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# Bioanalytical method development for the detection of Rac/Cdc42 inhibitor MBQ-167 in mouse tissue*

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## 1. Introduction

Breast cancer is the most common form of cancer in women in the United States and Puerto Rico. This disease is defined as the uncontrolled growth of cells in the lobules or ducts of the breast. Metastasis occurs when the tumor invades neighboring tissue and travels through lymph and blood vessels to new sites, most commonly the liver and lungs. The mortality rate of breast cancer has decreased 40% in the last 25 years due to advancement in treatment and diagnostics. However, it is estimated that 20-30% of all breast cancer patients will develop metastatic breast cancer.

We are developing targeted therapeutics to reduce cancer cell migration/invasion and thus, metastasis. The novel small molecule MBQ-167 inhibits Rac and Cdc-42, which are small GTPases involved in cancer cell migration and invasion. *In vivo* studies have shown that MBQ-167 inhibits mammary tumor growth and metastasis in immunocompromised mice by ~90%. To continue validating this drug for FDA approval, we studied the pharmacokinetic profile of the drug, which would elucidate the absoprtion, tissue distribution, metabolism, and excretion of the compound in the body. To accomplish this, a rapid and sensitive method to detect and quantify MBQ-167 in tissues was developed. Selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites are critical for the successful conduct of

preclinical studies. Previous data suggests that supercritical fluid chromatography coupled with electrospray ionization tandem mass spectrometry (SFC-MS/MS) is a method that can rapidly detect MBQ-167 in mouse plasma. However, further studies were done to validate the proposed method in tissues (liver, spleen, kidney, heart, lungs). The purpose of this study was to develop an analytical method for the quantification of MBQ-167 in mouse tissue, using SFC-MS/MS, with the objective of evaluating tissue distribution of the drug in mice.

Moreover, preliminary bioinformatic analysis of the structure of MBQ-167 shows high affinity binding sites of MBQ-167 with CYP3A4, a key enzyme in drug metabolism. Therefore, we studied the inhibitory effect MBQ-167 in the activity of this enzyme. Hence, an additional objective of this project was to assess the effect of MBQ-167 in CYP3A4 activity.

Overall, this study contributed to determination if the clinical development of MBQ-167 is feasible as an anti-metastatic therapy.

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## 2. Literature Review

### 2.1. Cancer

The National Cancer Institute (NCI) defines cancer as a conglomerate of diseases characterized by the uncontrolled division of cells[1]. This problem arises when one or more functions of the cell, the basic unit of life, are damaged. Cancer is a group of more than one hundred different pathologies, involving any tissue in the body and varying

types within the tissue. Also, cancer diseases can metastasize, meaning that the damaged cells can spread through lymph nodes and vessels toward new locations in the body. Early medical terminology from different parts of the word described cancer as malignant tumors and ulcers, using the word cancer that derives from the etymological Greek root of 'karkinos' or crab[2]. In other words, cancer was described as a crab-like disease.

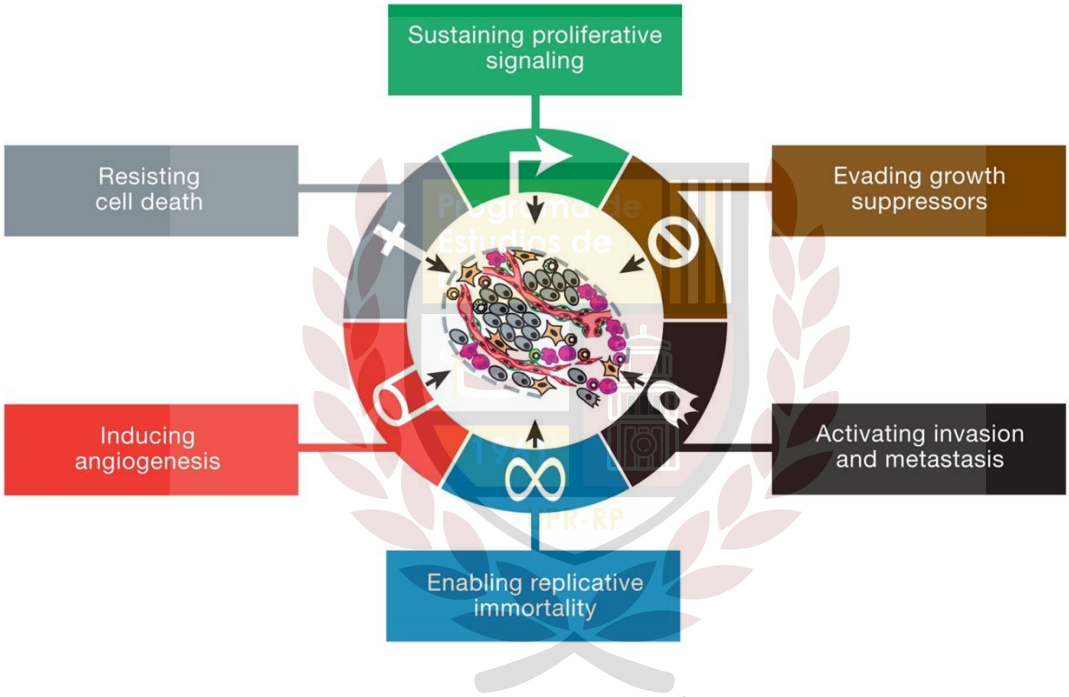



**Figure 1.** Six hallmarks of cancer from Hanahan and Weinberg [3]

The seminal article "Hallmarks of Cancer" by Douglas Hanahan and Robert Weinberg, 2000 describes and organizes the complexities of cancer into six major hallmarks (Figure 1)[3]. These hallmarks are sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, and resisting cell death. Ten years later, Yousef Fouad and Carmen Aanei published a research article with the objectives of revisiting, and

redefining the cancer hallmarks, adding two new hallmarks (reprogramming energy metabolism, and evading immune response)[4]. All hallmarks stand upon the assumption that normal cells will progressively become tumor-like and malignant, and/or have a sequential acquisition of traits (hallmarks). The authors emphasize the importance of studying and understanding the tumor microenvironment as an essential part of the different cancer cells involved in a heterotopic interaction during tumorigenesis.[3,4]

Metastatic cancer refers to the process in which cancer cells migrate towards new sites in the body and conquer them. This occurs when the tumor starts invading neighboring tissue, in the primary site, and some of these cells start a process of transformation called Epithelial-to-Mesenchymal transition. This process converts the cells from an attached manner (epithelial) to a migratory manner (mesenchymal). Once this transition has been completed successfully, these cells start migrating through lymph and blood vessels. Some of these cells, once in a new site, start the process of mesenchymal-to-epithelial transition. In this process, the cells convert from a migratory status to an attached status. Finally, the cells that attach have conquered new tissue and can start to forma metastasis tumor.

## 2.2. **Breast cancer**

Over the last decades, breast cancer in women has steadily increased, with older women (>50 years old) at higher risk. This type of

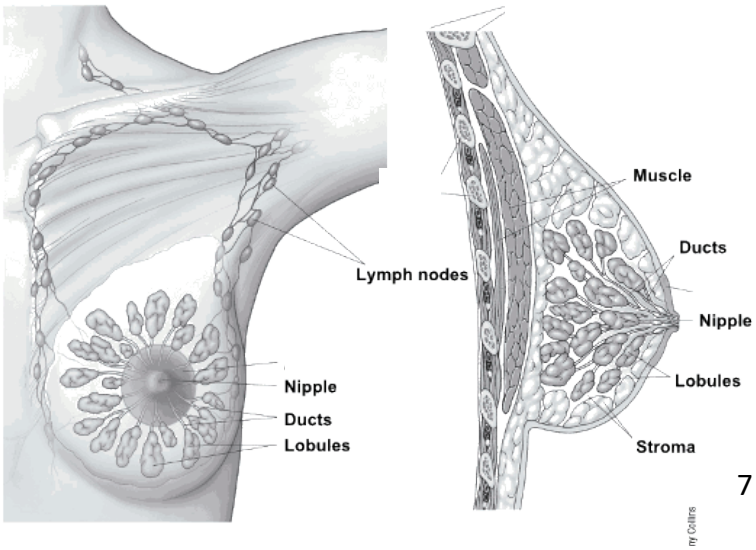



**Figure 2:** Normal breast tissue[2]

cancer could start either in the breast ducts that carry milk to the nipples or in the glands that produce milk (Figure 2).[2] However, other types of breast cancer, such as sarcomas or lymphomas, can initiate in muscular tissue of the breast. Similar to other cancer types, breast cancer markers can be inherited or caused by lifestyle choices. Breast cancer progression leads to metastasis, which results in cancerous cells losing their affinity for the growing tumor and entering either the venous or lymphatic system.[1,2,5] While the blood system distributes the cancer cells through arteries and veins, the lymphatic system distributes the cells trough the lymph vessels that carry waste material and immune system components[6].

In general, cancer is induced by proto-oncogenes (genes that activate cell division), but it is limited through tumor suppression genes (genes that slow cell division, repair DNA mistakes, and induce apoptosis). The most relevant proto-oncogene that has been discovered in breast cancer is c-erbB-2, which encodes a transmembrane growth factor receptor protein that when upregulated leads to cancer[7,8]. Tumor suppressor genes that are widely associated with breast cancer are p53, BRCA1, and BRCA2[7], which are involved in cell cycle control, gene transcription regulation, DNA damage repair, apoptosis and other important cellular processes[9]. Women with certain genetic mutations in BRCA have a higher lifetime risk of the disease. It's estimated that 55 – 65% of women with the BRCA1 mutation will develop breast cancer before age 70[9]. In fact, *BRCA* associated breast cancers are often triple negative and have higher mortality. On the other hand, p53 gene is also a tumor suppressor cell cycle checkpoint gene capable of eliminating the excessive proliferation of cancer cells. Different from the BRCA gene, p53 gene mutations are found in most cancer types.

One of the main problems in breast cancer is that 20-30% of patients will develop metastatic breast cancer with a five-year survival rate of 22%[6]. Currently, the most common treatments for these patients is surgical removal of the breast mass that may or may not include the preservation of the breasts. Later, patients are treated with radiation, chemotherapy, and/or hormones. However, these procedures fail to eliminate completely damaged portions of the affected area and are accompanied with many side effects[11]. On the other hand, the most successful treatments involve the targeted delivery of chemotherapies and hormone therapy towards overexpressed receptors in breast cancer, such as progesterone, estrogen, and HER2 (erbB2). Nonetheless, about 15-20% of breast cancer patients are triple negative; hence, they don't express the mentioned receptors, making this type of treatment useless[11]. Therefore, research has been focused in identifying other potential therapeutic targets downstream of HER2, such as the Rho GTPases family.

### 2.2.1. Rho GTPases in breast cancer metastasis

The Rho GTPases family is implicated in the development of cancer metastasis, especially breast cancer. These proteins are G-Proteins that play an important role in regulatory pathways of the cells, including cytoskeleton rearrangement. Also, these proteins are involved in the regulation of cellular adhesion, migration, proliferation, survival, differentiation, and

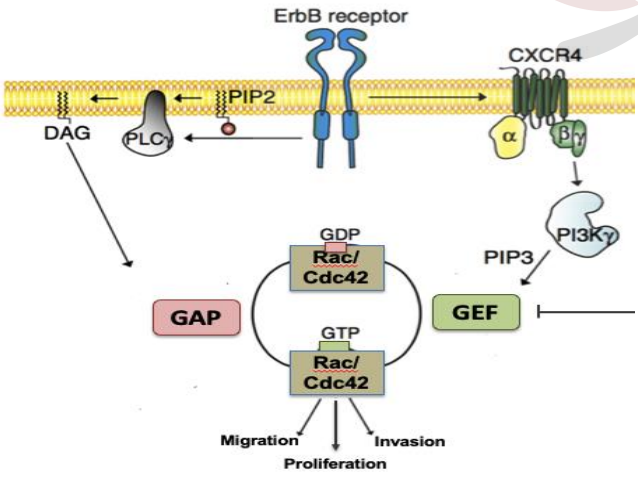



**Figure 3.** Targeting Rac/Cdc42 activation with the small molecule MBQ-167 (adapted from Wertheimer *et a12[3])*

malignant transformation[12]. Therefore, it is not surprising that deregulation of these proteins can cause devastating effects in cells and promote cancer. The Rho GTPase family is divided into six classes, Rho (RhoA, RhoB RhoC), Rac (Rac1, Rac2, Rac3, and RhoG), Cdc42 (Cdc42, Tc10, TCI, Chp/Wrch-2, and Wrch-1), RhoBTB, Rnd, and RhoT[13]. They are regulated by Guanine nucleotide Exchange Factors (GEFs) and GTPase Activation Proteins (GAPs). GEFs activate the Rho-GTPases by binding them to GTP, while the GAPs work towards the inactivation resulting in the GDP-bound form[12,13].

The Rac subfamily (Rac1, Rac2, Rac3) and the homologous protein Cdc42 are highly expressed in cancer. Specifically, Rac1 has been identified as overexpressed or hyperactivated in breast cancer[13]. Rac3 has also been shown to be overexpressed in brain and breast cancer. Moreover, Cdc42 has been found to activate > 20 downstream effector and adaptor proteins, including actin-associated proteins, phospholipases, kinases and adaptor proteins, among others; and its overexpression/hyperactivation in breast tissue can drive cancer[14].

### 2.2.2. Signaling pathways

Rac can be activated by different stimuli, such as growth factors and GPCR ligands. In general, as shown in Figure 3, cell surface receptors, such as growth factor receptors and GPCRs, can induce a series of downstream messengers that lead to either activation (via GEFs), or inactivation (via GAPs)[12] of Rac and Cdc42. Rac and Cdc42 Cdc42, can also be regulated by mechanosensitive receptors (such as stress or change in pressure), integrins, and glucose transporters (GLUT)[16].

The Rac/Cdc42 downstream effector p-21 activated kinases (PAK) belongs to a family of serine/threonine protein kinase that are present in most breast cancers and stimulates proliferative signaling, resisting cell death, activation of invasion, metastasis, and angiogenesis[6,15,16].

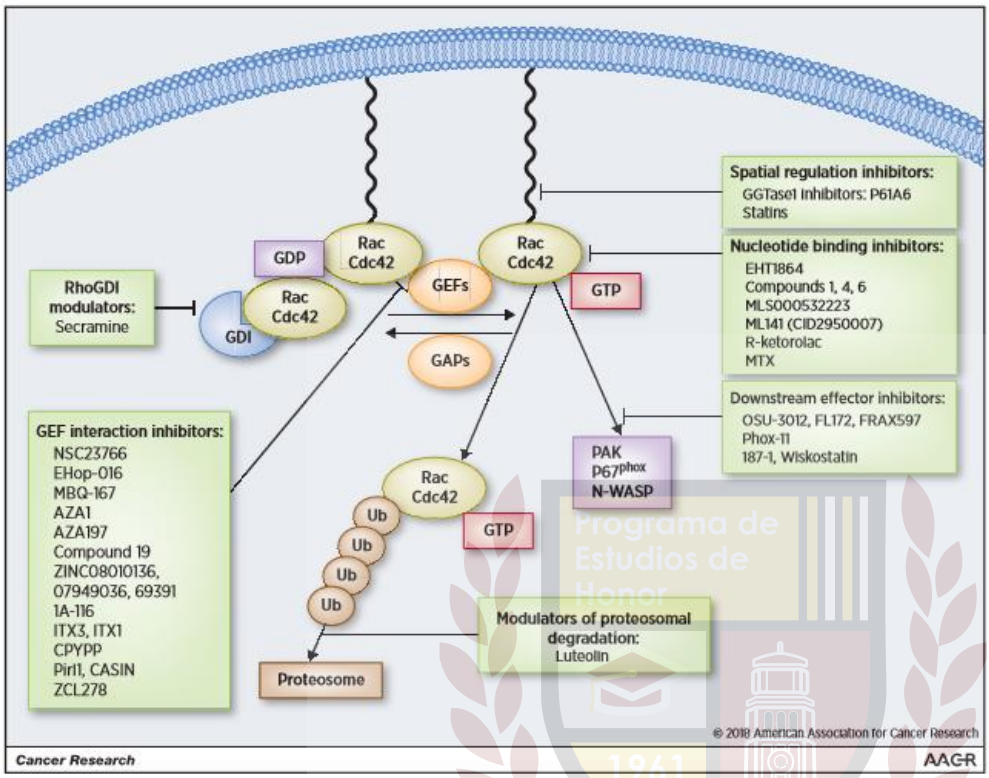



**Figure 4.** Available inhibitors of Rac and Cdc42[13]

Since PAK is downstream of Rac and Cdc42, inhibition of these two proteins can also lead to PAK inactivation[17].

Multiple strategies have been evaluated with the purpose of targeting Rac and Cdc42. These include the use of GEF interaction inhibitors, RhoGDI modulators, spatial regulation inhibitors, nucleotide binding inhibitors, and induction of proteasomal degradation (Figure 4)[13]. The laboratory of Dr. S. Dharmawardhane is focused in developing targeted therapeutics to reduce cancer cell migration and metastasis by inhibiting the Rac/Cdc42-GEF interactions.

## 2.3. **MBQ-167 as a Rac/Cdc42 inhibitor**

To prevent Rac/Cdc42 hyperactivity and cancer metastasis, the small molecule MBQ-167 (Figure 5) was developed as a a derivative of the Rac1 inhibitor EHop-016 (Figure 6), which has an $IC_{50}$= 1.1 µM for Rac. MBQ-167 is currently the most potent Rac/Cdc42 inhibitor reported in the literature with a half maximal inhibitory concentration ($IC_{50}$) of 0.1 µM for Rac and 0.08 µM for Cdc42[5,13].

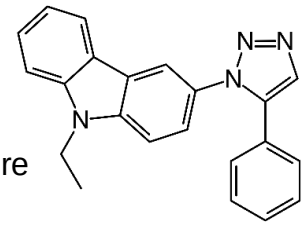



**Figure 5.** MBQ-167 structure

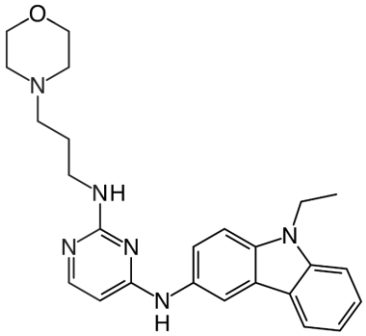



**Figure 6.** Ehop-016 structure[18]

Humphries *et al.* demonstrated through immunofluorescence that MBQ-167 reduces breast cancer cell polarity by studying the actin dynamics and focal adhesions of both Rac and Cdc42[5]. These results were also observed in other types of cancer like pancreatic cancer, gastric cancer and ovarian cancer. Considerable Rac and Cdc42 inhibition was obtained after treatment with 500 nM MBQ-167 along with ~90% cell detachment. When compared to EHop-016, MBQ-167 was deemed 10x more potent inhibitor of Rac and a 100x more potent inhibitor of Cdc42, which resulted in enhanced inhibition of cancer malignancy[5]. In addition to inhibiting Rac and Cdc42, MBQ-167 also inhibits its downstream effector PAK, which regulates actin filaments dynamics during cell division. Finally, MBQ-167 decreases the mammosphere-forming efficiency gland/breast metastatic cancer cells (MDA-MB-231) and reduces mammary fat pad tumor size[5]. Furthermore, MBQ-167 inhibits mammary tumor progression in nude mice. However, the data shows that there was no tumor regression, and treated mice did have a small percentage of tumor growth.

All the previously mentioned results of MBQ-167 are limited to cancer cells that have undergone epithelia-to-mesenchymal transition (EMT) but not to epithelial or non-cancer cells. In terms of toxicity, the concentrations of MBQ-167 that were tested proved to be non-toxic in cells and mice[5]. In general, the study of MBQ-167 demonstrated high efficacy towards reducing breast cancer metastasis in mice.

### 2.4. **Pharmacokinetics/ Pharmacodynamics**

In this thesis, a series of studies were performed on MBQ-167 to evaluate its pharmacodynamics and pharmacokinetics. Pharmacodynamics is defined as "the relationship between the concentration of drug at the site of action and the biochemical and physiological effect"[16]. Waller and Sampson, authors of the Medical Pharmacology and Therapeutics book, describe pharmacokinetics as the "movement of drugs into, though, and out of the body"[17], while Turfus *et. al*, authors of Pharmacognosy book, write that pharmacokinetics "is used to describe the absorption, distribution, metabolism, and excretion of a compound"[19]. Figure 8 illustrates the pharmacokinetic pathway for a drug.

First, there are various routes of administration that must be considered when developing a novel molecule as a potential drug. Among them: sublingual, oral (most common), rectal, intravenous injection, intra-arterial injection, intrathecal injection, intramuscular injection, subcutaneous injection, topical application, and inhalation. Each of them poses a specific advantage and disadvantage, therefore it's important to consider the drug variables such as solubility, polarity, size, among others[17,20,21].

The study of absorption consists in evaluating how biological matrixes uptake the drug, for example, through passive aqueous/lipid diffusion or by facilitated transport. It also measures the rate in which the drug enters the tissues and cells, and in order to do so, local blood flow must be taken into account[17,19,20].

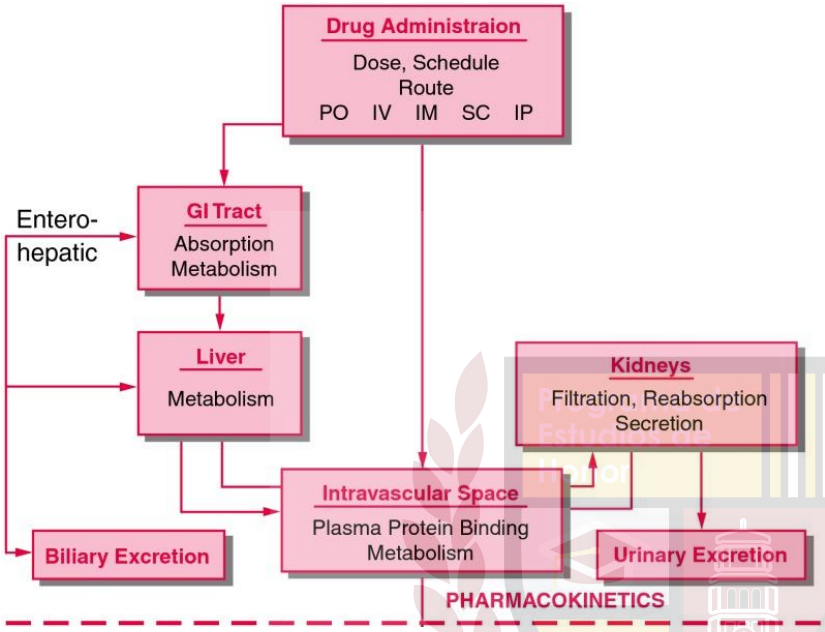



**Figure 8.** Pharmacokinetic pathway of a drug[16]

Drug distribution studies the mechanism the drug utilizes to disseminate through the compartments of the body. First, the drug arrives at the central compartment, which consists of well-perfused organs (heart, blood, liver, brain, and kidneys). Then, the drug is directed towards the peripheral compartments that are not as perfused (adipose and skeletal muscle). Finally, drugs may gain access to special compartments, like the central nervous system through the blood-brain barrier[17,19,20].

After distribution, drugs are metabolized through a series of chemical reactions that prepares them for elimination. In fact, kidneys are only capable of eliminating drugs that are polar, hydrophilic, ionized, and of low molecular weight. That is why, a drug must be converted into its metabolites, which are derivatives of the parent drug and usually less active. However, sometimes, a metabolite may be the active compound. The liver is the primary organ for drug metabolism. Nonetheless, lungs, kidneys, intestines, skin, and placenta also have metabolic functions. The most common enzymes involved in drug

metabolism are members of the Cytochrome P450, a family of isozymes that metabolizes drugs through a variety of processes like oxidation, hydrolysis, hydroxylation. Molecules that inhibit or induce the activity of the CYP may interact with drugs that are metabolized by these enzymes in a clinical setting, leading to unwanted effects. There are two types of metabolic reactions: Phase I and Phase II. Phase I reactions increase drug polarity by adding hydroxyl groups, amino groups, thiol groups, etc. However, if further manipulation is needed for the drug to be eliminated, Phase II reactions add bigger polar groups, such as glucuronic acid, sulfuric acid, acetic acid, or amino acids[17,19,20]

Finally, drug elimination is a slower process due to the possible drug interactions with cell and tissue proteins. Drugs are mostly excreted by either renal glomerular filtration, hepatic metabolism, or renal tubular secretion[17,19,20].

## 3. Theory & Hypothesis

**The Hypothesis** is that MBQ-167 is a physiologically relevant anticancer drug with acceptable pharmacokinetics/pharmacodynamics.

**The specific aims** of our project is:

I. to develop a bioanalytical sample preparation method to selectively separate MBQ-167 from endogenous material in mouse tissue;

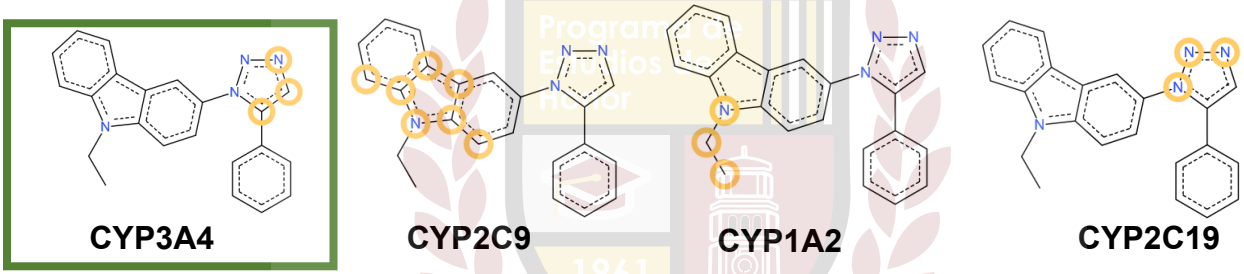
II. to determine potential interactions of MBQ-167 with CytP450 enzymes.

**Figure 9.** SFC MS/MS

**Aim I**. The successful development of a method to extract MBQ-167 from tissue would allow us to determine its tissue distribution *in vivo* which is essential for development of pre-clinical trials. This procedure will be divided in three main stages: tissue homogenization, MBQ-167 extraction, and drug detection by supercritical fluid chromatography-tandem mass spectrometry (SFC-MS/MS). Two methods will be evaluated for the extraction of MBQ-167 from tissues: liquid-liquid extraction and protein precipitation. Liquid-Liquid extraction involves the separation on a biological matrix through an aqueous and organic solvent; where the drug MBQ-167 will migrate selectively to the organic phase due to its non-polar structure. As part of the method, a basic environment is created upon addition of sodium hydroxide, to ensure the molecule remains in its neutral state, therefore, increasing the solubility in the organic phase. Various organic solvents will be compared due to their difference in polarity and interaction with our molecule of interest. Moreover, protein precipitation is a common procedure used to separate proteins, contaminants, and other components in the biological matrix. In this case, proteins and other endogenous tissue components will agglomerate and precipitate upon addition of an excess of organic solvent acetonitrile; hence, the small molecule MBQ-167 can then be recovered in the supernatant. We believe that MBQ-167 can be extracted with one of the methods proposed with a high and consistent recovery. The molecule of interest will be detected by supercritical fluid chromatography coupled with tandem mass spectrometry (SFC-MS/MS) (Figure 9) and method validation will be done following the FDA guidelines. Validation ensures that the

method is reliable and reproducible by evaluating accuracy, precision, selectivity, sensitivity, reproducibility, and stability[21].

**Aim II**: The study of the metabolism of a drug is a very important aspect of its pharmacodynamics. Preliminary bioinformatics data suggests that the small molecule MBQ-167 may have an inhibitory effect on CYP450 enzymes (Figure 10), specifically in the CYP3A4 isozyme. To further study this, we will incubate various concentrations of MBQ-167 with human recombinant CYP3A4 and evaluate the resulting effect on enzyme activity.




In general, this investigation proposes to develop a method for the detection of MBQ-167 in tissues for pharmacokinetic applications and to evaluate the effect of this drug in a major metabolic enzyme.

**Figure 10.** Potential binding sites of MBQ-167 with different CYPs. Analysis by WhichCyp version 1.2

## 4. Justification

MBQ-167 is a novel small molecule that has been demonstrated to be highly effective against metastatic breast cancer. Further validation of this drug for clinical purposes is required to elucidate the pharmacokinetics (PK) profile of MBQ-167. Currently there are no methods for the detection and quantification of MBQ-167 in biological matrixes. By developing a novel, rapid, and efficient way of detecting this

small molecule, the PK profile of the drug can be studied per the Product Development under the Animal Rule of the Food and Drug Administration (FDA)[23]. To accomplish this, protein precipitation and liquid-liquid extraction procedures will be compared, both of which are widely used in separations for biological matrix. Both methods require small amounts of solvents, are highly versatile, and are widely used in the pharmaceutical industry. Furthermore, supercritical fluid chromatography coupled with electrospray ionization tandem mass spectrometry (SFC- MS/MS) will be used for detection and quantification of MBQ-167 in mice tissues. The use of this technology poses many benefits for its high sensitivity, rapid analysis times and, most importantly, requires fewer organic solvents than other approaches. Hence, this technology contributes to the first principle of Green Chemistry of Prevention, in this case, reduction of organic solvents used.

Additionally, the study of the metabolism is also a fundamental part for the validation of this drug following FDA guidelines. It is essential to study the possible inhibitory (or inductory) effect of MBQ-167 in the liver cytochrome enzymes, specifically CYP3A4, which is widely involved in the metabolism of drugs. The CYP enzymes are responsible for most of the metabolism of the non-polar compounds in the body, making them water soluble, and able to be excreted from the body usually in the urine. If the drug MBQ-167 has an effect (inductor or inhibitory) in CYP3A4 activity, it may interfere with other compounds being processed, and produce side effects.

## 5. Materials and Methodology

### 5.1. Detection of MBQ-167

The method development for detection of MBQ-167 in tissues was done with mice livers. First, stored organs were thawed, weighted (100 mg), and homogenized using the Polytron PT 2100 instrument in pH 7.4 saline (1:4 w/v). \ Two extraction methods were compared, protein precipitation (with acetonitrile as the solvent) and liquid-liquid extraction (with acetonitrile, methanol, ethyl acetate, toluene, and/or heptane as the solvents) (Figure 11). Solvent ratios, pH, concentrations, and extraction times were evaluated. The small molecule EHop-0036 was used as an internal standard due to its structure similarity with MBQ-167. Experimental samples had a known concentration of MBQ-167 and a known concentration of internal standard, which was added before extraction.

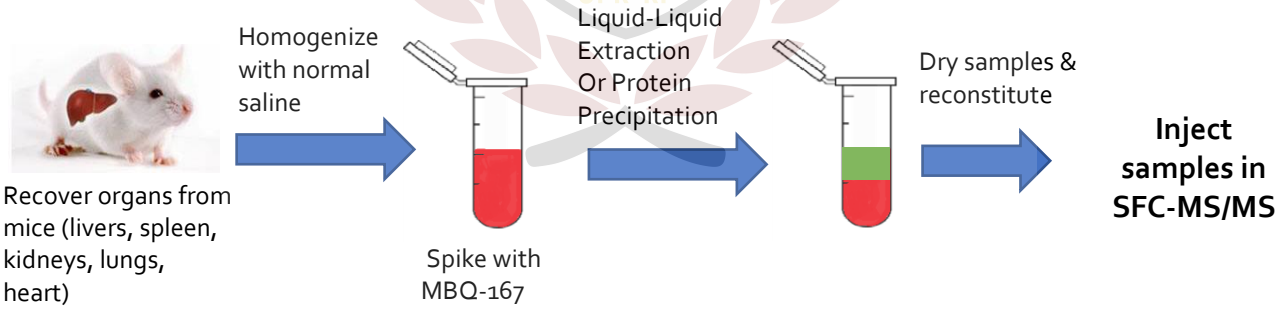



**Figure 11.** Bioanalytical method development for the detection of MBQ-167 in mice tissues.

Unextracted samples and blank samples (without internal standard nor MBQ-167) were used as experimental controls.

Tissue samples were extracted by liquid-liquid extraction method. The tissue homogenate (100 μL) was then transferred to another tube, followed by addition

of internal standard (10 μL from 4,500 ng/mL stock) and vortexed for 30 secs. Subsequently, 100 μL of NaOH 0.5 M were added to the mixture and vortexed for 5 minutes. A liquid-liquid extraction was performed using 790 μL of heptane: ethyl acetate (1:1). Samples were then vortexed for 10 minutes and later centrifuged for 5 minutes at 510 x g. The upper layer was recovered, and solvent was evaporated for one hour in a Labconco Centrivap console at room temperature. Afterwards, samples were reconstituted with 100 μL of methanol, vortexed for ten minutes, and centrifuged at 1000 x g for 1 minute. Samples were then transferred to an autosampler vial to be analyzed by SFC-MS/MS.

The analysis was performed on an Acquity UPC$^2$ system (Waters Corp., Milford, MA, USA), coupled to a triple quadrupole MS/MS (Figure 12). The separation was performed on an Acquity UPC 2TM BEH (3.0 × 100 mm, 1.7 μm) with $sCO_2$ and methanol/0.01% formic acid (95:5, v/v) as the mobile phase at a rate of 1.0 mL/min, while 0.01% formic acid in methanol was used as the make-up solvent at a flow rate of 0.15 mL/min. The gradient elution of A (sCO 2 ) and B (methanol/0.01% formic acid ) was performed as follows: 5% B maintained at 0–1.5 min, 5%-12% B at 1.5–2 min, 12% B at 2–3 min, 12–20% B at 3–3.5 min, 20% B at 3.5–4.5 min, 20%- 5% B at 4.5–5 min, 5%B at 5–6 min. The backpressure of the system was kept at 2200 psi and the column temperature was maintained at 40°C. The auto sampler was conditioned at 4°C. The total run time for analysis was 6 minutes and the injection volume were 1.0 μL. MBQ-167 with precursor ion $[M+H]^+$ = 339.12 was used as analytical standards monitoring the MRM transitions $[M+H]^+$ = 179.9 and 282.1. After the experimentation

procedure was validated with livers (three technical replicates), other organs were evaluated including kidneys, spleens, hearts, and lung.

## 5.2. Data Analysis

The recovery percentage of the extracted samples were compared using the following formula:

$$\text{Recovery } \% = \frac{\text{area under the curve of extracted samples}}{\text{area under the curve of unextracted samples}} \times 100$$

Also, the effect that other endogenous tissue components (including cell constituents) might have had in the

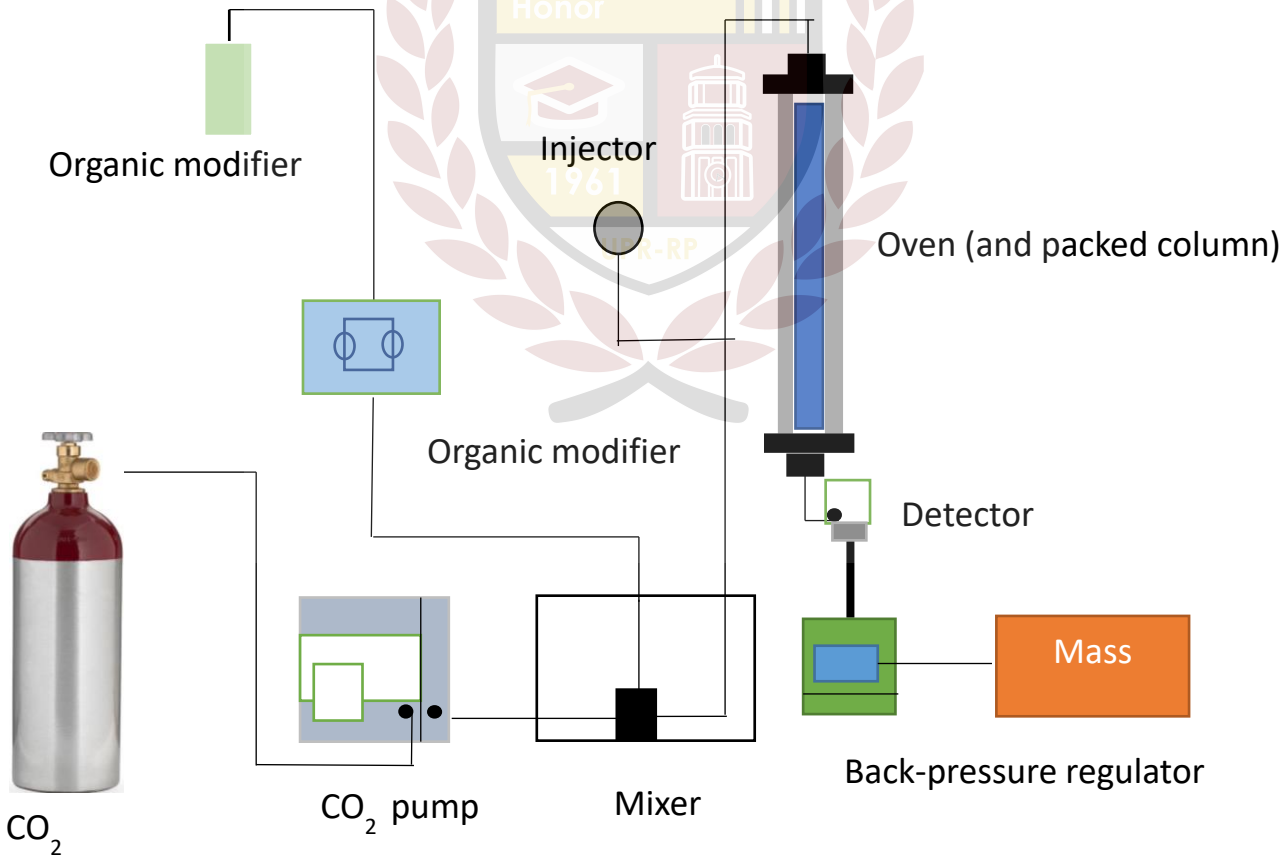



**Figure 12.** SFC-MS/MS instrument setup.

detection of MBQ-167 by SFC/MS/MS was evaluated by the following formula:

$$\text{Matrix } \% = \frac{\text{area under the curve of unextracted samples}}{\text{area under the curve of neat samples}} \times 100$$

## 5.3. Method Validation

Bioanalytical method validation of recovery and matrix was performed in mouse tissue matrixes according to the guidelines specified by the U.S. Department of Health and Human Services Guidance for Industry. A calibration curve was prepared at various concentrations. Also, quality controls were prepared for low, medium and high concentrations depending on the limits of detection and quantification of the SFC-MS/MS. Following the FDA guidelines, the short-term and long-term stability were evaluated in mouse tissue. For the short-term stability, quality control samples were measured after 3 hours on ice. Long term stability was measured after thirty days in -80ºC storage. After each condition, the samples were extracted and submitted to evaluation by the SFC-MS/MS for detection and quantifications.

## 5.4. *In vivo* Tissue Distribution study

To evaluate tissue distribution, a pharmacokinetics *in vivo* study was performed, where mice were administered with a set concentration of MBQ-167 (10mg/kg BW) via intraperitoneal route (I.P.) and sacrificed at different time intervals (10, 30, 60, and 180 minutes) post dosage. After, that blood and tissues (heart, lungs, liver, spleen, and kidneys) were recovered. MBQ-167 was extracted from

samples and the concentrations was detected and quantified (with respect to an internal standard) using SFC-MS/MS.

### 5.5. Human recombinant CYP3A4 enzyme activity assays

Commercially available human recombinant CYP3A4 was purchased from Promega and enzyme activity assays were performed using different concentrations of MBQ-167 (0.001μM -100μM). First, the drug, enzyme, and luminogenic substrate were pre-incubated. Then, reaction was started upon addition of the NADPH regeneration system. Product formation was monitored by luminescence of the resulting luciferin analog (Figure 13). The activity of the enzyme was compared with three controls: (1) untreated, (2) treated with a known CYP3A4 inhibitor (ketoconazole), and (3) incubated with control unreactive membrane. Half-maximal inhibitory concentrations ($IC_{50}$) were determined using GraphPad Prism. Data was analyzed by performing a linear regression analysis of luminescence from standards to generate a standard curve. Reaction rates were determined by comparing known values of reaction rates of the positives and negative controls. Inhibition or induction was determined by comparing the affinity constant ($K_m$) of the experimental sample with the control sample.

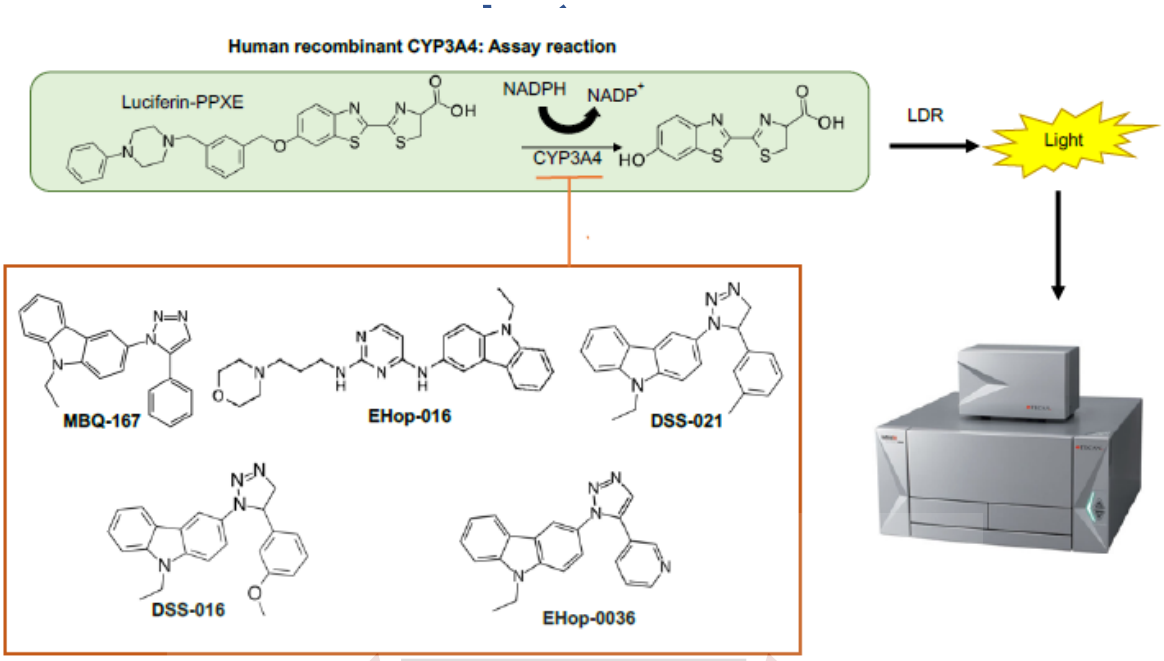



**Figure 13**. Human recombinant CYP3A enzyme activity assay reaction.

## 6. Results

### 6.1. Detection of MBQ-167 by SFC-MS/MS

The molecule of interest MBQ-167 was rapidly and selectively detected in mouse livers by SFC-MS/MS. The SFC-MS/MS can detect MBQ-167 fragment ions of 179.99 and 282.13, from the precursor ion [M + H] = 339.12. Our data indicates that the molecule was detected with a retention time of aproximately 2.37 minutes. Figure 14 shows the total ion chromatogram of MBQ-167 extracted from endogenous tissue material. MBQ-167 is observed as a single defined peak with a magnitude of 1.75 x $10^5$. This chromatogram demostrates a clean detection with a low retention time. This retention time is benefitial because a greater amount of

samples could then be analyzed rapidly at the SFC-MS/MS. Also, comparing the MBQ-167 chromatogram with a blank chromatogram we confirm the singularity and definition of the peak, given that a blank liver sample produced higher noise. It is important to point out that the full chromatogram of the blank sample presented in Figure 15 has a magnitude of 1.02 x $10^3$, which is two magnitudes below the MBQ-167 detection chromatogram. Hence, this supports our hypothesis that SFC-MS/MS is a rapid and selective method for the detection of MBQ-167 in mouse tissue.

## 6.2. Optimization of MBQ-167 extraction procedures

Liquid-liquid extraction and protein precipitation extraction procedures were performed with various organic solvents and compared using recovery percent, standard deviation percent, and

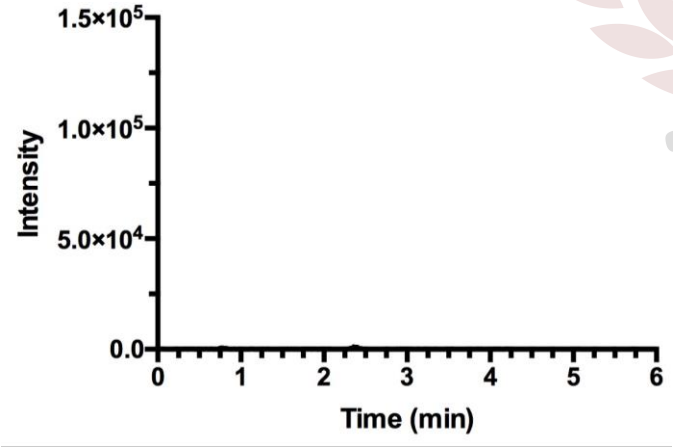



**Figure 14.** SCF-MS/MS total ion chromatogram of MBQ-167 at 100nmg/mL in mouse liver

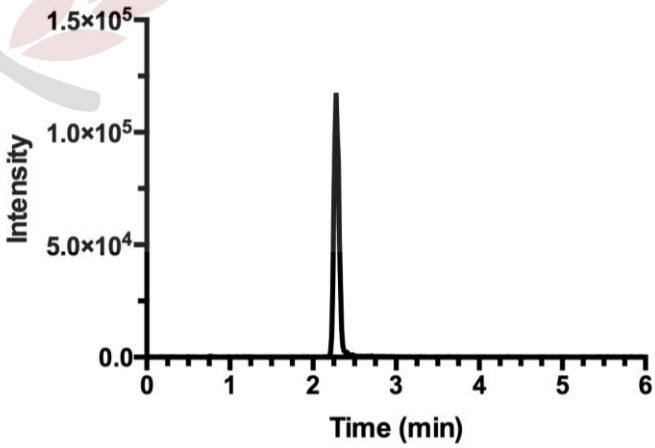



**Figure 15.** SFC-MS/MS total ion chromatogram of a blank mouse liver. There is a defined peak, suggesting a selective selection of MBQ-167 by SFC-MS/MS.

matrix effect. As seen in Figure 16, the highest recovery percentages of MBQ-167 were obtained by liquid-liquid extraction using isopropanol-toluene (1:1), Heptane-Ethyl acetate (1:1), and by protein-protein precipitation with acetonitrile resulting in 64%, 66% and 58%, respectively (Table 1). However, a higher variability was observed with protein-protein precipitation (standard deviation = 23 %), while the organic solvent mixtures mentioned earlier have a standard deviation of 6-7 %. Hence, protein-protein precipitation was discarded as the best method for tissue and MBQ-167 separation. Furthermore, isopropanol-toluene (1:1) was discarded due to the toxicity of toluene, which requires working in a chemical hood at all times. Therefore, the solvent mixture Ethyl acetate:Heptane (1:1) was determined as the best method for the separation of MBQ-167 from endogeneous mouse tissue due to its simplicity, high recovery, and small variability. Moreover, for the Ethyl acetate:Heptane (1:1), a minimum matrix effect was observed at 142% (Table 1). This percentage means that the cellular and other biological components had little effect in the detection of MBQ-167 by SFC-MS/MS.

**Table 1**: Evaluation of matrix and recovery effects of MBQ-167 in mouse livers

| **Solvents** | **Recovery (%)** | **Standard deviation (%)** | **Matrix (%)** |
|---|---|---|---|
| Heptane (100%) | 43 | 29 | 100 |
| Ethyl acetate (100%) | 63 | 6 | 140 |
| Toluene (100%) | 35 | 25 | 157 |
| Heptane-Ethyl acetate (1:1) | 66 | 6 | 142 |
| Heptane-isopropanol (1:1) | 24 | 2 | 143 |
| Heptane-toluene (1:1) | 2.5 | 1 | 139 |
| Ethyl acetate-toluene (1:1) | 14 | 4 | 146 |
| Isopropanol-toluene (1:1) | 64 | 7 | 154 |
| Heptane-Ethyl acetate-toluene (1:1:1) | 20 | 5 | 76 |
| Heptane-Ethyl acetate (1:2) | 37 | 4.1 | 83 |
| Acetonitrile (100%) | 44 | 19 | 110 |
| Protein-protein precipitation | 58 | 23 | 78 |

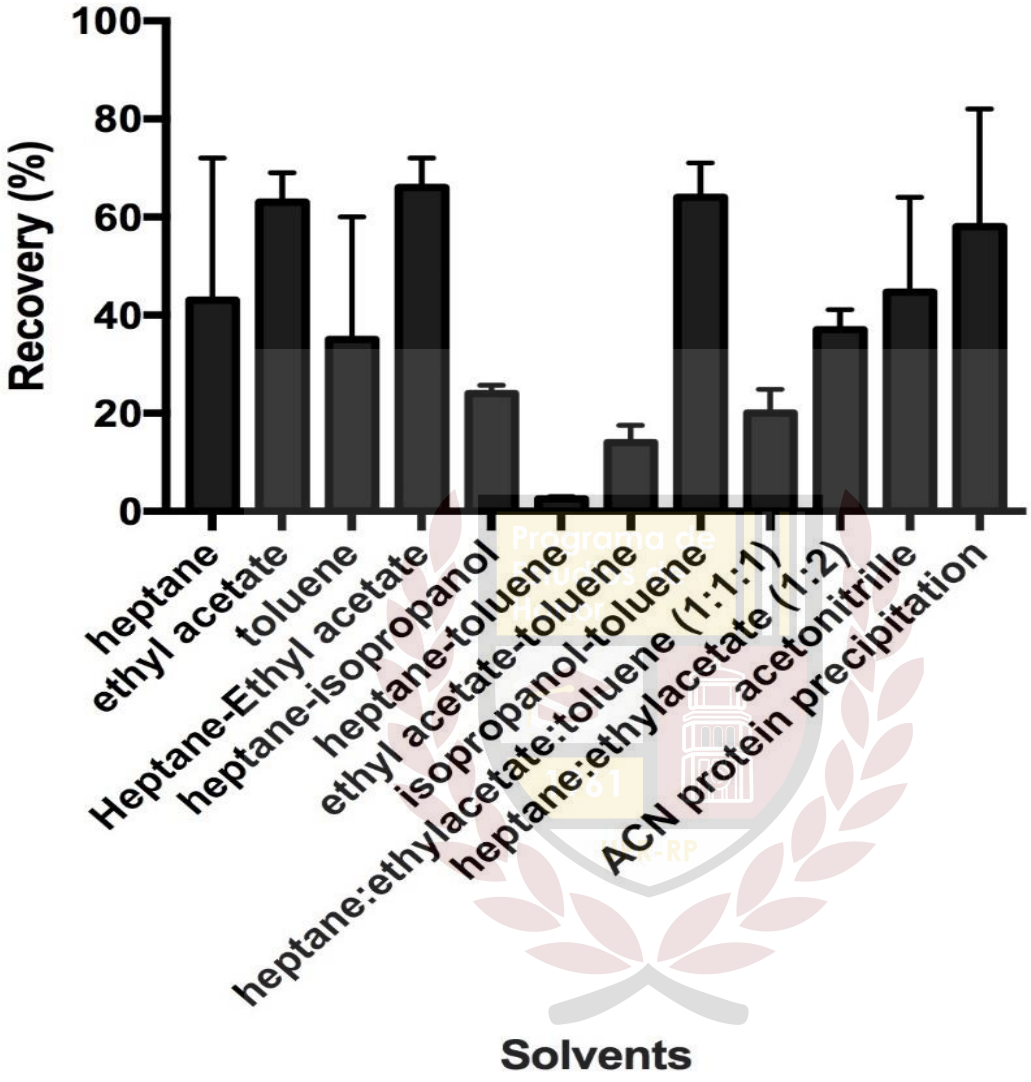



**Figure 16.** Recovery of MBQ-167 in liver after liquid-liquid extraction with various organic solvents and protein precipitation. using different solvents and mixtures of solvents for Liquid-Liquid extraction and Protein precipitation. The highest recovery was obtained with Liquid-Liquid extraction using Heptane: Ethyl Acetate (1:1).

### 6.2.1. Purification of MBQ-167 from tissues

Liquid-liquid extraction with Heptane-Ethyl acetate (1:1) was also conducted with homogenates of different mouse organs, such as kidneys, lungs, and heart (Table 2). These organs were spiked with the same concentration of MBQ-167, since the purpose of this experiment was to confirm that consistent recovery of MBQ-167 could be obtained using liquid-liquid extraction in different biological matrixes. As shown in Table 2, similar recovery percentages were obtained from all the organs, varying from 54.9 – 66.0 %. The lungs had the least recovery, and the livers had the most recovery. With these results, we concluded that liquid-liquid extraction with Heptane-Ethyl-Acetate (1:1) is the most efficient way to extract MBQ-167 from endogenous tissue resulting in high and consistent recoveries with low matrix effect.

Table 2: Recovery of MBQ-167 in different organs with liquid-liquid extraction

Heptane: Ethyl Acetate (1:1)

| Organ | Recovery (%) |
|---|---|
| **Liver** | 66.00 |
| **Kidneys** | 65.41 |
| **Lungs** | 54.96 |
| **Heart** | 57.41 |

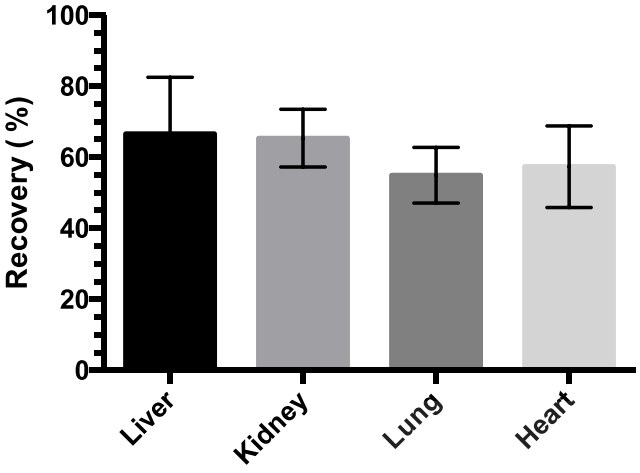



**Figure 17.** Recovery % of different mice organs using Liquid-Liquid extraction with Heptane: Ethyl Acetate (1:1) solvents. Similar recovery was observed at all organs.

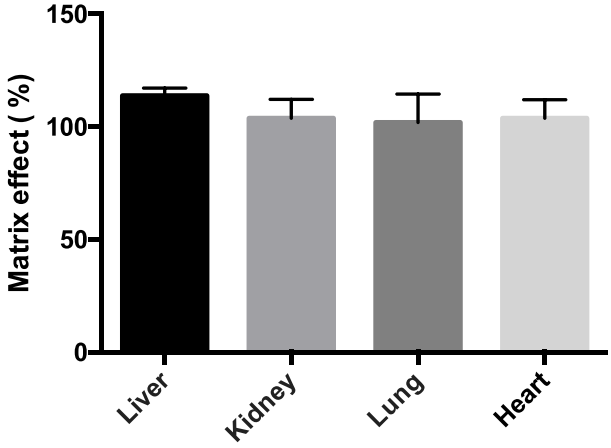



**Figure 18.** Matrix effect % of different mice organs using Liquid-Liquid extraction with Heptane: Ethyl Acetate (1:1) solvents. Similar recovery was observed at all organs.

## 6.3. Method Validation

### 6.3.1. Linearity

In order to be able to quantify the MBQ-167 concentration in tissue samples after extraction, a calibration curve was prepared. These experiments correlate the intensity of detected MBQ-167 and compare it to a set concentration of our internal standard (EHop-0036). This procedure is done for different known concentrations of MBQ-167 in order to correlate relative response to MBQ-167 concentration (ng/mL). Linearity if this curve was determined to be able to reliable determines the concentration of an unknown sample. Linearity was determined using eight calibrant concentrations (10, 20, 40, 62.5, 125, 250, 500, and 1000 ng/mL) of MBQ-167 in mouse livers. The dynamic range

established was 10 – 1000 ng/mL (Figure 17) with a determination coefficient ($R^2$) greater than 0.99 in al validation runs.

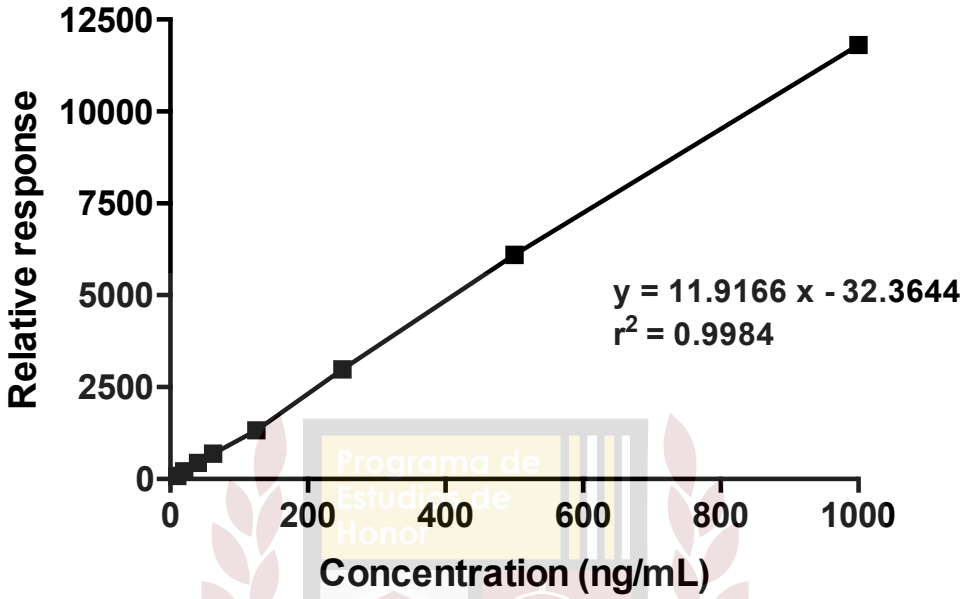



**Figure 19.** Calibration curve of MBQ-167 in mouse livers. Linearity was observed at 10-1000 ng/mL

### 6.3.2. Recovery and matrix effect

Three different concentrations (low, medium, high) of MBQ-167 in mouse livers were evaluated for recovery and matrix effects (Table 3). All three concentrations had similar recovery % and matrix (%), leading us to conclude that the concentrations within the calibration curve can be detected and quantified with similar recovery and matrix percenteges.

**Table 3:** Evaluation of matrix and recovery effect of liquid-liquid extraction of MBQ-167 in mouse livers

| **Spiked level (ng/mL)** | **15** | **450** | **950** | **CV%** |
|---|---|---|---|---|
| **Matrix%** | 111.00% | 98.00% | 94.00% | 8.90% |
| **Recovery %** | 73.10% | 60.70% | 64.80% | 9.60% |

### 6.3.3. Short term stability

The short term stability of MBQ-167 was evaluated after leaving the samples (15 and 950 ng/mL) during three hours inside an ice bucket. In order to pass this analysis, the accuracy (relative error %) and precision (relative standard deviation) should be ≤ 15%, as described in the FDA bioanalytical method validation guidelines. As shown in Table 4, MBQ-167 was stable in accuracy and precision at both concentrations.

### 6.3.4. Long term stability

As shown in Table 4, samples of MBQ-167 demostrated to be stable after 15 days stored at -80ºC (RE and RSD ≤ 15%). Further experimentation must be conducted to elucidate the long term stability parameters of MBQ-167 after 30 days of storage.

**Table 4:** Stability of MBQ-167 in mouse livers

| Conditions | Concentration (ng/mL) | | Accuracy (RE %) | Precision (RSD %) |
|---|---|---|---|---|
| | Nominal | Measured (Mean ± S.D) | | |
| Post-preparative (3 Hrs in ice bucket) n = 6 | 15 | 11.4 ± 1.7 | 8.0 | 7.8 |
| | 950 | 634.0 ± 37.8 | 15.2 | 3.5 |
| Long term (-80ºC) *(15 days)* n=4 | 15 | 13.3 ± 0.8 | 6.5 | 13.6 |
| | 950 | 962.0 ± 73.0 | 9.2 | 12.6 |



### 6.4. Tissue distribution of MBQ-167

Tissue distribution of MBQ-167 after *in vivo* intraperitoneal (IP) administration was determined. The tissues (liver, kidneys, spleen, hearts, and lungs) were removed following administration of 10 mg/kg BW MBQ-167 at different times, and stored at -80ºC. Later, the tissues were homogenized with normal saline, and extracted through Liquid-Liquid extraction using Heptane: Ethyl Acetate (1:1). The samples were reconstituted with methanol and analyzed on SFC-MS/MS. A total of five samples were analyzed (n=5) per organ. The results (Figure 18) demonstrate the tissue distribution of MBQ-167, and it can be observed that the highest concentration was present in the kidneys and liver at thirty-minute time point. The kidneys presented the highest concentration during all time points. The second

organ with the highest concentration was the liver at 10, 30, and 60 minutes after drug administration. At 10 minutes the spleen, heart, and lungs had similar concentration of MBQ-167, while the liver had almost four times higher concentration, and the kidneys had the highest concentration. At 30 minutes, MBQ-167 was found with the least concentration in the heart, followed by the lungs, spleen, livers, and kidneys respectively. After one hour, MBQ-167 was found with the least concentration at the heart, followed by the lungs, livers, spleen, and kidneys respectively. Finally, after two hours of administration, the lowest concentration of MBQ-167 was found in the hearts, then the lungs, spleen, livers, and kidneys respectively.

In general, during most time points, the concentration of MBQ-167 followed the same pattern of distribution, the least concentrated being the heart, followed by the lungs, spleen, livers, and kidneys respectively. Since the livers and kidneys are involved in drug metabolism it was expected that the highest concentration of the drug MBQ-167 was found in these organs. Moreover, the presence of MBQ-167 in all organs is consistent with the pharmacodynamics studies of MBQ-167 as an anti-metastatic compound. Overall, the results demonstrate an extensive distribution of MBQ-167 though the mouse organs studied.

Tissue distribution of MBQ-167 after intraperitoneal (I.P) administration

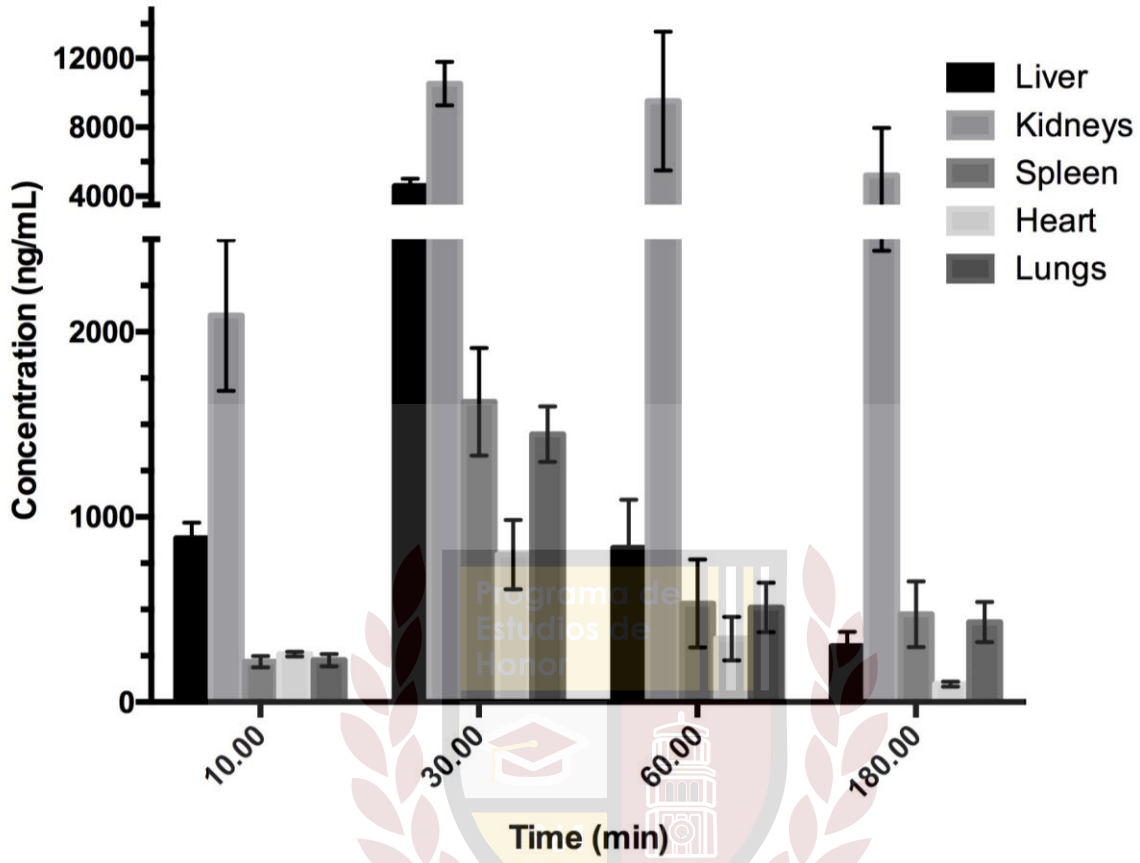



**Figure 20** Tissue distribution of MBQ-167 after intraperitoneal (I.P) administration of a single dose (10 mg/kg) of MBQ-167 in mice (N =5). Highest concentration of MBQ-167 was observed in kidneys and livers at all time points.

## 6.5. Effect of MBQ-167 and derivatives on CYP3A4 activity

To understand the effect of MBQ-167 and derivates on the metabolic enzyme CYP3A4, equal amounts of compounds (10μM) were added to a known concentration of enzyme to evaluate an inhibitory or inductory effect (Figure 19). Results demonstrated an inhibitory effect by MBQ-167 and all of the derivatives. The negative control, or vehicle, was used to demonstrate 100% activity by the CYP3A4. The positive control, Ketoconazole, demonstrated high inhibition that reduced the activity of the

enzyme by ~95%. These controls were important to understand the effect MBQ-167 and derivatives had on CYP3A4. The highest inhibition was observed by MBQ-167, and the lowest inhibition was observed by EHop-016. MBQ-167 inhibited CYP3A4 to less than 25% enzyme activity. Moreover, all of the inhibitors reduced the activity of CYP3A4 to a 10-40% activity. This experiment validated the hypothesis that MBQ-167 inhibits CYP3A4.

Screening for Human recombinant CYP3A4 inhibition

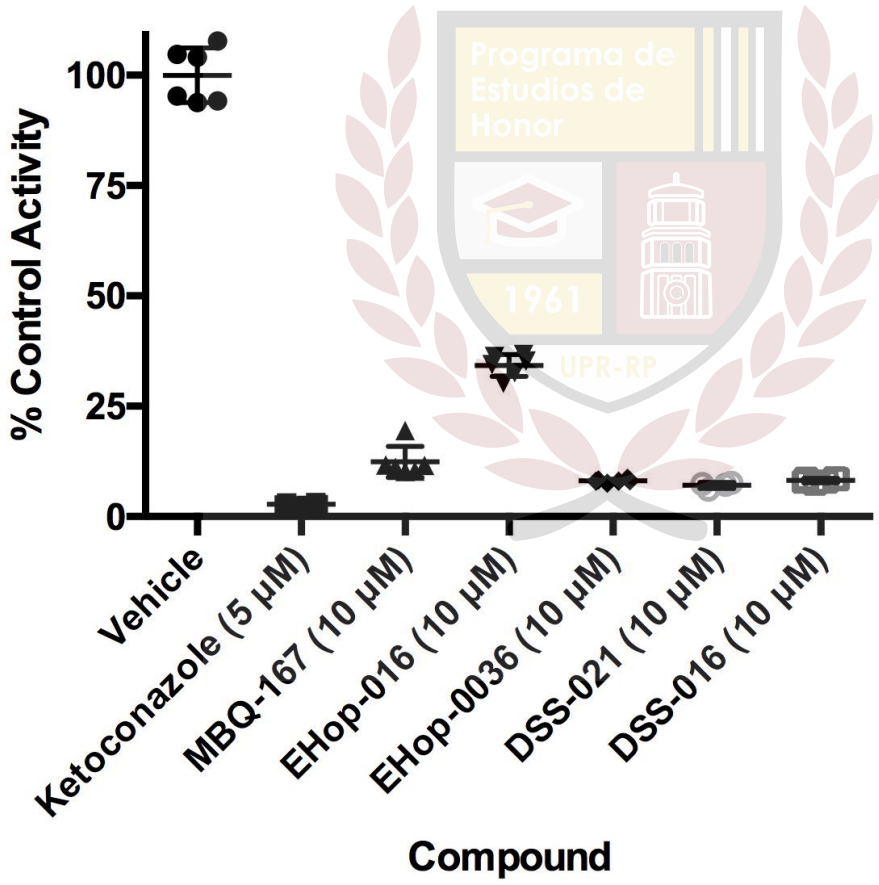



**Figure 21.** Percent of control activity of CYP3A4 under the presence of MBQ-167 and other derivatives. Highest inhibition was observed with MBQ-167.

### 6.5.1. $IC_{50}$ for MBQ-167 and derivatives in CYP3A4

To further elucidate the inhibitory effect MBQ-167 and derivatives on CYP3A4, the $IC_{50}$s for each compound were generated. To accomplish this, different concentration of the compounds, between 0.01μM and 100 μM, were tested on CYP3A4 activity. The $IC_{50}$ of MBQ-167 was approximately 0.28μM, EHop-036 was 8.04μM, EHop-016 was 3.31, DSS-016 was 3.18μM, and DSS-021 was 15.03μM, while Ketoconazole (positive control) was 0.09μM. The positive control Ketoconazole demonstrated the highest inhibition as expected, to corroborate the efficiency of the assay. The next highest inhibition was observed by MBQ-167 and the lowest inhibition was observed by EHop-016. This information is useful because it can educate the researcher and medical specialist of the possible drug-drug interactions of MBQ-167 and derivatives with metabolic enzymes. This information can be used to evaluate possible interactions in combined therapy.

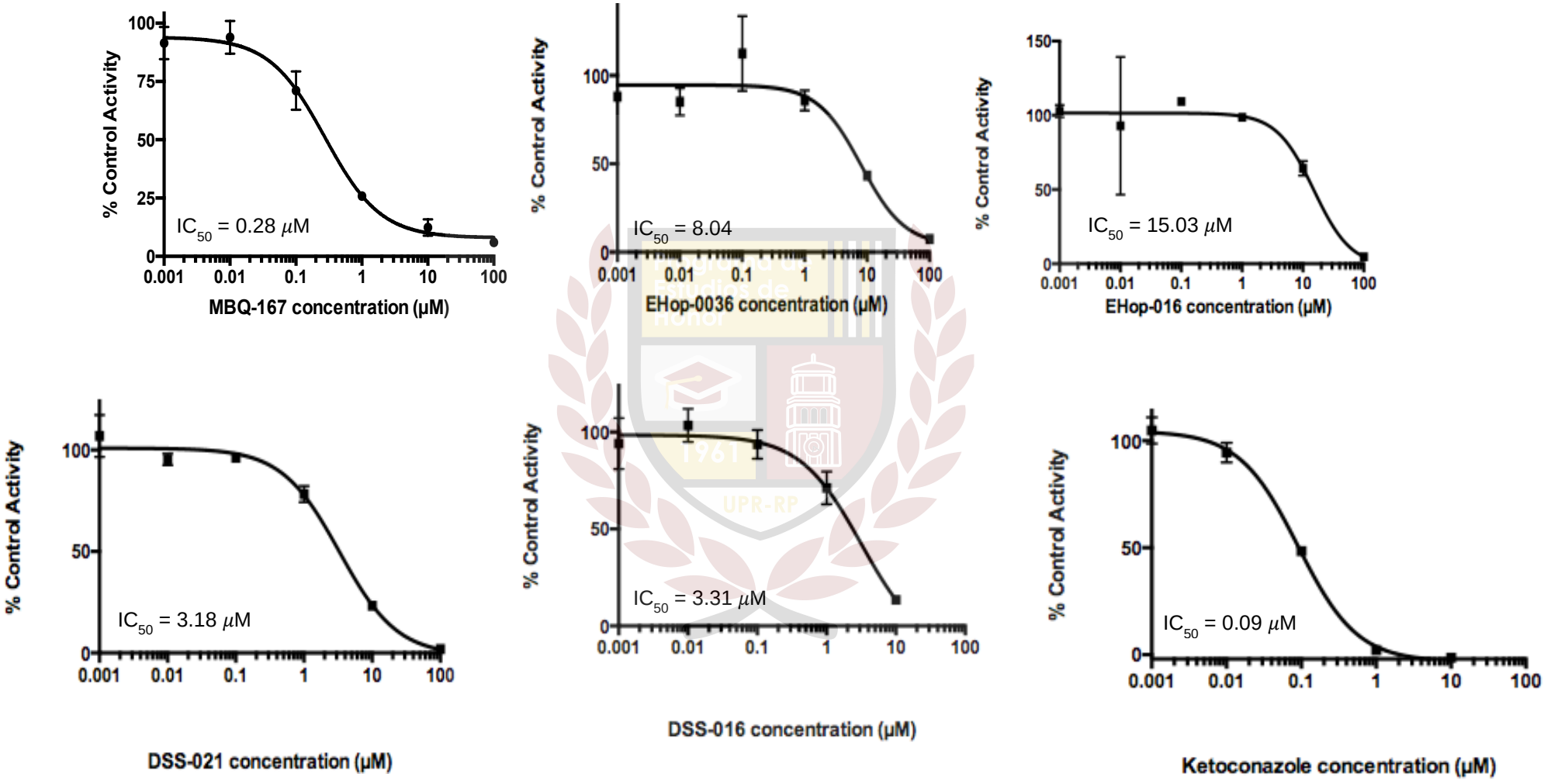



**Figure 22**. $IC_{50}$ curves of MBQ-167 and derivatives for CYP3A4. The $IC_{50}$ for MBQ-167 was 0.28 uM

### 6.5.2. Kinetics of MBQ-167 on CYP3A4

The next objective was to understand the type of inhibition MBQ-167 had on the CYP3A4 metabolic enzyme. To accomplish this, different concentrations of enzymes were incubated with different concentrations of MBQ-167 to evaluate the activity of CYP3A4 at different concentrations. The Michaelis-Menten inhibition curves are shown in Figure 21. This curve shows higher activity of CYP3A4 when there was 0 MBQ-167. As the concentration of MBQ-167 was increased, the activity of the enzyme was decreased. To better understand the kinetics behind the inhibition of MBQ-167, the Michaelis-Menten curves were converted to Lineweaver-Burk plots (Figure 22). This information was used to ascertain the changes in $K_m$ and $V_{max}$ of the enzyme CYP3A4. The kinetics values (Table 5) demonstrate a slow increase in affinity ($K_m$) with a slow decrease in maximum velocity ($V_{max}$). These values suggest a mixed inhibition of CYP3A4 by MBQ-167. The analysis was performed using GraphPad Prism 6.

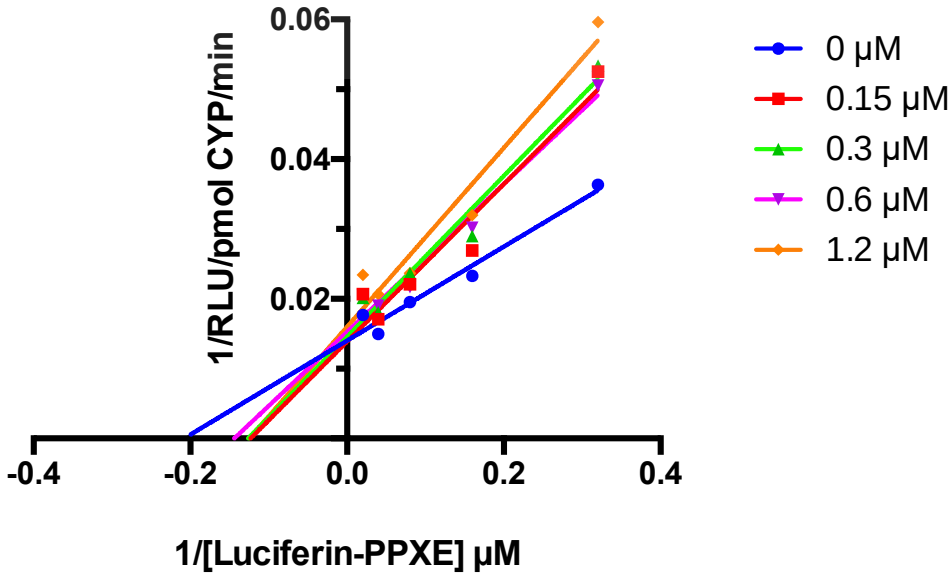



**Figure 23.** Michaelis-Menten inhibition of CYP3A4 activity by MBQ-167.

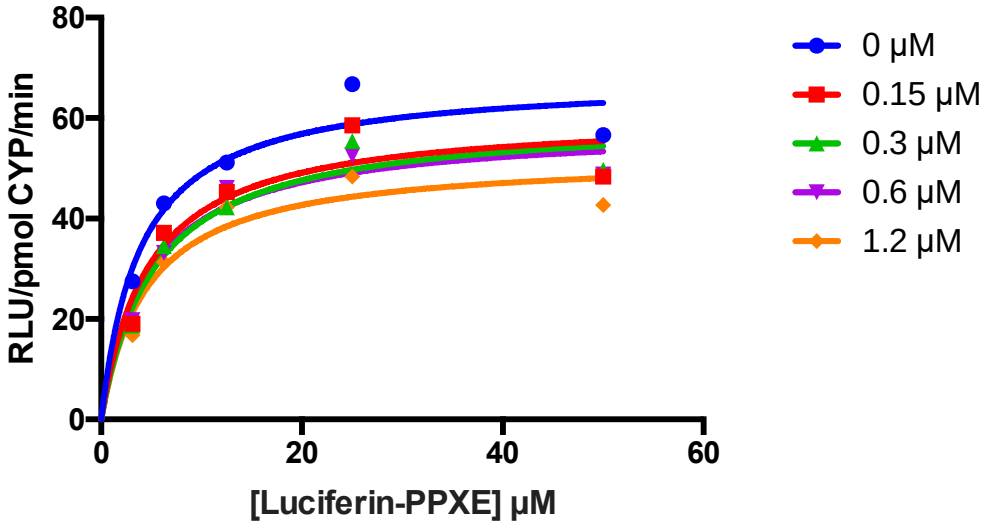



**Figure 24.** Lineweaver-Burk inhibition of CYP3A4 activity by MBQ-167.

**Table 5.** Inhibition of CYP3A4 activity by MBQ-167

| Michaelis-Menten values | Control | 0.15 µM | 0.3 µM | 0.6 µM | 1.2 µM |
|---|---|---|---|---|---|
| Vmax (RLU/pmol CYP/min) | 67.98 ± 6.4 | 60.58 ± 7.6 | 60.20 ± 5.7 | 58.42 ± 4.9 | 52.43 ± 5.7 |
| Km (µM) | 3.904 ± 1.5 | 4.658 ± 2.2 | 5.276 ± 1.8 | 4.737 ± 1.5 | 4.560 ± 1.9 |
| R square ($r^2$) | 0.8703 | 0.8393 | 0.9124 | 0.9194 | 0.8708 |

## 7. Conclusions

This project is an integral part in the evaluation of pharmacokinetics of the novel anti-metastatic drug MBQ-167 and is necessary for the continued development of this drug as a potential anti-cancer therapeutic. A rapid, sensitive and simple liquid-liquid extraction method was developed for the analysis of MBQ-167 in mouse tissue (livers, lungs, hearts, kidneys). This is the first analytical method that has been developed for the detection of MBQ-167 in this type of biological matrix. Successful detection and

quantification of the drug was achieved by SFC-MS/MS, which offers advantages such as high resolution and short analysis times. Ethyl acetate-Heptane demonstrated a higher recovery (~60%) in livers, heart, spleen, lungs, and kidneys. Recovery and matrix effects were consistent among different concentrations. Linearity was observed between 10 ng/mL -1000 ng/mL. The data obtained will be used to evaluate the pharmacokinetics of the novel anti-metastatic drug MBQ-167.

Next, MBQ-167's potential to inhibit metabolizing enzymes such as the cytochrome P450 (CYP450) family of enzymes was evaluated. Inhibition of CYP3A4 was observed after treatment of MBQ-167 and derivatives. MBQ-167 was the most potent inhibitor when compared to other derivatives, while EHop-016 was the least potent compound. Michaelis-Menten and Lineweaver-Burk plots suggest a mixed inhibition mechanism of CYP3A4 following MBQ-167 treatment. Hence, MBQ-167 could contribute to drug-drug interactions when orally co-administered with drugs metabolized by CYP3A4 in a clinical setting.

Future work will be focused on elucidating the pharmacokinetics of MBQ-167 in mammary fat pad tumors. Also, to continue studying the pharmacology of MBQ-167, specifically understanding the effect MBQ-167 on other the principal metabolic enzyme, such as the CYP2D6, CYP2C19, CYP2C9, and CYP1A2 will also be conducted.

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# 9. Annex

## 9.1. Abstract: Experimental Biology Conference 2019

***Bioanalytical method development for the detection of the Rac/Cdc42 inhibitor MBQ-167 in mouse tissue***

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Metastatic breast cancer is the second most common cancer in US women. The mortality rate has decreased approximately 40% in the last 25 years due to advancement in treatment and diagnostics. However, it is estimated that 20-30% of all breast cancer patients will develop metastatic breast cancer. Therefore, there is a need to develop targeted therapeutics to reduce cancer cell migration and subsequent metastasis. The novel small molecule MBQ-167 inhibits Rac and Cdc-42, proteins involved in cancer cell growth, migration and invasion. *In vivo* studies have shown that MBQ-167 inhibits mammary tumor growth and metastasis in immunocompromised mice by ~90% (Humphries-Bickley, *et al.,* 2017). However, to continue validating this drug for FDA approval, it is necessary to study the pharmacokinetic profile of the drug, which includes elucidating the tissue distribution of the compound in the body. Hence, it is necessary to develop a rapid and sensitive method to quantify MBQ-167 in tissues. The purpose of this study was to develop and validate a method for the quantification of MBQ-167 in mouse tissues using Supercritical fluid chromatography coupled with electrospray ionization tandem mass spectrometry (SFC-MS/MS). Mouse livers were homogenized, spiked with MBQ-167, and liquid-liquid extractions were performed with different organic solvents (heptane, ethyl acetate, 2-propanol, toluene, and acetonitrile), in order to determine the optimal sample preparation method to selectively separate the drug MBQ-167 from endogenous material in mouse tissue. Separation was performed on an ACQUITY UPC$^2$ BEH (3.0 x 100 mm, 1.7 µm) column at 40 ºC with a mobile phase consisting of $CO_2$ and methanol 0.1 % formic acid (95:5, v/v) at a flow rate of 1 mL/min. A tandem triple quadrupole mass spectrometer was operated in multiple reaction monitoring (MRM) mode. High recovery of MBQ-167 (> 65%) and lower matrix effects were observed upon extraction with Heptane:Ethyl Acetate (1:1). Consistent recovery was observed in other tissues such as kidney, lungs and heart. The method was sensitive with a lower limit of quantification (LLOQ) of 10 ng/mL. Linearity was observed over the concentration range of 10-1000 ng/mL. The data obtained supports the use of SFC-MS/MS as a highly specific method to evaluate the tissue distribution of MBQ-167 in mice. This study is supported by MARC grant NIH/NIGMS 5T34GM007821-39 (to GRG) and NIGMS-RISE 25 GM061838 (to MM).

## 9.2. **Abstract: Annual Biomedical Research Conference for Minority Students (ABRCMS) 2019**

### ***Effect of the metastatic cancer inhibitor MBQ-167 and derivatives on human drug metabolizing enzyme CYP3A4***

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Metastatic breast cancer is the second most common cancer in US women. Even though the mortality rate has decreased in the last 25 years, it is estimated that 20-30% of all breast cancer patients will develop metastatic breast cancer. Therefore, there is a need to develop targeted therapeutics to reduce cancer cell migration and subsequent metastasis. The novel small molecule MBQ-167 inhibits Rac and Cdc42, proteins involved in cancer cell growth, migration, and invasion. *In vivo* studies have shown that MBQ-167 inhibits mammary tumor growth and metastasis in immunocompromised mice by ~90%. However, to continue validating this drug for FDA approval, it is necessary to evaluate MBQ-167's potential to inhibit metabolizing enzymes such as the cytochrome P450 (CYP450) family of enzymes. CYP450 enzymes are hemeproteins, primarily found in the liver, which are responsible for the metabolism and clearance of xenobiotics, such as drugs. Molecules that inhibit or induce the activity of these CYPs may interact with drugs that are metabolized by these enzymes in a clinical setting, potentially leading to adverse effects. Bioinformatics analysis of the structure of MBQ-167 shows high-affinity binding sites of MBQ-167 with CYP3A4, a key CYP enzyme in Phase I drug metabolism that is responsible for the metabolism of about half of all drugs in the market. The objective of this study was to evaluate the potential inhibitory effect of MBQ-167 and derivatives on CYP3A4 activity. Luminescent assays were performed *in vitro* using human CYP3A4 recombinant enzymes. Enzyme reactions were started with the addition of NADPH and product formation was monitored by the luminescence of the resulting luciferin analog. $IC_{50}$ values and enzyme kinetics parameters were determined using GraphPad Prism 6. Preliminary results show that MBQ-167 inhibited CYP3A4 activity with an $IC_{50}$ of 0.28 $\mu$M. Four derivatives with similar structures to MBQ-167 also inhibited CYP3A4 activity with $IC_{50}$'s ranging from 3.2 $\mu$M to 15 $\mu$M. Lineweaver-Burk plot analysis suggested inhibition through mixed mechanisms. Hence, MBQ-167 could contribute to drug-drug interactions when orally co-administered with drugs metabolized by CYP3A4 in a clinical setting. This study is supported by MARC (NIH/NIGMS 5T34GM007821-39) for GRG, MBRS-RISE (R25GM061838) for MM, and NIH/NIGMS GM084824 to SD.

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