,222.2	99.4%
Resveratrol 100μM + AGTR1	30.72	1.06	31.78	635,555.6	96.7%
Resveratrol 200μM + AGTR1	31.00	0.44	31.44	628,888.9	98.6%
Resveratrol 50μM + Ang II + AGTR1	30.39	0.89	31.28	625,555.6	97.2%
Resveratrol 100μM + Ang II + AGTR1	31.22	0.89	32.11	642,222.2	97.3%
Resveratrol 200μM + Ang II + AGTR1	31.72	0.50	32.22	644,444.4	98.5%
Pterostilbene 20μM + Ang II + AGTR1	30.67	0.27	30.94	618,888.9	99.1%
Pterostilbene 20μM + Ang II + AGTR1	31.11	1.06	31.17	643,333.3	99.8%

Table 8. Cell Density and Viability Trial 2. Number (#) of average viable, nonviable, and combined cells (average # of cells) per square for control, AGTR1, pGL4.54, Ang II (43 nM) with AGTR1, losartan (133 3nM) with AGTR1, Ang II + losartan with AGTR1, resveratrol 50 μM, 100 μM, 200 μM with AGTR1, Ang II + resveratrol 50 μM,100 μM, 200 μM with AGTR1, pterostilbene 20 μM with AGTR1 and Ang II + pterostilbene 20 μM with AGTR1. Calculated cell density and cell viability included. See appendix for formulas.

	Average # of Viable Cells (per square)	Average # of Non-Viable Cells (per square)	Average # of Cells (per square)	Cell Density (cells/ml)	Cell Viability %
Control	30.17	0.88	31.06	621,111.1	97.1%
AGTR1	30.89	0.61	31.50	630,000	98.0%
pGL4.54	30.28	0.66	30.94	618,888.9	97.9%
Ang II + AGTR1	30.89	1.06	31.94	638,888.9	96.7%
Losartan + AGTR1	30.06	0.61	30.67	613,333.3	98.0%
Ang II + Losartan + AGTR1	30.89	0.61	31.50	630,000	98.0%
Resveratrol 50μM + AGTR1	33.11	1.11	34.22	684,444.4	96.8%
Resveratrol 100μM + AGTR1	32.50	1.56	34.06	681,111.1	95.4%
Resveratrol 200μM + AGTR1	30.72	1.17	31.89	637,777.8	96.3%
Resveratrol 50μM + Ang II + AGTR1	30.27	0.77	31.06	621,111.1	97.5%
Resveratrol 100μM + Ang II + AGTR1	32.61	0.83	33.44	668,888.9	97.5%
Resveratrol 200μM + Ang II + AGTR1	33.44	1.17	34.61	692,222.2	96.6%
Pterostilbene 20μM + Ang II + AGTR1	29.70	0.50	30.22	604,444.1	98.3%
Pterostilbene 20μM + Ang II + AGTR1	32.00	0.50	32.50	650,000	98.5%

Table 9. Cell Density and Viability Trial 3. Number (#) of average viable, non,viable, and combined cells (average # of cells) per square for control, AGTR1, pGL4.54, Ang II (43 nM) with AGTR1, losartan (1333 nM) with AGTR1, Ang II + losartan with AGTR1, resveratrol 50 μM,100 μM, 200 μM with AGTR1, Ang II + resveratrol 50 μM,100 μM, 200 μM with AGTR1, pterostilbene 20 μM with AGTR1 and Ang II + pterostilbene 20 μM with AGTR1.Calculated cell density and cell viability included. See appendix for formulas.

	Average # of Viable Cells (per square)	Average # of Non-Viable Cells (per square)	Average # of Cells (per square)	Cell Density (cells/ml)	Cell Viability %
Control	30	1.11	31.11	622,222.2	96.4%
AGTR1	30.72	1.27	32.00	640,000.0	96.0%
pGL4.54	30.39	0.55	30.94	618,888.9	98.2%
Ang II + AGTR1	34.6	1.50	36.11	722,222.2	95.8%
Losartan + AGTR1	32.10	0.94	33.05	661,111.1	97.1%
Ang II + Losartan + AGTR1	30.20	1.50	31.77	635,555.6	95.3%
Resveratrol 50μM + AGTR1	33.83	1.06	34.89	697,777.8	97.0%
Resveratrol 100μM + AGTR1	32.50	1.27	33.78	675,555.6	96.2%
Resveratrol 200μM + AGTR1	30.56	0.55	31.11	622,222.2	98.2%
Resveratrol 50μM + Ang II + AGTR1	30.39	0.50	30.89	617,777.8	98.3%
Resveratrol 100μM + Ang II + AGTR1	31.17	0.94	32.11	642,222.2	97.1%
Resveratrol 200μM + Ang II + AGTR1	30.39	0.88	31.27	625,555.6	97.2%
Pterostilbene 20μM + Ang II + AGTR1	31.22	1.17	32.89	647,777.8	94.9%
Pterostilbene 20μM + Ang II + AGTR1	30.44	1.72	32.17	643,333.3	94.6%

Table 10. Cell Density and Viability Trial 4. Number (#) of average viable, nonviable, and combined cells (average # of cells) per square for control, AGTR1, pGL4.54, Ang II (43 nM) with AGTR1, losartan (1333 nM) with AGTR1, Ang II + losartan with AGTR1, resveratrol 50 µM, 100 µM, 200 µM with AGTR1, Ang II + resveratrol 50 µM,100 µM, 200 µM with AGTR1, pterostilbene 20 µM with AGTR1 and Ang II + pterostilbene 20 µM with AGTR1.Calculated cell density and cell viability included. See appendix for formulas.

	Average # of Viable Cells (per square)	Average # of Non-Viable Cells (per square)	Average # of Cells (per square)	Cell Density (cells/ml)	Cell Viability %
Control	32.83	0.66	33.16	663,333.3	99.0%
AGTR1	34.0	0.94	34.94	698,888.9	97.3%
pGL4.54	30.83	0.50	31.33	626,666.7	98.4%
Ang II + AGTR1	32.44	0.83	33.28	665,555.6	97.5%
Losartan + AGTR1	31.50	1.11	32.61	652,222.2	96.6%
Ang II + Losartan + AGTR1	33.33	1.00	34.33	686,666.7	97.0%
Resveratrol 50µM + AGTR1	31.22	0.77	32.00	640,000	97.5%
Resveratrol 100µM + AGTR1	30.78	1.11	31.89	637,777.8	96.5%
Resveratrol 200µM + AGTR1	34.55	0.66	35.22	704,444.4	98.1%
Resveratrol 50µM + Ang II + AGTR1	29.89	0.38	30.83	616,666.7	97.0%
Resveratrol 100µM + Ang II + AGTR1	32.00	0.94	32.94	658,888.9	97.1%
Resveratrol 200µM + Ang II + AGTR1	30.89	0.77	31.67	633,333.3	97.5%
Pterostilbene 20µM + Ang II + AGTR1	32.06	1.50	33.55	671,111.1	95.6%
Pterostilbene 20µM + Ang II + AGTR1	30.50	1.33	31.83	636,666.7	95.8%

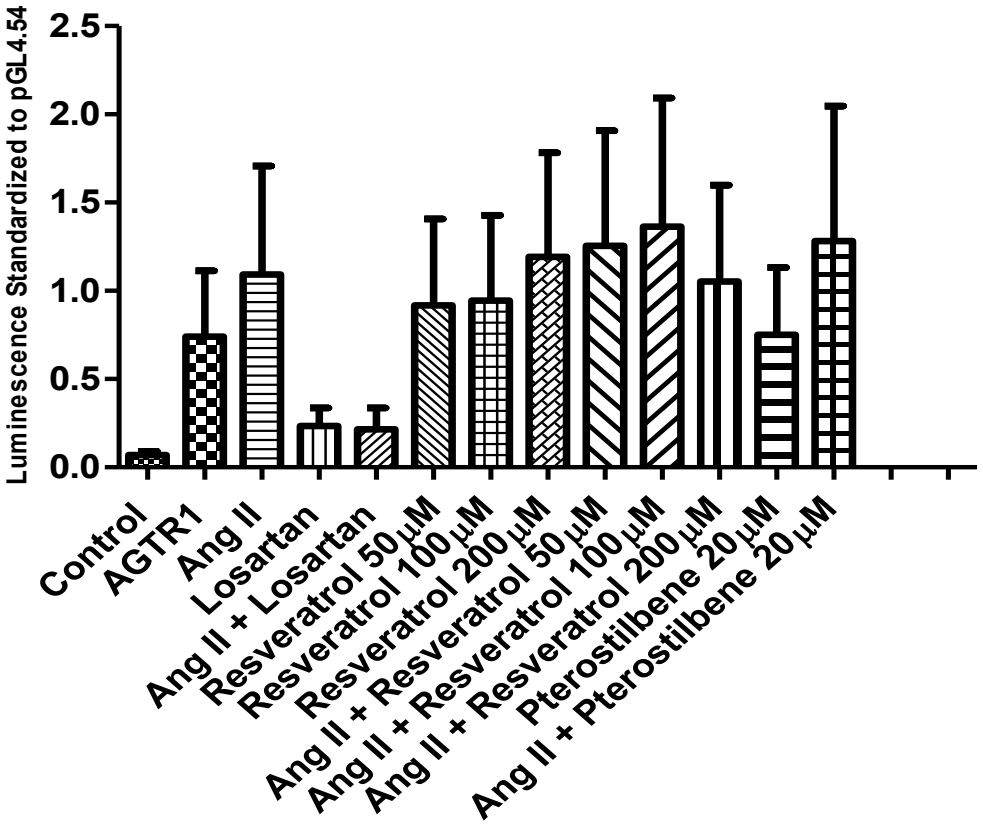


Figure 23. Effect of resveratrol and pterostilbene on AGTR1 activation. Average luminescence reading standardized to pGL4.54 of 0.1 μg AGTR1, Ang II, 50 μM, 100 μM, 200 μM resveratrol treatments with AGTR1, Ang II + resveratrol 50 μM, 100 μM, 200 μM with AGTR1, pterostilbene 20 μM with AGTR1 and Ang II + pterostilbene 20 μM with AGTR1. Error bars represent the mean ± SEM of four independent experiments.

Chapter 4: Discussion

Hypertension is mediated through the RAAS pathway [1]. The RAAS, through a variety of other systems, allows blood pressure homeostasis to be controlled [15]. Ang II, the major product of the pathway, works through two GPCRs, namely, AGTR1 and AGTR2 [44]. Ang II acts as an agonist to AGTR1, promoting vasoconstriction which allows for increased blood pressure [44].

One of the major antihypertensive medications that target the RAAS is the Ang receptor blockers, which block the binding of Ang II [10]. Losartan is an Ang II blocker that acts as an antagonist to AGTR1 [44]. Losartan limits the binding of Ang II to AGTR1, allowing for a decrease in blood pressure [44]. Although medications are available some drugs can cause unwanted side effects and might not fully block [5, 10, 11]. Therefore, naturally occurring compounds were studied as an alternative

Two naturally occurring compounds belonging to the polyphenol family thought to reduce blood pressure are resveratrol and pterostilbene [36, 38, 42]. Resveratrol in clinical and animal studies has been shown to reduce blood pressure [36]. Pterostilbene is an understudied compound in the cardiovascular system, with only one study showing that pterostilbene supplements can reduce the blood pressure of patients with high cholesterol [42]. It is thought that pterostilbene has better bioavailability because of the slightly altered structure from resveratrol but also might have a more toxic effect [38]. Although both compounds have the ability to lower blood pressure, neither compound had been examined specifically for its effect on the AGTR1 receptor. In fact, resveratrol and pterostilbene have yet to be studied at the receptor level regarding hypertension because interactions between GPCRs and ligands are not easily detectable.

The PRESTO-TANGO method developed by the Bryan Roth lab group is a revolutionary system that eased the detection of receptor and ligand activation without interference [26]. The most promising aspect of this system is that it facilitated the scanning of the entire human genome with a compound/ligand at once, allowing new activities of GPCRs to be determined. Constructs of GPCRs were designed specifically for this system, including AGTR1. This system allowed researchers to study the effect of these polyphenols role in hypertension by looking at their effect on AGTR1 activation.

The PRESTO-TANGO system, although a revolutionary system for studying GPCRs and ligand interaction, was still a new system. This meant that the PRESTO-TANGO system had to be optimized to look at AGTR1 with the compounds of resveratrol and pterostilbene. Currently, limited methods are available for this system, but they have never been optimized specifically for AGTR1. To optimize this study, the limited amount of available information on the PRESTO-TANGO was combined with knowledge from the TANGO method [26, 45].

4.1 Optimization of System

The system first had to be investigated to determine whether this system would produce a luminescence reading on Day 3 using the FLUOstar OPTIMA. This experiment was carried out in six-well plates to optimize experiment. When cells reached approximately 90% confluency, a volume of 200 µL of cells was plated/well. This was an experimental value based on the size of a six-well plate. Cells were incubated at 37° C, 5% CO_2, and 100% humidity for optimal cell growth [26]. Cells, except control, were then transfected 24 hours later (Day 1) with a 6:1 ratio of FuGENE 6 to AGTR1 based on the recommended guidelines by the FuGENE 6 transfection agent, allowing AGTR1 to

be transected inside the cells. The media was removed, and treatment or supplemented media was added after being transfected for 24 hours (Day 2). This was a change to the PRESTO-TANGO method based on the methods for the PRESTO-TANGO system [26].

Finally, after another 24 hours (Day 3), luminescence readings were taken using the FLUOstar OPTIMA machine. This involved removing media, adding 100 μL of Bright-Glo, incubating for 5 minutes (in the dark) on plate shaker and then transferring to a 96-well plate to read in the machine. These experimental steps were based on previous steps in the literature [26, 45]. The setting that was chosen for a snapshot reading was one cycle with a cycle time of 1 second and a measurement interval time of 4.06 seconds. This again was an experimental setting. It should be noted that the time frame of 24 hours between each day was selected based on previous literature on transfection, TANGO, and the PRESTO-TANGO methods [26, 45].

When this experiment was first run following the aforementioned guidelines, it showed that AGTR1 had almost a luminescence reading 40 times higher than that of the nontransected control cells (Figure 8). However, when combined with losartan, the luminescence reading was decreased (Figure 9), indicating that AGTR1 was being activated on its own (Figure 9). Because AGTR1 was being activated, the concern was that it would be difficult to examine the effect of treatments on AGTR1 if AGTR1 on its own was being activated and produced such a high luminescence (Figures 8 & 9).

The next step in the optimization process was to reduce AGTR1's luminescence readings. It was first thought that an ingredient in the supplemented media might have been activating the AGTR1 receptor, specifically, the FBS. Although FBS provides nutrients to cells, it can contain unknown ingredients because it has been poorly defined

[46]. An unknown compound in the FBS was thought to be what might have caused an increase in AGTR1 activation because it was the most undefined ingredient in the supplemented media.

Reducing the amount of FBS in the supplemented media showed that the luminescence readings of AGTR1 decreased (Figures 10 & 11). However, with the decrease in AGTR1's luminescence readings, there also was a decrease in cell density. As shown in Table 1, the cell density decreased as there was a decrease in the FBS percentage in the supplemented media, but the viability of the cells was high. Because the cells were largely unaffected as viability of all cells being over 93%, it was thought that the cells were growing slower because fewer nutrients were being added to the supplemented media. To keep the nutrients high, with 10% FBS being added to the media, filter sterilized and dialyzed media was tested. The literature suggested that this may remove small undefined compounds from FBS [45, 47]. However, these methods did not reduce AGTR1 luminescence values; instead, they produced an increased response (12 & 13), indicating that AGTR1 was being activated still.

The next step in the optimization process was to reduce the amount of AGTR1 since the amount of FBS could not be reduced as it was thought to be affecting cell growth. Previously, the amount of 1 μg of AGTR1 was being used based on plate size and FuGENE 6 guidelines. However, reducing the amount of AGTR1 plasmid showed that lower amounts could reduce AGTR1 luminescence readings (Figures 14 & 15). While this was done, the settings on the FLUOstar OPTIMA also were changed to see if time affected the readings. Previously, only a snapshot reading had been taken, but by taking the reading over 10 min for 20 cycles with a cycle time of 30 seconds and a

measurement interval time of 1 seconds, a true reading could be shown. However, the half-life of the Bright-Glo™ Luciferase Assay System is 30 minutes and the readings over the 10 minutes indicated a constant decrease over the 10 minutes (Figure 15) [48]. This data suggested no difference between a snapshot reading or the average over the 10 min, as shown in Figures 14 and 15, in which the trends between different AGTR1 amounts were the same between the two figures. Therefore, the remainder of the experiments were carried out with a snapshot reading. In addition, based on the data, 0.1 μg of AGTR1 was selected to be used for the remaining experiments because it reduced AGTR1 activation levels. Therefore, trials were transfected at a 60:1 ratio of FuGENE 6 to AGTR1.

The third step of optimization was to ensure that the system would work correctly by using the known agonist of Ang II and the known antagonist of losatan in the system. Both well-known compounds are used in the regulation of blood pressure and so were selected for this experiment. This system facilitated the detection of agonist of GPCRs through an increased luminescence signal. Thus, Ang II should have produced a luminescence greater than that of AGTR1, suggesting that AGTR1 was being activated by Ang II. In the case of losartan, no increased signal should have been displayed. First, Ang II was examined at the different time points to determine whether leaving the treatment on for longer periods of time would have affected the luminescence reading. As shown in Figure 16, Ang II luminescence readings did not fluctuate much between 6 and 36 hours. It should also be noted that on Day 0, 250,000 cells were plated per well based on HEK293 cells, plate size and experimental values to reach 90% confluency by Day 3. Again, this was demonstrated in Figure 17, which indicated that the system was working

based on the luminescence values observed. Ang II had an increased average luminescence over AGTR1; losartan and the combination of Ang II had decreased values, suggesting that Ang II was activating the receptor and that it was working as the agonist. The decreased luminescence observed for the losartan treatments suggested that losartan was able to block the effect of Ang II and was working as an antagonist. The concentrations for Ang II and losartan showed good viability (Table 2).

The final step was optimization of the treatments within the PRESTO-TANGO system. Optimization involved two steps: The first step was determination of resveratrol and pterostilbene concentration, and the second step was normalization of the cell densities. Based on the system thus far, the experiment was tried with various concentrations of resveratrol that included 50, 100, and 200 μM. The results of using various concentrations suggested that resveratrol did not produce activation when combined with AGTR1, but may have when combined at low dose concentrations (50 μM) with Ang II (Figure 18). A cell count indicated that the cells that received treatment had a decreased cell count compared to that of the control and AGTR1. Therefore, the effect of resveratrol could not be determined at that time. The viability of the cells was still greater than 94%, suggesting that cell concentrations were not killing the cells (Table 3). This same experiment was conducted again with various concentrations of pterostilbene, but because pterostilbene was a newer compound, there was less information available. Therefore, a range of concentrations suggested from other cell lines was created that contained the concentrations of 20, 40, 60, and 100 μM [49, 50]. Concentrations of 60 μM and 100 μM were eliminated because of the dramatic change in cell morphology. Based on a similar morphology and cell density to AGTR1, the

concentration of 20 μM was selected (Table 4). Based on Figures 19 and 20, average luminescence readings suggested that pterostilbene did not cause AGTR1 activation at 20 μM, but with the addition of Ang II, pterostilbene did (Figure 20). Cell densities decreased with treatments compared to that of AGTR1 (Table 5). Therefore, the effect of pterostilbene could not be determined at the time. The viability of the cells was still greater than 95%, suggesting that cell concentrations were not killing the cells at 20 μM.

The final part of the optimization was normalization of cell densities with treatments. As indicated previously, the effect of resveratrol and pterostilbene could not be determined because cell densities were not similar between AGTR1 and treatments. If the cell counts were not similar, results might not have been accurate because fewer cells were producing luminescence in the treatment wells. This could not confirm whether the receptor was being or not being activated. It was originally thought that based on the viability of the cells being high, treatment wells were growing slower. To confirm this hypothesis, a BrdU test was performed, which allows the proliferation of cells to be examined. These results indicated that the proliferation of treatments was higher than that of the control (see Appendix). This meant that the cells might have been growing faster and thus detaching in the treatment groups. Therefore, cell counts could have been lower for the treatment groups because the cells could have been lost during the counting stage. The experiment was optimized to BrdU format, growing cells in 100 x 20 mm dishes and splitting the cells between Day 2 and Day 3 into six-well plates to reach a 80% confluency by Day 3 (see Methods section). This experiment was run once more based on the Methods section, which produced cell densities similar between treatments, AGTR1 and control with a viability of greater than 97% (Table 6). It also was verified

that it could run in the FLUOstar OPTIMA with 80% confluency on Day 3 (Figure 21). Luminescence values were read using the FLUOstar for one cycle with a cycle time of 1 second and a measurement interval time of 4.06 sec. The average luminescence for control had a value of 13,002, whereas AGTR1 had an increased value of 57,624 (Figure 21). This indicated that the addition of AGTR1 to the cells created a higher luminescence reading, although in close proximity to the control. Ang II had an increased average luminescence reading of 98,656, as did resveratrol at 100 μM (Figure 21). This indicated that Ang II and resveratrol activated the AGTR1 receptor. The highest increased average luminescence was resveratrol + Ang II, with a value of 163,183 (Figure 21), thus confirming the activation of AGTR1. Losartan showed a decreased average luminescence to AGTR1 at a value of 26,601 (Figure 21), indicating that it did not activate AGTR1.

4.2 Optimized System

Once the system had been optimized with AGTR1 and treatments, it was run four times with triplicates for each condition. The optimized steps, as indicated in the Methods section, included using 10% FBS containing media, using a AGTR1 plasmid concentration of 0.1 μg, splitting cells between Day 2 and Day 3 for optimal confluency, and taking a snapshot reading. The conditions included control, AGTR1, Ang II, losartan, resveratrol 50 μM, resveratrol 100 μM, resveratrol 200 μM, resveratrol 50 μM + Ang II, resveratrol 100 μM + Ang II, resveratrol 200 μM + Ang II, pterostilbene 20 μM, and pterostilbene 20 μM+ Ang II,. In addition to the treatment groups, pGL4.54, a control plasmid that is constantly expressed using Bright-Glo, was included. As shown in Figure 22, 0.1 μg of pGL4.54 was selected for this experiment because it was a middle number in the dilution series.

Tables 7, 8, 9, and 10 indicated that for all four trials, cell densities were similar. Luminescence readings were standardized to pGL4.54 for each of the four trials. The average of each group was then taken for each trial, and then those averages were combined to produce Figure 23. AGTR1 (0.73) increased from control (0.07). The treatment groups, in comparison to AGTR1, showed an increase with Ang II (1.09), a decrease with losartan (0.23), and a decrease with Ang II + losartan (0.21). This result indicated that Ang II activated AGTR1, having an agonist effect, and that losartan did not activate the receptor, but could block Ang II from binding, having an antagonist effect. However, the results were not statistically significant.

Resveratrol groups of 50 μM (0.92), 100 μM (0.94), and 200 μM (1.2) showed an increase above AGTR1, with 200 μM being slightly higher than others (Figure 23). This increase indicated that resveratrol on its own actually activated AGTR1, thus having an agonist effect. Resveratrol 50 μM (1.27), 100 μM (1.36), and 200 μM (1.05) + Ang II groups showed an increase above AGTR1 and were higher then resveratrol groups, with the exception of Ang II + 200 μM, thus confirming that resveratrol activated the receptor, having an agonist effect (Figure 23). Resveratrol 50 μM and 100 μM with Ang II were higher than Ang II alone indicating an increased activation. However, the results were not statistically significant.

Pterostilbene (0.75) remained similar to AGTR1, indicating that pterostilbene at 20 μM did not activate the receptor (Figure 23). When pterositlbene was combined with Ang II, it had a large increase above AGTR1 (1.28), indicating an agonist effect (Figure 23). The combination also was higher than Ang II alone, indicating that pterostilbene,

when combined with an agonist, produced an increased activation. However, the results were not statistically significant.

These results suggested that resveratrol and pterostilbene did not produce a decreased luminescence compared to that of AGTR1. In fact, the results suggested that they activated AGTR1 when combined with Ang II. Although not statistically significant, these results suggested that resveratrol and pterostilbene do not block Ang II and do not work through the AGTR1 receptor to reduce blood pressure. Therefore, they may be working through another GPCR to reduce blood pressure if they do indeed reduce blood pressure. Based on the results obtained, pterostilbene may be more toxic to the cells, but both resveratrol and pterotilbene promote AGTR1 activation in combination with ANG II.

Chapter 5: Conclusion

Hypertension is a serious medical condition that can have detrimental consequences. Investigating alternative treatment options with the natural compounds of resveratrol and pterostilbene offered the possibility of reducing the side effects associated with drugs. GPCRs, are important targets for drugs, so understanding how these compounds work at the receptor level will help to explain how they can reduce blood pressure. This study examined resveratrol and pterostilbene at the receptor level using the PRESTO-TANGO method for the first time, focusing on the angiotensin II receptor type 1 (AGTR1). Results indicated that that resveratrol at concentrations of 50 μM, 100 μM, and 200 μM activate the receptor and also when combined with Ang II, indicating an agonist effect. Ang with resveratrol 50 μM and 100 μM had a greater activation then Ang II alone. It was also found that pterostilbene 20 μM combined with Ang II activated AGTR1 and has a greater activation then Ang II alone. Although the results were not statistically significant, the trends suggested that resveratrol and pterostilbene do promote AGTR1 activation. Future studies will be needed to investigate these compounds further with receptors involved with hypertension.

Chapter 6: References

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Chapter 7: Appendix

7.1 Formulas

7.1.1 Cell Density

Cell Density (cells/ml) = Average count per square x dilution factor x 10^4 [51]

*Average count = # of cells combination (viable + non-viable)

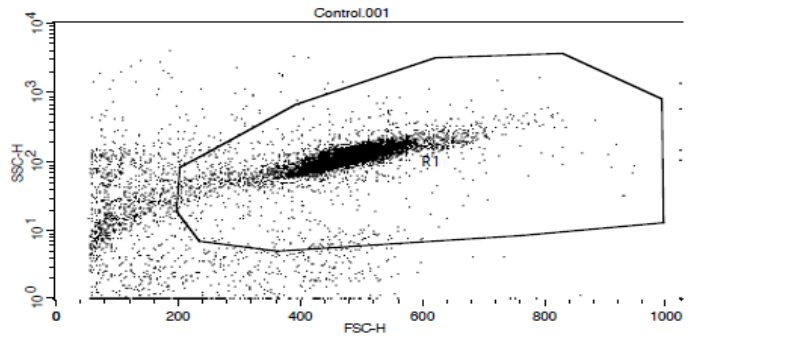
7.1.2 Cell Viability

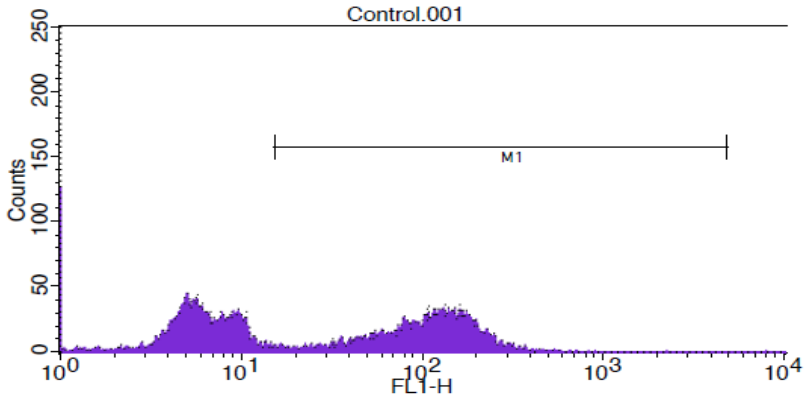
Cell Viability (%) = Average # of viable cells / total cells (viable + non-viable) x 100 [52]

7.2. BrdU Results

All supplementary data is reported as n = 1 for BrdU results.

7.2.1 Control





Histogram Statistics

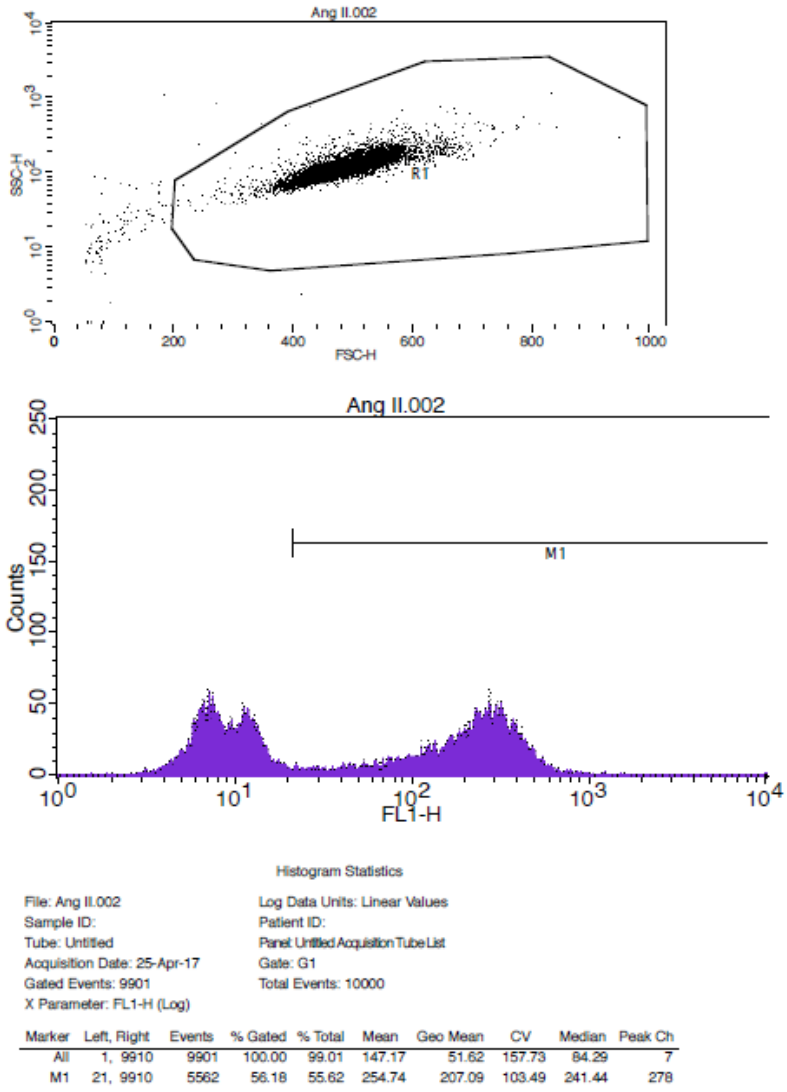
File: Control.001
Sample ID:
Tube: Untitled
Acquisition Date: 25-Apr-17
Gated Events: 8232
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: G1
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	8232	100.00	82.32	69.08	24.20	232.68	22.27	1
M1	15, 4783	4233	51.42	42.33	124.40	102.72	66.22	113.42	130

Figure 1. BrdU result for control.

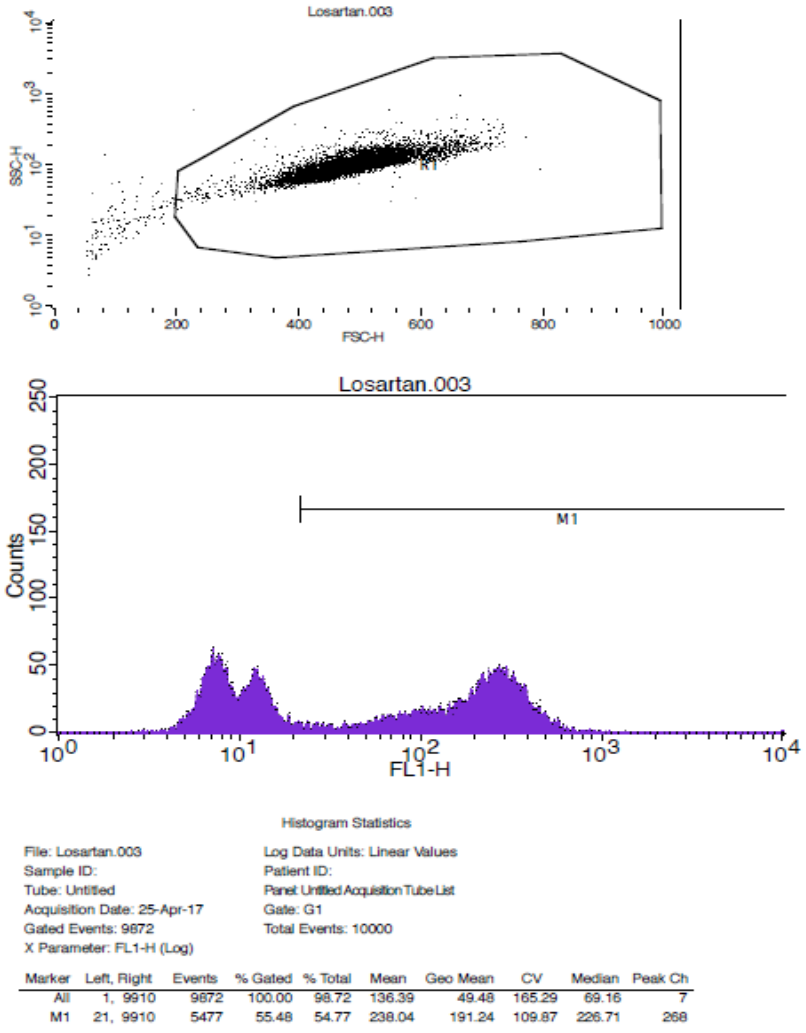
7.2.2 Angiotensin II



Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	9901	100.00	99.01	147.17	51.62	157.73	84.29	7
M1	21, 9910	5562	56.18	55.62	254.74	207.09	103.49	241.44	278

Figure 2. BrdU result for Ang II with AGTR1.

7.2.3 Losartan



Histogram Statistics

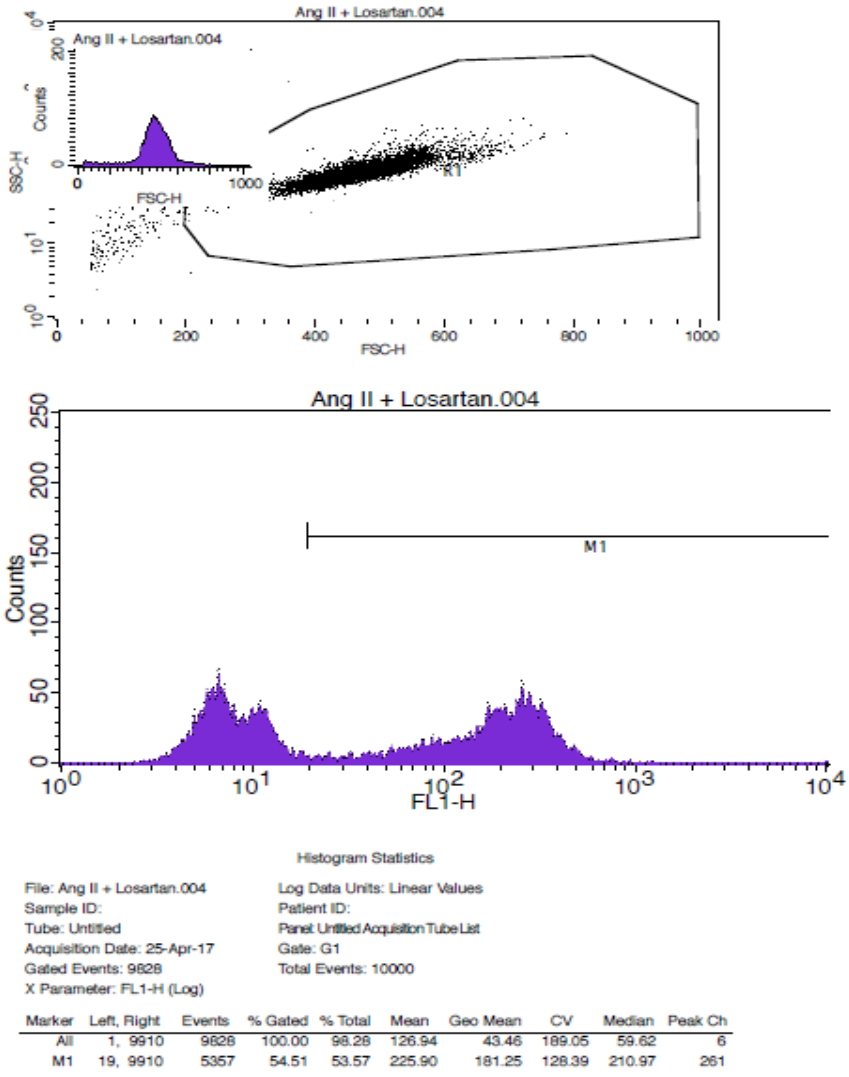
File: Losartan.003
Sample ID:
Tube: Untitled
Acquisition Date: 25-Apr-17
Gated Events: 9872
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: G1
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	9872	100.00	98.72	136.39	49.48	165.29	69.16	7
M1	21, 9910	5477	55.48	54.77	238.04	191.24	109.87	226.71	268

Figure 3. BrdU result for losartan with AGTR1.

7.2.4 Angiotensin II + Losartan



Histogram Statistics

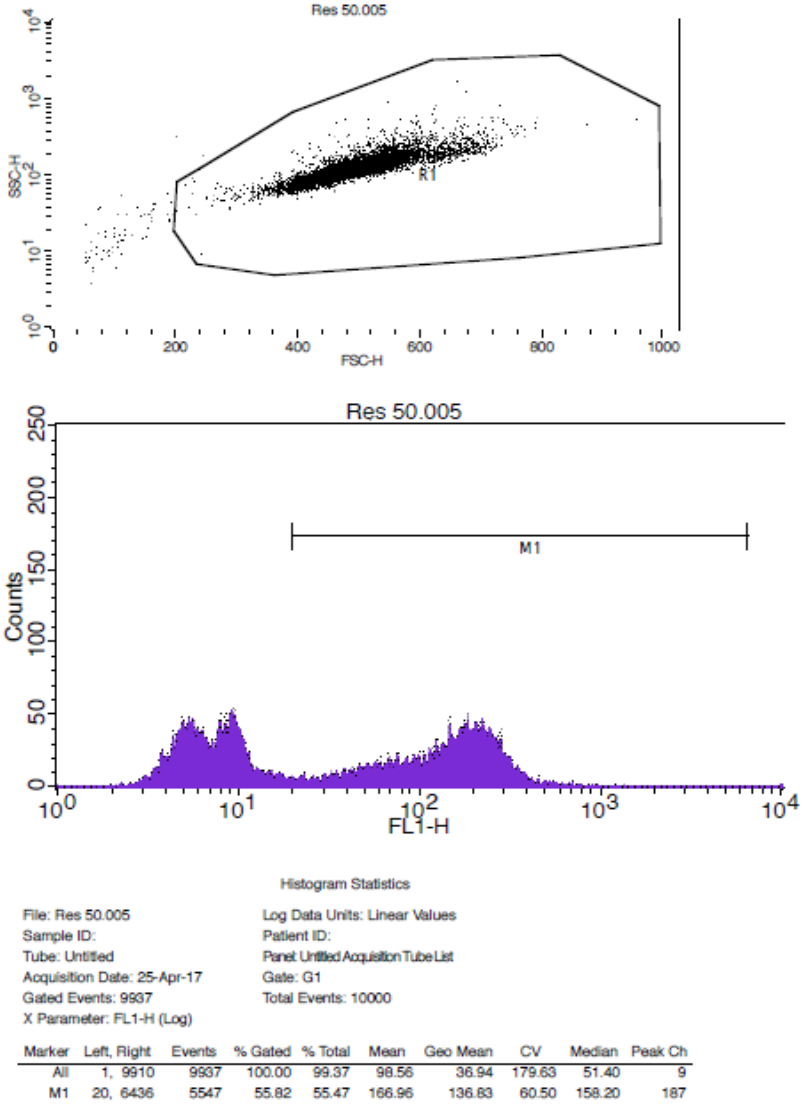
File: Ang II + Losartan.004
Sample ID:
Tube: Untitled
Acquisition Date: 25-Apr-17
Gated Events: 9828
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel Untitled Acquisition Tube List
Gate: G1
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	9828	100.00	98.28	126.94	43.46	189.05	59.62	6
M1	19, 9910	5357	54.51	53.57	225.90	181.25	128.39	210.97	261

Figure 4. BrdU result for Ang II + losartan with AGTR1.

7.2.5 Resveratrol 50 µM



Histogram Statistics

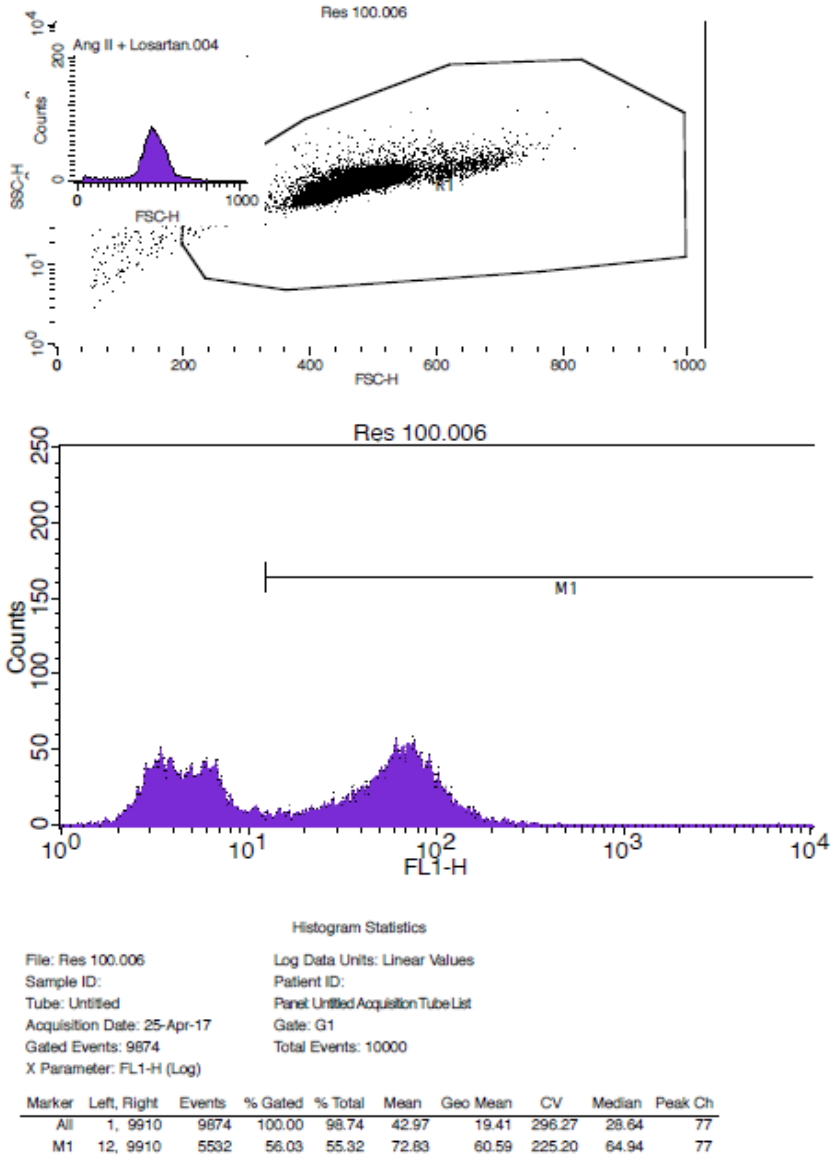
File: Res 50.005
Sample ID:
Tube: Untitled
Acquisition Date: 25-Apr-17
Gated Events: 9937
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: G1
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	9937	100.00	99.37	98.56	36.94	179.63	51.40	9
M1	20, 6436	5547	55.82	55.47	166.96	136.83	60.50	158.20	187

Figure 5. BrdU result for resveratrol 50 µM with AGTR1.

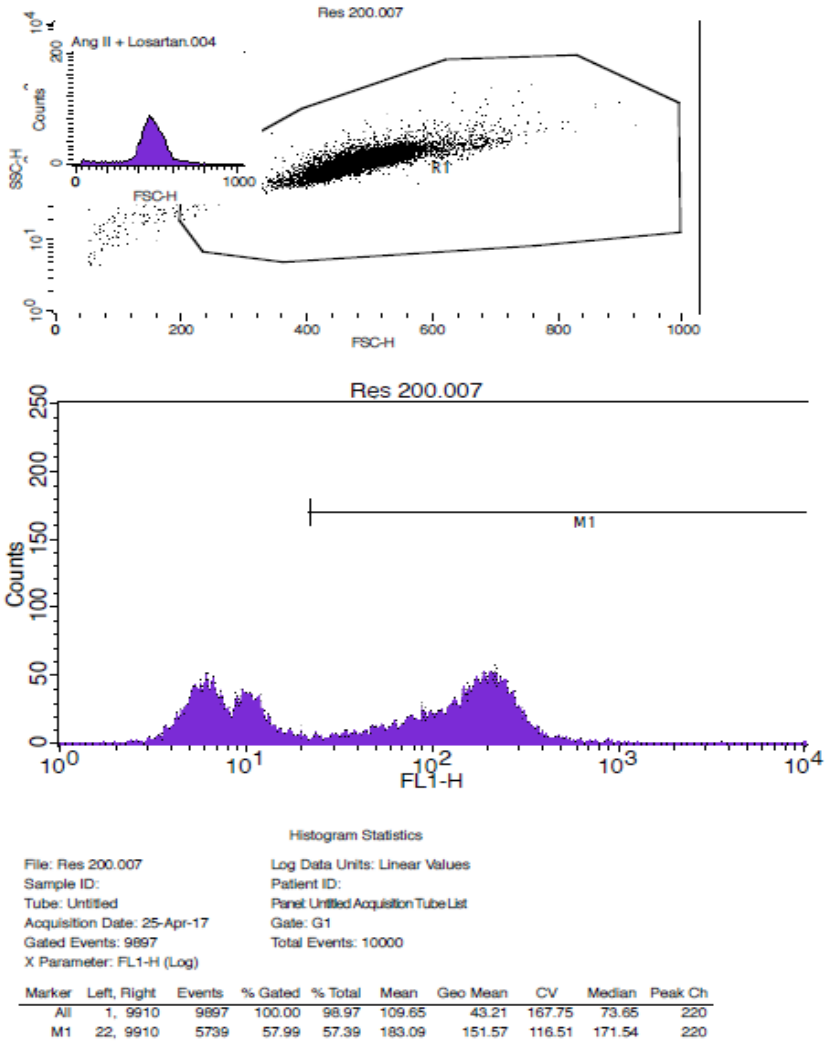
7.2.6 Resveratrol 100 µM



Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	9674	100.00	96.74	42.97	19.41	296.27	28.64	77
M1	12, 9910	5532	56.03	55.32	72.83	60.59	225.20	64.94	77

Figure 6. BrdU result for resveratrol 100 µM with AGTR1.

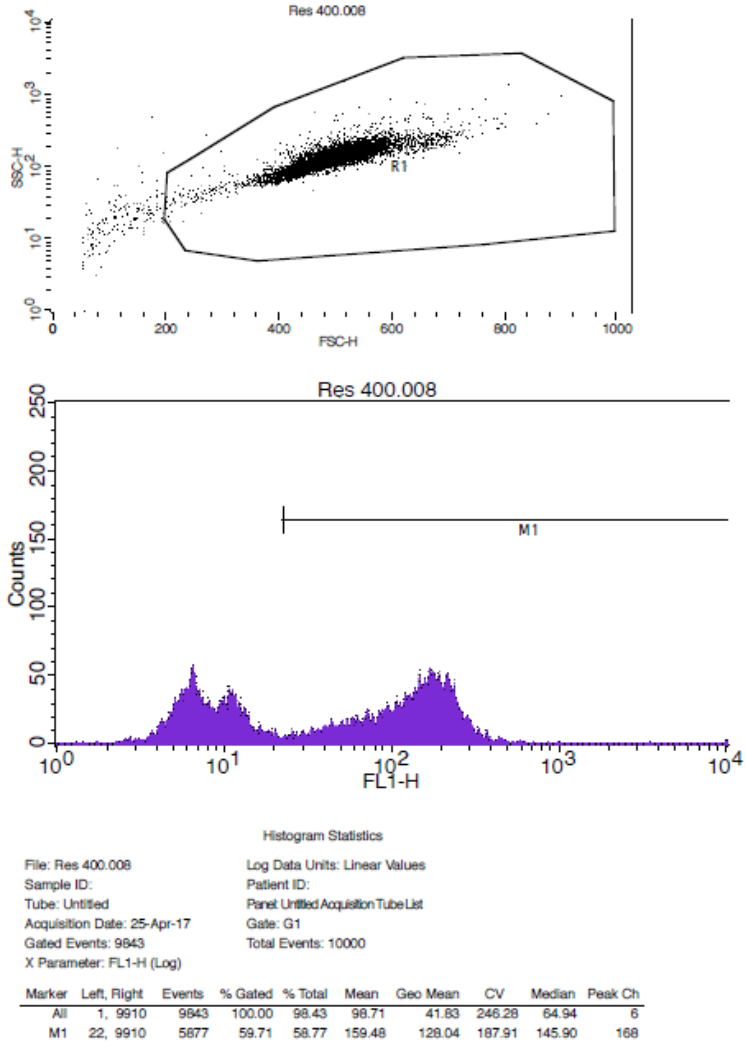
7.2.7 Resveratrol 200 µM



Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	9897	100.00	98.97	109.65	43.21	167.75	73.65	220
M1	22, 9910	5739	57.99	57.39	183.09	151.57	116.51	171.54	220

Figure 7. BrdU result for resveratrol 200 µM with AGTR1.

7.2.8 Resveratrol 400 µM



Histogram Statistics

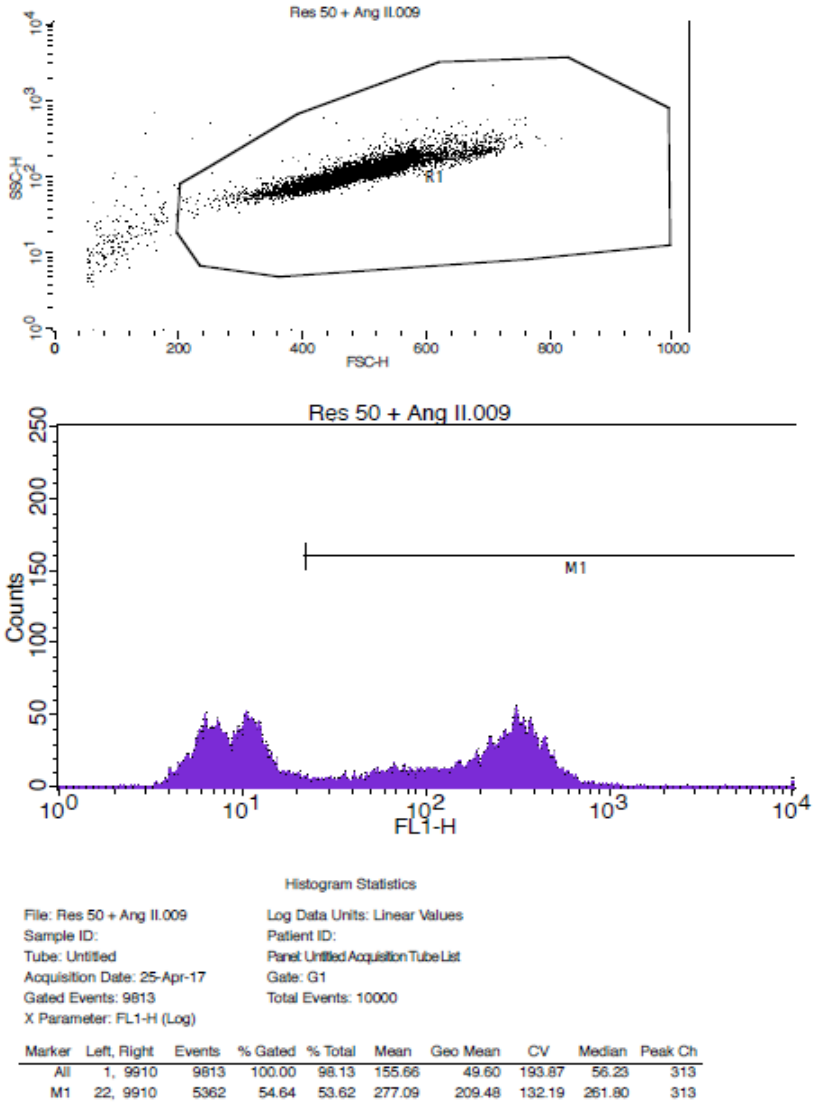
File: Res 400.008
Sample ID:
Tube: Untitled
Acquisition Date: 25-Apr-17
Gated Events: 9843
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel Untitled Acquisition Tube List
Gate: G1
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	9843	100.00	98.43	98.71	41.83	246.28	64.94	6
M1	22, 9910	5877	59.71	58.77	159.48	128.04	187.91	145.90	168

Figure 8. BrdU result for resveratrol 400 µM with AGTR1.

7.2.9 Angiotensin II + Resveratrol 50 µM



Histogram Statistics

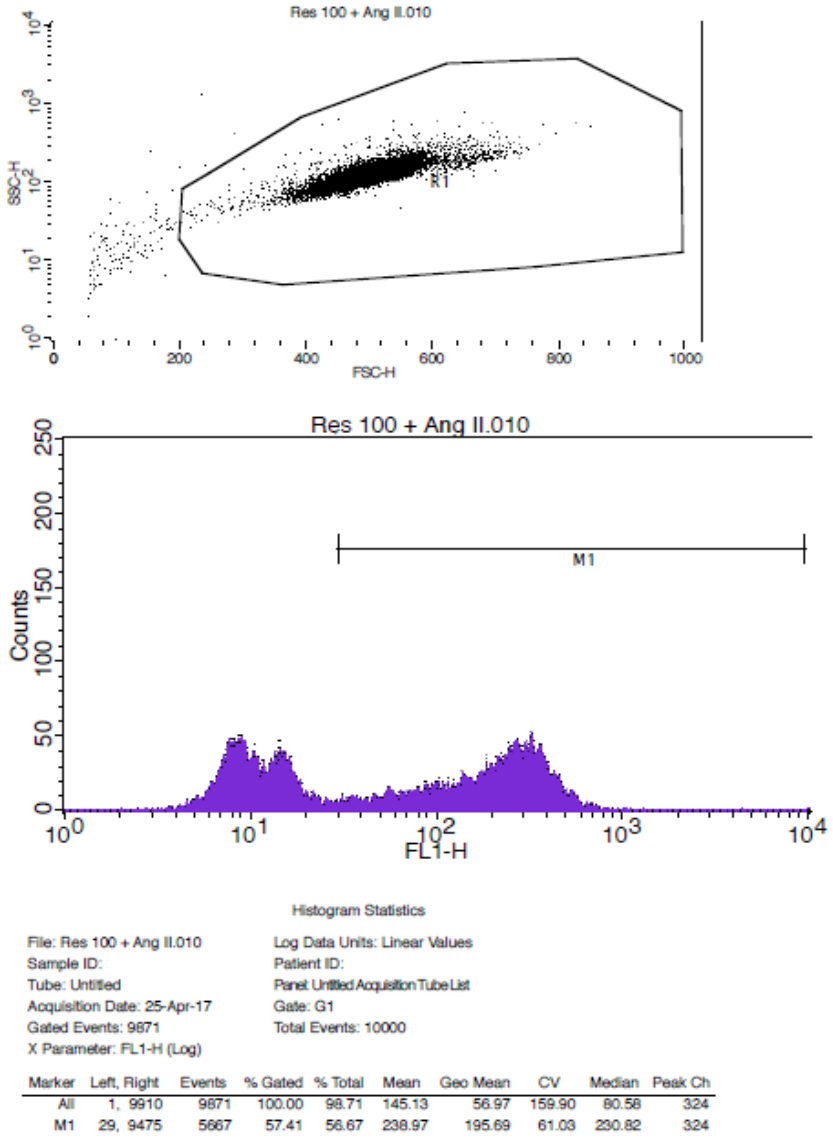
File: Res 50 + Ang II.009
Sample ID:
Tube: Untitled
Acquisition Date: 25-Apr-17
Gated Events: 9813
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel Untitled Acquisition Tube List
Gate: G1
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	9813	100.00	98.13	155.66	49.60	193.87	56.23	313
M1	22, 9910	5362	54.64	53.62	277.09	209.48	132.19	261.80	313

Figure 9. BrdU result for Ang II + resveratrol 50 µM with AGTR1.

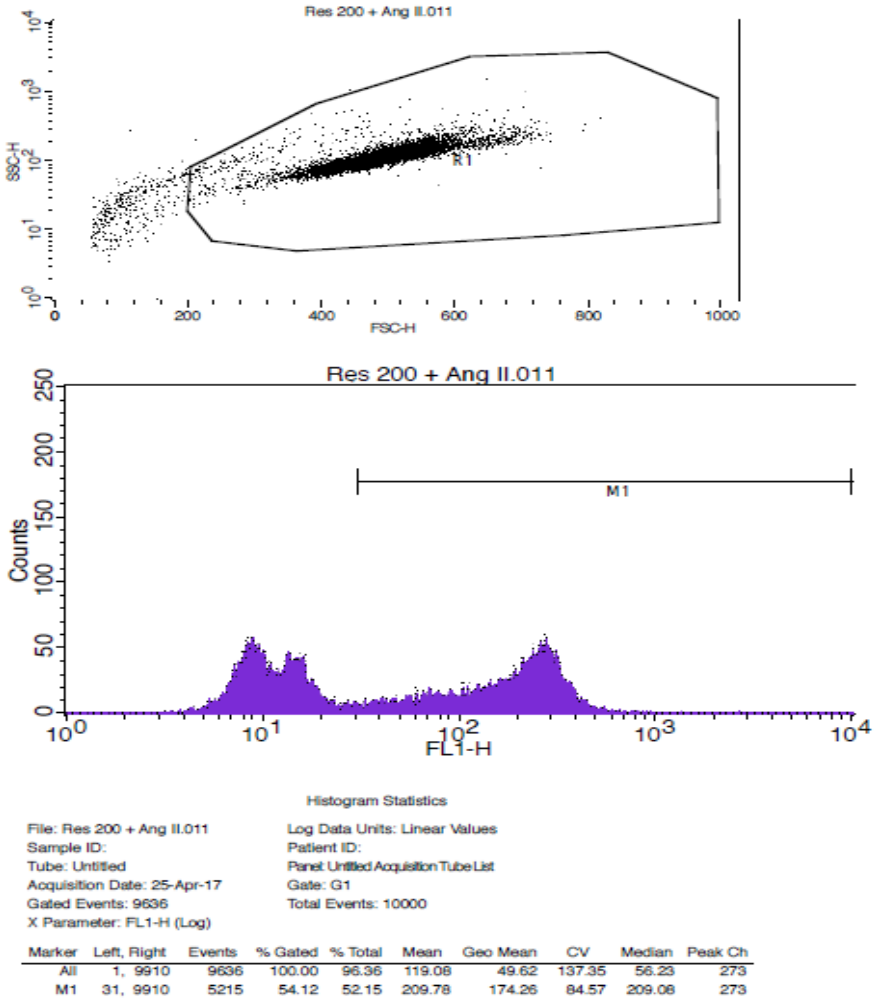
7.2.10 Angiotensin II + Resveratrol 100 µM



Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	9671	100.00	96.71	145.13	56.97	159.90	80.58	324
M1	29, 9475	5667	57.41	56.67	238.97	195.69	61.03	230.82	324

Figure 10. BrdU result for Ang II + resveratrol 100 µM with AGTR1.

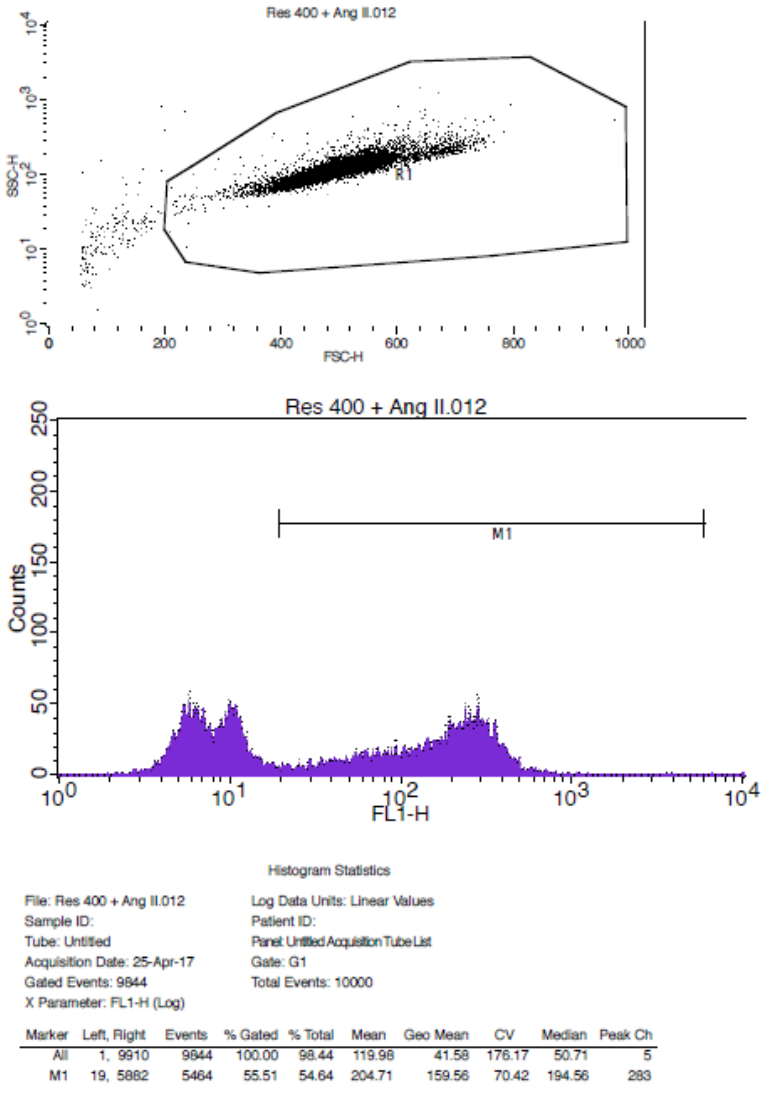
7.2.11 Angiotensin II + Resveratrol 200 µM



Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	9636	100.00	96.36	119.08	49.62	137.35	56.23	273
M1	31, 9910	5215	54.12	52.15	209.78	174.26	84.57	209.08	273

Figure 11. BrdU result for Ang II + resveratrol 200 µM with AGTR1.

7.2.12 Angiotensin II + Resveratrol 400 µM



Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	9844	100.00	98.44	119.98	41.58	176.17	50.71	5
M1	19, 5882	5464	55.51	54.64	204.71	159.56	70.42	194.56	283

Figure 12. BrdU result for Ang II + resveratrol 400 µM with AGTR1.