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Investigating the Effect of Rutaecarpine on the Benzo[a]pyrene-Induced DNA Damage in vitro

You Li
University of the Pacific, y_li33@u.pacific.edu

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INVESTIGATING THE EFFECT OF RUTAECARPINE ON THE
BENZO[A]PYRENE-INDUCED DNA DAMAGE *IN VITRO*

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You Li

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INVESTIGATING THE EFFECT OF RUTAECARPINE ON THE
BENZO[A]PYRENE-INDUCED DNA DAMAGE *IN VITRO*

by

You Li

APPROVED BY:

Advisors: Zhu Zhou, Ph.D. & Miki Susanto Park, Ph.D.

Committee Member: Liang Xue, Ph.D.

Department Chair: William K. Chan, Pharm.D., Ph.D.

Dean of Graduate School: Thomas Naehr, Ph.D.

DEDICATION

To my family and friends with love and thanks.

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Investigating the Effect of Rutaecarpine on the
Benzo[a]pyrene-induced DNA Damage *in vitro*

Abstract

by You Li

University of the Pacific

2019

Benzo[a]pyrene (BaP), is one of the most potent mutagens and carcinogens known. It requires metabolic activation through cytochrome P450 (CYP)1A1 to yield the ultimate carcinogenic metabolite, benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE). BPDE can bind to DNA and form predominantly covalent (+) trans adducts at the N2 position of guanine causing DNA damage. Rutaecarpine (RTC) is an herbal medicine that has been used to treat several diseases such as headache, hypertension, gastrointestinal disorders, amenorrhea, and anti-inflammation. It has also been reported as a potent inducer of CYP enzymes, including CYP1A1, and CYP1A2. The mechanisms underlying up-regulation of CYP1A1 by RTC is dependent on aryl hydrocarbon receptors. Meanwhile, RTC can inhibit the activity of CYP1A1, CYP1A2 and CYP1B1.

To investigate the effect of RTC on the BaP-induced DNA damage, we analyzed the CYP1A1 enzyme activity and DNA damage level in two cell lines, namely mucoepidermoid pulmonary carcinoma cells (H292) and hepatocellular carcinoma cells (Hep3B). The cells either were treated with only 5 μM BaP or 1.25, 2.5, 5 and 10 μM RTC, respectively; or were co-administrated 5 μM BaP and one of the four concentrations of RTC for 24 hours.

Ethoxyresorufin-O-deethylase (EROD) assay was used to detect CYP1A1 enzyme activity. The results showed that both BaP and RTC significantly ($p<0.05$) induced CYP1A1 enzyme activity when administered separately, with RTC induction exhibiting a concentration-dependent manner. Interestingly, co-administration of RTC with BaP, especially at high concentration (10 μM) of RTC, induced less CYP1A1 enzyme activity compared to either only RTC or BaP administration. Muse[TM] Multi-Color DNA Damage kit was used to evaluate the DNA damage level in cells. The data showed that the DNA damage induced by BaP alone was about 2-fold higher ($p<0.05$) than that by concurrent administration of RTC and BaP.

In conclusion, our data showed that although both RTC and BaP are inducers of CYP1A1 enzyme, their co-administration will reduce CYP1A1 enzyme activity compared with BaP administration alone. The DNA damage kit results supported that there is a potential protective effect of RTC against BaP-induced DNA damage in both H292 and Hep3B cells.

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LIST OF ABBREVIATIONS

BaP	benzo[a]pyrene
BCA	bicinchoninic Acid Assay
BNF or βNF	β-naphthoflavone
BPDE	benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide
BSA	bovine serum albumin
CYP1A1	cytochrome P450 1A1 enzyme
CYP450	cytochrome P450 enzymes
EMEM	eagle's minimal essential medium
EROD	ethoxyresorufin-O-deethylase
FBS	fetal bovine serum
MBI	mechanism-based inhibition
NADPH	β -Nicotinamide adenine dinucleotide phosphate
NSCLC	non-small-cell lung carcinoma
PAHs	polycyclic aromatic hydrocarbons
PBS	phosphate buffered saline
RFU	relative fluorescence units
RLM	rat liver microsome
RTC	rutaecarpine
SCLC	small-cell lung carcinoma
TDI	time-dependent inhibition
1-EP	1-ethynylpyrene

Chapter 1: Introduction

1.1 Statement of Purpose

Benzo[a]pyrene (BaP) is one of the polycyclic aromatic hydrocarbons (PAHs) that are known to be potent human carcinogens and its important role in cancer, especially lung cancer, makes it an interesting target to study. The carcinogenic mechanism of BaP stems from its diol epoxide metabolite, benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE), which can alkylate DNA at the N2 position of guanine bases to cause DNA damage and initiate mutations. CytochromeP450 (CYP) 1A1 enzyme is the main enzyme known to be involved in the metabolic pathway of BaP, therefore, regulation of CYP1A1 is a key factor for the study of the carcinogenicity of BaP.

Rutaecarpine (RTC) is a pentacyclic indolopyridoquinazolinone alkaloid isolated from Evocardia rutaecarpa, an herb that has been used in traditional medicine to treat various diseases. It has been reported that RTC has a complex modulatory effect on CYP1 family, including CYP1A1 and CYP1A2 enzymes. Since CYP1A1 contributes to the carcinogenicity of BaP and RTC could regulate CYP1A1 activity, it is of significance to investigate the effects of RTC on the carcinogenicity of BaP.

Chapter 1 introduces the background of lung cancer, BaP, BaP metabolic pathway, CYP enzymes, and RTC.

1.2 Lung Cancer

Lung cancer is a group of diseases characterized by abnormal growths that originate from the lungs. It is by far the leading cause of cancer death among both men and women each year.

The American Cancer Society estimates that in 2019 there will be about 228,150 new cases of lung cancer in the U.S. and around 142,670 deaths would be expected due to the disease [1].

1.2.1 Types of lung cancer

There are three main types of lung cancer: small-cell lung carcinoma (SCLC), non-small-cell lung carcinoma (NSCLC) and lung carcinoid tumor.

SCLC is also called oat cell cancer. It constitutes around 10% to 15% of lung cancers. This type of lung cancer is characterized by its ability to spread quickly.

NSCLC is the most common type of lung cancer. Subtypes of NSCLC include squamous cell carcinoma, large cell carcinoma and adenocarcinoma. Though the subtypes start from different types of lung cells, they are grouped as NSCLC because the treatment and prognosis of the various subtypes are often similar. About 25% to 30% of all lung cancers are squamous cell carcinomas. These start in early versions of squamous cells, which are flat cells that line the inside of the airways in the lungs. They are often linked to a history of smoking and tend to be found in the central part of the lungs, near the bronchus.

Adenocarcinomas make up around 40% of lung cancers. Adenocarcinomas start in early versions of the cells that would normally secrete substances such as mucus. Even though this type of lung cancer occurs mainly in current or former smokers, it is also the most common type of lung cancer seen in non-smokers. Furthermore, it occurs more often in women than in men, and compared to other types of lung cancer, it is also more likely to occur in younger people. Studies have shown that it tends to grow slower than other types of lung cancers thus more likely to be discovered early, though this varies from patient to patient. Adenocarcinoma is most often found in outer parts of the lung. Large cell carcinoma accounts for about 10% to 15% of lung

cancers. It tends to grow and spread rapidly, thus making it harder to treat. It can appear in any part of the lung. A subtype of large cell carcinoma, known as large cell neuroendocrine carcinoma, is a fast-growing cancer that is very similar to small cell lung cancer [2].

Fewer than 5% of lung cancers are lung carcinoid tumors, which are also sometimes called lung neuroendocrine tumors. A majority of these tumors grow slowly and are rather confined in their place of origin. Lung carcinoid tumors originate from neuroendocrine cells, a special kind of cells found in the lungs. Neuroendocrine cells are also found in other areas of the body, but only cancers that form from neuroendocrine cells in the lungs are called lung carcinoid tumors. Neuroendocrine tumors are most commonly found in the digestive while being the second most common in the lungs. Lung carcinoid tumors can be separated into 2 types: typical carcinoids and atypical carcinoids. Typical carcinoids tend to grow slowly and rarely spread beyond the lungs. About 9 out of 10 lung carcinoids are typical carcinoids. Furthermore, there appears to be no link between typical carcinoids and smoking. Atypical carcinoids grow a little faster and are slightly more likely to spread to other organs. They have more cells undergoing division and resemble a fast-growing tumor. However, they are much less common than typical carcinoids and are linked to smoking [3].

1.2.2 Risk factors of lung cancer

Tobacco smoke. Smoking is the most dominant risk factor for lung cancer. Around 80% of lung cancer deaths can be attributed to smoking. The risk for lung cancer among smokers is many folds higher than that of non-smokers, and such a risk is directly increased with the duration of smoking and the number of packs smoked.

Cigar smoking and pipe smoking. These other forms of smoking are almost equally likely to cause lung cancer as cigarette smoking. Smoking low-tar or "light" cigarettes increases lung cancer risk at a level similar to regular cigarettes. Menthol cigarette smokers may have an even higher risk since the menthol allows smokers to inhale more deeply.

Secondhand smoke Indirect exposure to a smoking environment, breathing in the smoke of others (called secondhand smoke or environmental tobacco smoke) can also increase the risk of developing lung cancer. Secondhand smoke is thought to cause more than 7,000 deaths from lung cancer each year.

Exposure to radon. Radon is a naturally occurring radioactive gas that results from the breakdown of uranium in soil and rocks. It exhibits no visible characteristic, taste, or smell. Surprisingly, according to the US Environmental Protection Agency (EPA), radon is the second leading cause of lung cancer and is the leading cause among non-smokers. There are very low amounts of radon outdoors that it is not likely to be dangerous, but radon can become more concentrated indoors. Breathing in radon exposes the lungs to small amounts of radiation, which may increase a person's risk of lung cancer. Homes and other buildings in nearly any part of the United States can have high levels of radon (especially in basements).

Exposure to asbestos. People who work in environments containing asbestos (i.e., mines, mills, textile plants, places where insulation is used, and shipyards) are many times more likely to die of lung cancer. The risk of lung cancer risk is compounded in workers exposed to asbestos who also smoke. There is no consensus on what degree of low-level or short-term exposure to asbestos might raise lung cancer risk.

Exposure to other cancer-causing agents in the workplace. There are various carcinogens can be found in some workplaces thus increasing lung cancer risk. These may include radioactive ores such as uranium, inhaled chemicals such as arsenic, beryllium, cadmium, silica, vinyl chloride, nickel compounds, chromium compounds, coal products, mustard gas, chloromethyl ethers, diesel exhaust, arsenic in drinking water and certain dietary supplements.

Previous radiation therapy to the lungs. Prior radiation therapy to the chest for the treatment of other cancers increases the risk of lung cancer, which is even further compounded if combined with smoking. For instance, treatments for Hodgkin disease or radiation after a mastectomy for breast cancer can increase the risk of lung cancer. However, women who have radiation therapy to the breast after a lumpectomy do not appear to have a higher than expected risk of lung cancer.

Air pollution. Air pollution appears to raise the risk of lung cancer slightly. Though such a risk is far less than the risk caused by smoking, yet some researchers estimate that worldwide about 5% of all deaths from lung cancer may be due to outdoor air pollution.

Personal or family history of lung cancer. Previous occurrences of lung cancer will also mean having a higher risk of developing another lung cancer. Close relatives and children of people who have had lung cancer may have a slightly higher risk of lung cancer, especially if the relative was diagnosed at a younger age. Currently, it is not clear to what extent this risk might be due to shared genes and how much this might be from shared living environments (such as tobacco smoke or radon) [4].

1.3 Benzo[a]pyrene

Smoking, particularly of cigarettes, is by far the main contributor to lung cancer. Cigarette smoke contains at least 73 known carcinogens, including benzo[a]pyrene (BaP), NNK, 1,3-butadiene, etc. [5] BaP is the most well-studied among those carcinogens. It is one of the polycyclic aromatic hydrocarbons (PAHs), a group of cancer-inducing substances, listed as a Group 1 carcinogen by the IARC [6, 7].

BaP is primarily found in gasoline, cigarette smoke, coal tar, coal tar pitch, and charcoal-broiled foods. It also can be formed during other incomplete combustion of organic matter.

1.3.1 Metabolism of benzo[a]pyrene

BaP is metabolized by both phase-I and phase-II enzymes to form a series of arene oxides, dihydrodiols, phenols, quinones and their polar conjugates with glutathione, sulfate, and glucuronide [8]. Benzo[a]pyrene-7,8-diol is a key metabolite that is formed by the action of epoxide hydrolase on benzo[a]pyrene-7,8-epoxide. This dihydrodiol can be further metabolized by cytochrome P450s (CYPs) to form a series of benzo[a]pyrene-7,8-diol-9,10-epoxides, which constitutes one class of the ultimate carcinogenic metabolites of BaP. The major CYPs involved in the formation of diols and diol epoxides are CYP1A1 and CYP1B1. Cytochrome P450s are

inducible by BaP and other PAHs through binding to the aryl hydrocarbon-receptor (AhR)

nuclear complex, leading to changes in gene transcription of CYPs and phase-II enzymes [9].
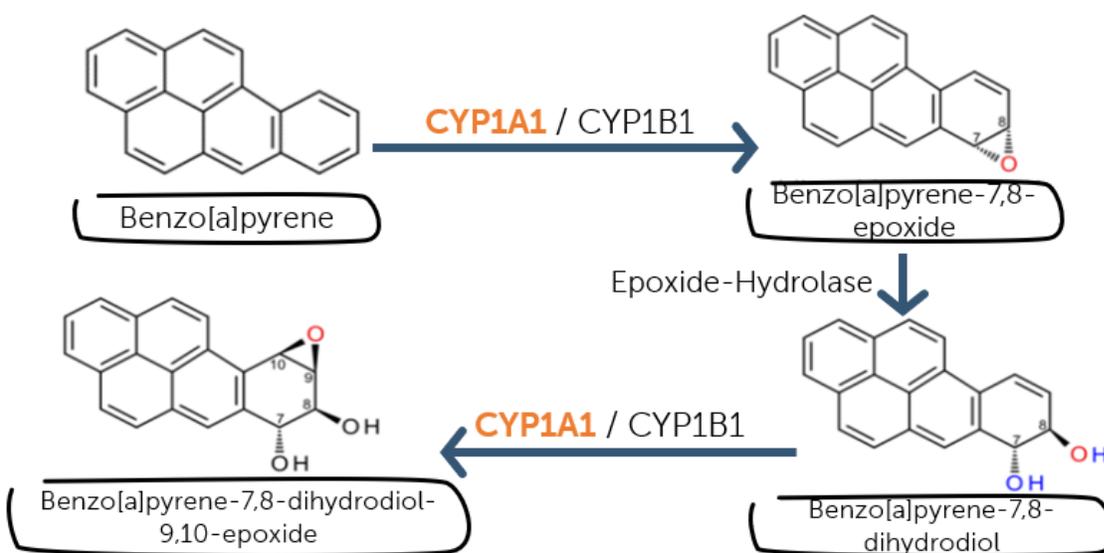
Figure 1. 1 Metabolic Process of BaP

1.3.2 Carcinogenicity of benzo[a]pyrene

The current understanding of the mechanisms underlying BaP-induced carcinogenesis in

experimental animals is almost solely based on its diol-epoxides.

The diol-epoxide mechanism for BaP features a sequence of metabolic transformations:

benzo[a]pyrene → benzo[a]pyrene-7,8-oxide (by CYP1A1 and CYP1B1) → benzo[a]pyrene-

7,8-diol (by epoxide hydrolase) → benzo[a]pyrene-7,8-diol-9,10-epoxides (by CYP1A1 and

CYP1B1). Each class of metabolic intermediate is genotoxic and carcinogenic. The

stereochemistry of the metabolic transformation of BaP to diols and diol-epoxides is an important component of this mechanism of action. Due to the creation of chiral carbons during the metabolic conversions, many of the metabolic intermediates of BaP have multiple stereochemical forms (enantiomeric and diastereomeric). As the metabolism proceeds, the complexity of the stereochemical forms increases, it eventually leads to four benzo[a]pyrene-7,8-diol-9,10-epoxides [(+)- and (-)-anti, (+)- and (-)-syn]. Diol-epoxides react mainly with the purines, deoxyguanosine, and deoxyadenosine of DNA, and each diol-epoxide can form both cis and trans adducts thus giving a total of 16 possible benzo[a]pyrene -7,8-diol-9,10-epoxide DNA adducts. However, in most cases, far fewer DNA adducts are observed. The most ubiquitous BaP adduct detected in isolated mammalian DNA after metabolic conversion in either metabolically competent mammalian cells in culture or in mammals, is the N2-deoxyguanosine adduct, (+)-N2-10S-(7R,8S,9R-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene)-yl)-2' deoxyguanosine, derived from 7R,8S-dihydroxy-9R,10R-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (anti-benzo[a]pyrene-7,8-diol-9,10-epoxide, or BPDE). This adduct was first fully identified after isolation from BaP-treated human and bovine bronchial explants. This diol-epoxide is considered to be an ultimate, DNA-reactive, metabolite of BaP[10, 11].

1.4 Cytochrome P450 1A1

Cytochrome P450 (CYP) enzymes are a large ubiquitous superfamily of enzymes, playing a significant physiological role in the detoxification of xenobiotics, and the biosynthesis of many endogenous compounds. P450 families 1, 2, and 3 contribute most extensively to the biotransformation of xenobiotics into more polar metabolites that are readily excreted. In humans and most mammals, P450 family 1 comprises three well-studied monooxygenases, 1A1, 1A2, and 1B1.

Cytochrome P450 1A1 (CYP1A1), a well-known aryl hydrocarbon hydroxylase, is implicated in the metabolic activation of environmental procarcinogens such as polycyclic aromatic hydrocarbons (PAHs) and polyhalogenated aromatic hydrocarbons (PHAHs). Human P450 1A1 is mainly expressed in extrahepatic tissues such as lung, gastrointestinal tract, placenta, and skin, and is present only at low levels in the liver. P450 1A1 is one of the most important enzymes involved in tumorigenesis initiated by environmental pollutants. Many epidemiological studies have shown that genetic variants of human CYP1A1 gene are significantly associated with the susceptibilities to lung and breast cancers. Because of the significant role of P450 1A1 enzyme in human carcinogenesis, modulation of P450 1A1 activity has been considered as a potential target for cancer chemoprevention [12].

1.5 Rutaecarpine

Rutaecarpine (RTC) is a main quinazolinocarboline alkaloid isolated from Evodia rutaecarpa (Wu-chu-yu), which has been used as an herbal medicine for the treatment of several diseases [13]. Evodia is used for digestion problems including diarrhea, dysentery, nausea, vomiting, abdominal pain, gastroesophageal reflux disease (GERD), stomach ulcers, and lack of appetite. It is also used for obesity, headache, high blood pressure, congestive heart failure, infections caused by viruses, Alzheimer's disease, cancer, and fluid retention. Women use Evodia to prevent pregnancy, start their periods, and treat bleeding after giving birth. Evodia root bark is used for infections caused by parasites such as tapeworm and pinworm [14].

As the alkaloid isolated from Evodia, RTC induces CYP1A, 2B, and 2E1 in mice, based on measuring CYP-associated enzyme activities, and increases the metabolism of caffeine by inducing CYP1A2 and CYP2E1 in rats [15]. It was reported that RTC strongly induces CYP1A1

and CYP1A2 in mouse liver [16, 17]. Furthermore, there are some other studies indicating that RTC significantly induces CYP1A1 gene through the AhR- and [Ca2+]-dependent mechanisms as an AhR ligand in Hepa-1c1c7 cells [18].

Meanwhile, RTC has also been reported as a selective inhibitor of CYP1A1 enzyme in mouse and human liver microsomes [19] and a mechanism-based inhibitor of CYP1A2 enzyme in human liver microsomes [20].

1.6 Hypothesis

Two possible processes for the regulation of CYP1A1 enzyme by RTC have been reported: induction of its expression and inhibition of its activity. In general, the induction of CYP1A1 means the mRNA expression, protein expression and enzyme activity of CYP1A1 are increased. According to the references [17, 18], RTC did increased CYP1A1 mRNA expression, protein expression and enzyme activity. On the other hand, the inhibition of CYP1A1 enzyme activity by RTC was reported by Ueng et al. 2002 [19], however, the mechanism of the inactivation is still unclear. Since the regulation of CYP1A1 enzyme by RTC is complicated, there are two possible net effects of RTC on the BaP-induced DNA damage. If the induction of CYP1A1 enzyme is greater than its inhibition, we can expect that co-administration of RTC and BaP will result in more CYP1A1 activity than BaP administration alone, thus promoting the metabolism of BaP and create more BPDE, thus causing more DNA damage. On the contrary, if the inhibition effect is greater than its induction, less activity of CYP1A1 enzyme could be expected by co-administration of RTC and BaP compares to BaP administration alone, leading to reduced DNA damage.

According to our lab's previous results on CYP1A2 enzyme, which is similar to the CYP1A1 enzyme, after RTC administration, CYP1A2 enzyme showed a higher activity in rats, thus suggesting that the induction effect of RTC on CYP1A2 is greater than its inhibition effect. Therefore, we expected a similar outcome for CYP1A1 enzyme.

We hypothesized that co-administration of RTC and BaP will increase BaP-induced CYP1A1 enzyme activity, which will result in a higher potential risk for DNA damage.

Chapter 2: Investigating the Effect of Rutaecarpine on Benzo[a]pyrene-Induced CYP1A1 Enzyme *in vitro*

2.1 Introduction of Cell Lines

To investigate the effect of BaP and RTC on CYP1A1 enzyme *in vitro* we used two cell lines. The first is the hepatocellular carcinoma cells, Hep3B, which is used as an experimental model because it is the most commonly available and well-characterized liver cancer cell lines [21]. The other is NCI-H292, the mucoepidermoid pulmonary carcinoma cells. H292 cells are sensitive to cigarette smoke and other agents that are harmful by inhalation, and there are data suggesting that it responds similarly as primary lung epithelial cells[22].

2.2 Material and Methodology

2.2.1 Materials

NCI-H292 cell line was a gift from Dr. John C. Livesey (University of the Pacific). Hep3B cell line was a gift from Dr. William K. Chan (University of the Pacific). Gibco™ RPMI-1640 mediums and Gibco™ TrypLE Express Enzyme were purchased from Fisher Scientific (Pittsburgh, Pennsylvania, USA). EMEMs were purchased from VWR International (Claremont, CA, USA). 1% antibiotic-antimycotic mixture of penicillin-G, streptomycin sulfate and amphotericin B were purchased from Sigma Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Gemini Biological Products (Calabasas, CA, USA). BaP was purchased from VWR International (Claremont, CA, USA). RTC was donated by Linnet Biopharmaceuticals. 7-ethoxyresorufin and phosphate buffered saline (PBS) were purchased from Fisher Scientific (Pittsburgh, Pennsylvania, USA). Culture plates (12-well and 6-well plates) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). 96 well UV and

fluorescence microplates were obtained from Spectrum Chemicals (Gardena, CA). Pierce™ Rapid Gold BCA Protein Assay Kit and 96-Well Plates for Pierce™ BCA-RAC Assay were purchased from Thermo Fisher Scientific (Grand Island NY, USA). TriStar LB 941 Multimode Microplate Reader was from Berthold Technologies (Oak Ridge, TN).

2.2.2 Ethoxyresorufin-O-deethylase (EROD) assay

H292 were cultured in RPMI-1640 and Hep3B cells were cultured in EMEM, and both types of mediums were supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were incubated in an incubator (5% CO_2, 37 °C) and were grown to reach confluence in a 6-well plate before experiments. Wells (n = 3/treatment) were incubated with 0.1% DMSO fresh media containing BaP (5 µM) and/or 10 µM of RTC for 24 h, followed by washing with fresh medium. Cells were then incubated in fresh media containing 5 µM 7-ethoxyresorufin and 10 µM dicumarol for 1 hour before 200 µl medium from each well was sampled and analyzed with a fluorimeter, using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. A resorufin calibration curve, ranging from 25 nM to 1600 nM, was prepared in duplicate and analyzed. The fluorescence readings were normalized by protein concentrations.

2.2.3 BCA protein assay

After EROD assay, cells were harvested by trypsinized and washed with PBS. Centrifuged the cells for 5 minutes at 400 g to collected cell pellet and resuspended cells in 20 µL lysis buffer. Through three times of freeze-thaw cycles and 30 minutes incubation on ice, cells were lysed and the protein were obtained by 10 minutes 16000 g centrifugation at 4°C. A standard calibration curve for bovine serum albumin (BSA) with seven concentrations, ranging from 0-1.5 mg/mL, were prepared in duplicate. All serial dilutions were made with 0.1 M

phosphate buffer. 20 µL of standards and 20 µL cell samples were added individually into each well of a 96-well plate. With a multi-channel pipette, 180 µL of Pierce reagent A and B mixture (50:1, respectively) was added to each well (containing samples or standards). The plate was incubated at 37 °C for 30 min, after which the reactions were analyzed at 562 nm using Tristar LB 941 multimode microplate reader.

2.2.4 Statistical analysis

GraphPad Prism (Version 7.0) software was used for statistical analysis. Analysis of variance (ANOVA) with post hoc Tukey's multiple comparisons test (compares means of each group with every other group) were performed on all the data sets. Statistical significance was determined at $P < 0.05$.

2.3 Results

2.3.1 BCA assay in H292 and Hep3B cell lines

Figure 2.1 shows the protein concentrations of H292 or Hep3B cells of each treatment. There were no significant differences in protein concentrations among the treatments, which meant cells under all treatments showed similar viabilities and had similar amounts of protein.
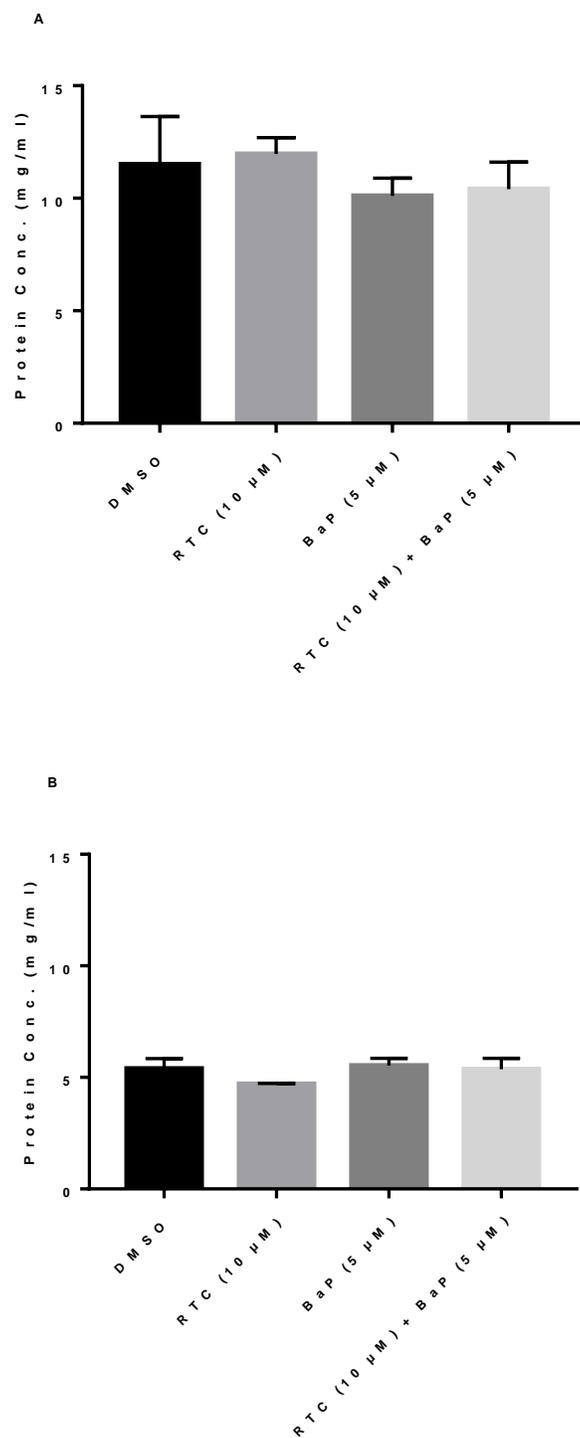
Figure 2. 1 Protein concentration of each treatment in (A). H292 cells and (B). Hep3B cells.

2.3.2 Enzyme activity of CYP1A1 in H292 cell line

CYP1A1 enzyme activities after RTC, BaP or RTC and BaP pretreatment in H292 cells were measured by EROD assay (Fig 2.2). The results of EROD activity showed that both 5 μM BaP and 10 μM RTC treatments significantly (p<0.0005 and P<0.0001, respectively) induced CYP1A1 enzyme activity when administered separately compared to DMSO treatment in H292 cell line, which is consistent with previously reported data [18, 23]. However, in contrast to our original hypothesis, the CYP1A1 activity in cells that received co-administration of RTC with BaP was significantly (P<0.0001) lower than in cells that were administered with BaP alone.

Figure 2. 2 CYP1A1 enzyme activity in 1% DMSO (vehicle), 10 μM RTC, 5 μM BaP or 10 μM RTC and 5 μM BaP treated H292 cells. ***p<0.0005, ****p<0.0001.

2.3.3 Enzyme activity of CYP1A1 in Hep 3B cell line

CYP1A1 enzyme activities after RTC, BaP or RTC and BaP pretreatment in Hep3B were measured by EROD assay (Fig 2.3). The results of EROD activity showed that both 5 μM BaP and 10 μM RTC treatments significantly ($P<0.0001$) increased CYP1A1 enzyme activity when administered separately compared to DMSO treatment in Hep3B cell line, which is consistent with previously reported data. Similar to the results in H292 cells, CYP1A1 activity in cells receiving co-administration of RTC with BaP was significantly ($P<0.0001$) lower when compared with CYP1A1 activity in cells that were pretreated by either compound alone.
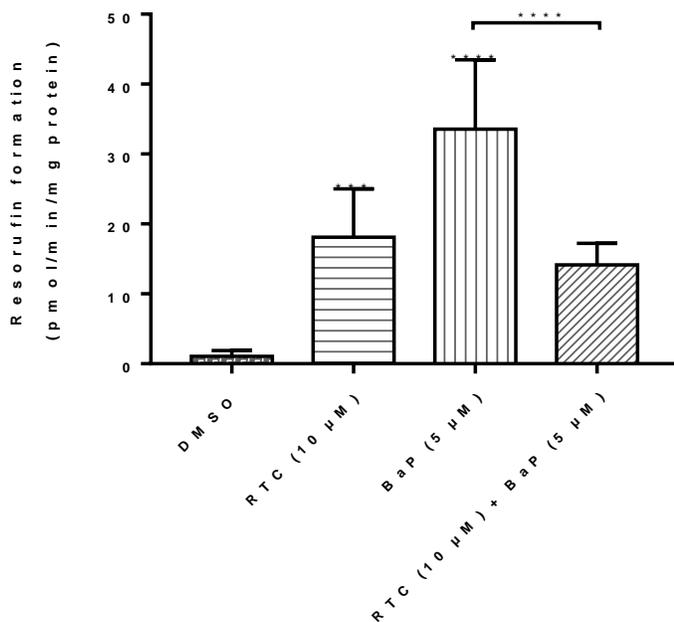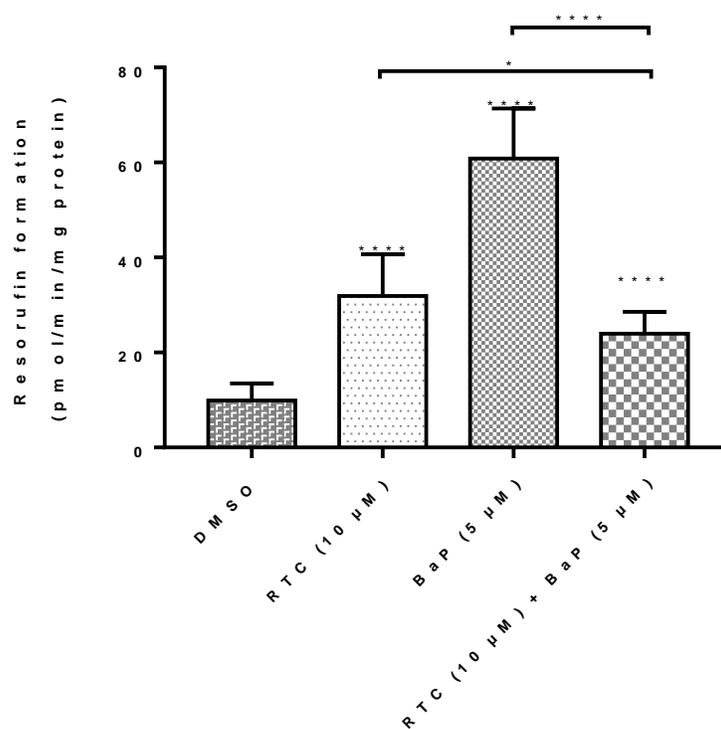
Figure 2. 3 CYP1A1 enzyme activity in 1% DMSO (vehicle), 10 μM RTC, 5 μM BaP or 10 μM RTC and 5 μM BaP treated Hep3B cells. ***p<0.0005, ****p<0.0001.

2.4 Discussions

Based on the results from this chapter, we concluded that CYP1A1 activity is induced by either RTC or BaP pretreatment *in vitro*. Meanwhile, co-administration of RTC and BaP induced less CYP1A1 enzyme activity than BaP administration alone in both H292 and Hep3B cells.

Chapter 3: Investigating the Effect of Rutaecarpine on BaP–Induced DNA Damage *in vitro*

3.1 Material and Methodology

3.1.1 Materials

Muse™ Multi-Color DNA Damage Kits were purchased from Millipore Sigma (Burlington, MA, USA). Muse® Cell Analyzer were obtained from Millipore (Hayward, CA, USA).

3.1.2 Measurement of DNA damage (Muse^{TM} multi-color DNA damage kit)

H292 were cultured in RPMI-1640 and Hep3B cells were cultured in EMEM, and both types of mediums were supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated in an incubator (5% CO2, 37 °C) and were grown to reach 70-80 % confluency in a 12-well plate before experiments. Wells (n = 3/treatment) were treated with 1 % DMSO (vehicle) or 5 μM BaP and /or 10 μM RTC, respectively, for 24 hours at 37 °C. Cell lines were sub-cultured by enzymatic digestion with TrypLE solution and collected. Cells were then washed twice with 1× PBS and re-suspended in assay buffer for DNA damage assay. DNA damage was measured with the Muse® Cell Analyzer using the Muse^{TM} Multi-Color DNA Damage kit which measures DNA damage by detecting the phosphorylation state of ATM and Histone H2A.X simultaneously. The phosphorylation of the two antibodies in response to DNA damaging reagents (e.g., etoposide) or UV light indicates that double-strand DNA breaks have occurred. Data was presented as proportional DNA damage (%).

3.2 Results

We studied the DNA damage level in H292 and Hep3B cells after 10 μM RTC, 5 μM BaP and co-administered 10 μM RTC and 5 μM BaP treatments. The results of Muse^{TM} Multi-Color

DNA Damage kit (Figure 3.1) showed that there were no significant differences of DNA damage between DMSO control treatment and 10 μM RTC treatment groups, which meant 10 μM RTC did not cause significant DNA damage in cells. The percentage of total DNA damage cells in the control and BaP treatment group was 7.9 and 52.4% in H292 cells, 4.8 and 65.1% in Hep3B cells, respectively. The DNA damage ratio of 6.63 (BaP to control treatment group) in H292 cells is comparable to the reported ratio of 6.68 in neonatal human dermal fibroblasts tested by TUNEL assay [24]. And the DNA damage caused by BaP alone was about 2-fold higher than that by co-administration of RTC and BaP in the two cell lines. We postulated that co-administration of RTC and BaP resulted in less DNA damage than BaP administration alone due to fewer metabolites (BPDE) being formed, due to lower CYP1A1 activities in RTC and BaP co-administered cells. These results are in agreement with the previous results in which the activity of CYP1A1 under co-administration of RTC and BaP was less than that of BaP alone.
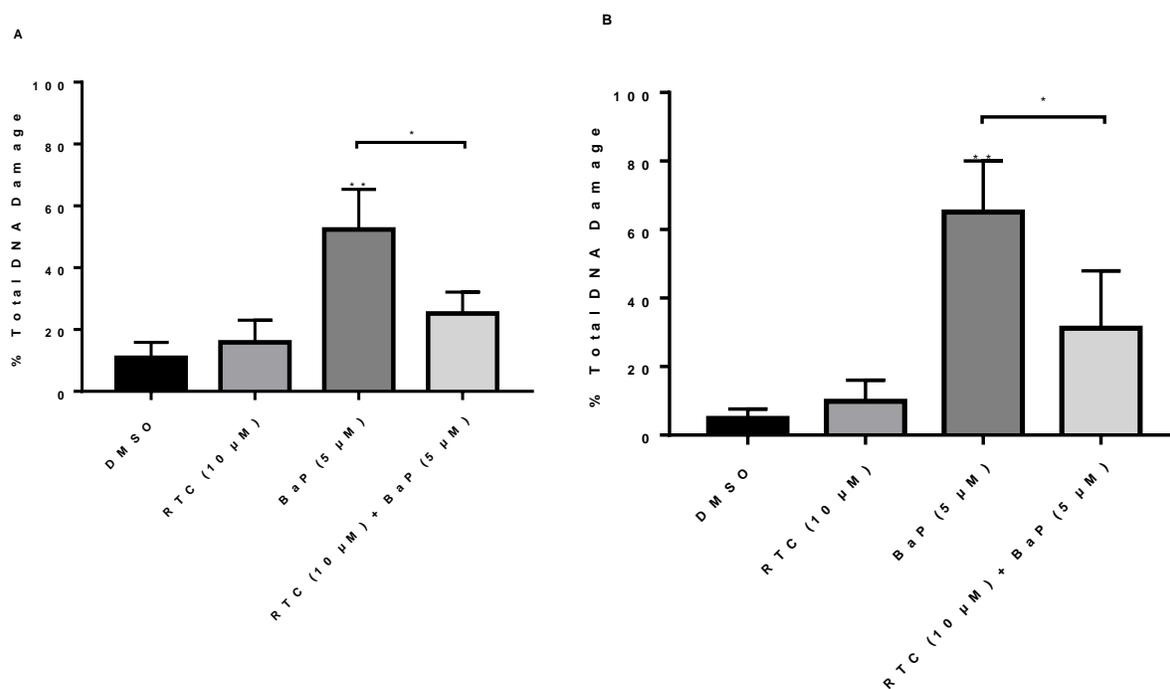
Figure 3. 1 DNA damage caused by different treatments: (DMSO (vehicle), 10 μM RTC, 5 μM BaP, 10 μM RTC and 5 μM BaP) in (A). H292 cells and (B). Hep3B cells. **p<0.005, *p<0.05

3.3 Discussions

During the metabolic process of BaP, reactive oxygen species (ROS) and metabolites will be produced via CYP1A1 enzyme [24]. These ROS and metabolites, mainly BPDE, can cause oxidative DNA damage and form adducts with DNA, starting the mutagenic chain of events responsible for tumor initiation [25]. Thus, DNA damage level could be regarded as an indicator for the formation of metabolites (BPDE) of BaP, then the reduction of DNA damage caused by BaP suggested less metabolism of BaP. The results of DNA damage studies further confirmed previous enzyme activity studies that co-administration of RTC and BaP decreases BaP-induced

CYP1A1 enzyme activity and showed that RTC has a potential protective effect on BaP-induced

DNA damage.

Chapter 4: Concentration-Dependent Induction of CYP1A1 by Rutaecarpine in the Presence of Benzo[a]pyrene in H292 Cells

4.1 Statement of Purpose

Due to the unexpected and unexplained results of the effect of RTC on the BaP-induced CYP1A1 enzyme activity in H292 and Hep3B cell lines, further enzymatic studies need to be undertaken in order to investigate the effect of RTC on CYP1A1 enzyme activity. This chapter introduces the concentration-dependent study of RTC on CYP1A1 and BaP-induced CYP1A1 enzyme activity in H292 cells conducted using EROD and BCA assay.

4.2 Methodology

4.2.1 Ethoxyresorufin-O-deethylase (EROD) assay

H292 were cultured in RPMI-1640 and the medium was supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated in an incubator (5% CO_2, 37 °C) and they were grown to reach confluence in a 6-well plate before experiments. Wells (n = 3/treatment) were incubated with BaP (5 μM) and/or varying concentrations of RTC (1.25 μM, 2.5 μM, 5 μM, and 10 μM) in fresh media containing 0.1% DMSO for 24 h, followed by washing with fresh medium. The cells were then incubated in fresh media containing 5 μM 7-ethoxyresorufin and 10 μM dicumarol for 1h before 200 μl medium of each well was sampled and analyzed with a fluorimeter, under an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The resorufin calibration curve, ranging from 25 nM to 1600 nM, was prepared in duplicate and analyzed. The fluorescence readings were normalized by protein concentrations.

4.2.2 BCA protein assay

After EROD assay, cells were harvested by trypsinized and washed with PBS.
Centrifuged the cells for 5 minutes at 400 g to collected cell pellet and resuspended cells in 20
μL lysis buffer. Through three times of freeze-thaw cycles and 30 minutes incubation on ice,
cells were lysed and the protein were obtained by 10 minutes 16000 g centrifugation at 4°C. A
standard calibration curve for bovine serum albumin (BSA) with seven concentrations, ranging
from 0-1.5 mg/mL, were prepared in duplicate. All serial dilutions were made with 0.1 M
phosphate buffer. 20 μL of standards and 20 μL cell samples were added individually into each
well of a 96-well plate. With a multi-channel pipette, 180 μL of Pierce reagent A and B mixture
(50:1, respectively) was added to each well (containing samples or standards). The plate was
incubated at 37 °C for 30 min, after which the reactions were analyzed at 562 nm using Tristar
LB 941 multimode microplate reader.

4.2.3 Statistical analysis

GraphPad Prism (Version 7.0) software was used for statistical analysis. Analysis of
variance (ANOVA) with post hoc Tukey's multiple comparisons test (compares means of each
group with every other group) were performed on all the data sets. The acceptance level of
statistical significance was P<0.05.

4.3 Results

As Figure 4.1 shows, the induction of CYP1A1 activity by RTC in H292 cells was
dependent on RTC concentrations. The lowest concentration of RTC (1.25 μM) shows the
highest induction of CYP1A1 enzyme activity which is significantly (p<0.005) higher than it by
5 μM BaP. When the concentration of RTC was doubled to 2.5 μM, there was a significant

(p<0.05) decrease in CYP1A1 activity. Although 5 μM RTC did not show a reduction of CYP1A1 activity in comparison to 2.5 μM, we can see that 10 μM RTC caused another significant (p<0.005) decrease in enzyme activity. These results suggest that as concentration of RTC increased, there was a decrease of CY1P1A1 induction by RTC.
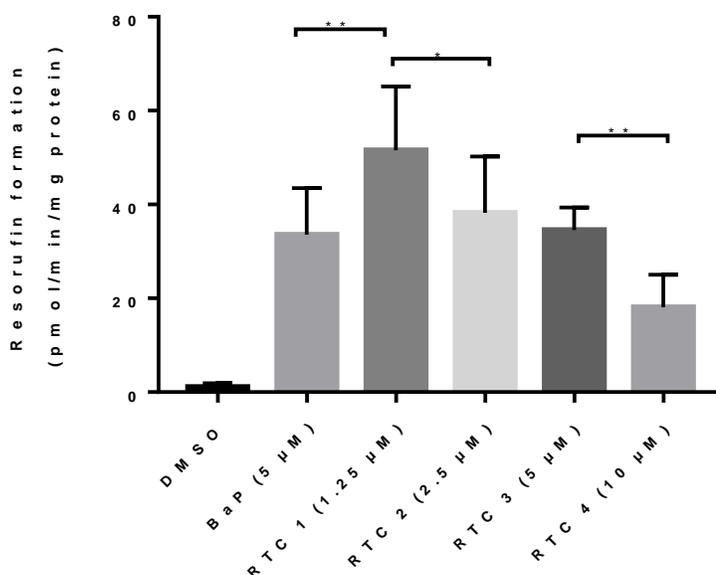

Figure 4. 1 CYP1A1 enzyme activity in 1% DMSO (vehicle) or 5 μM BaP or different concentrations (1.25 μM, 2.5 μM, 5 μM, 10 μM) of RTC treated H292 cells. *p<0.05, **p<0.005.

According to Figure 4.2, BaP-induced CYP1A1 enzyme activities in H292 cells were significantly decreased under co-administration of 5 μM and 10 μM RTC (p<0.005, p<0.0001,

respectively). The results show that a higher concentration of RTC exhibits stronger inhibition of BaP-induced CYP1A1 enzyme activities.
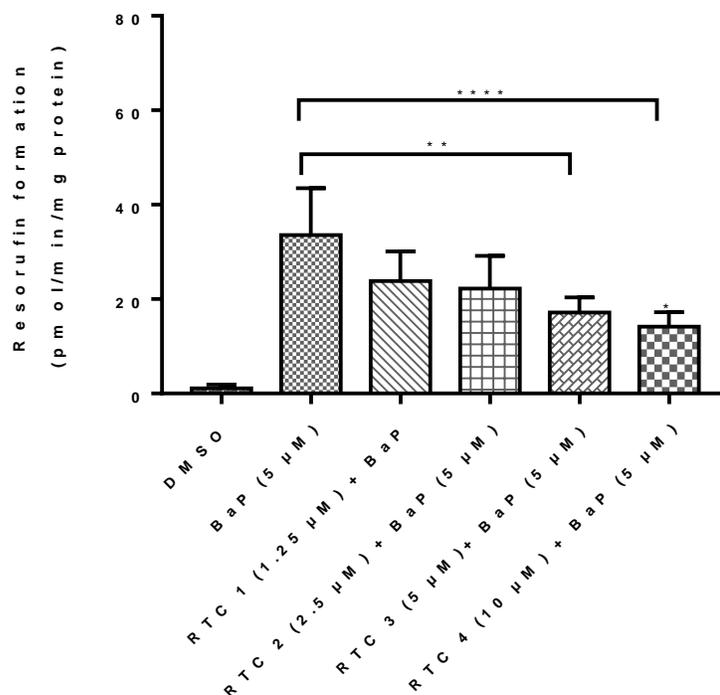
Figure 4. 2 CYP1A1 enzyme activity in 1% DMSO (vehicle) or 5 μM BaP or 5 μM BaP and different concentrations (1.25 μM, 2.5 μM, 5 μM, 10 μM) of RTC treated H292 cells. *p<0.05, **p<0.005, ****p<0.0001.

Interestingly, comparative analysis of the CYP1A1 enzyme activity induced by different concentration of only RTC and the CYP1A1 enzyme activity induced by co-administration of different concentrations of RTC and BaP (Figure 4.3) found that co-administration of 1.25 μM,

2.5 µM, and 5 µM RTC with 5 µM BaP decreased the induction of CYP1A1 (****$p<0.0001$, **$p<0.005$, ***$p<0.0005$). It appears that the inhibition of CYP1A1 enzyme activity by RTC is greater than its induction when co-treated with BaP in H292 cells.
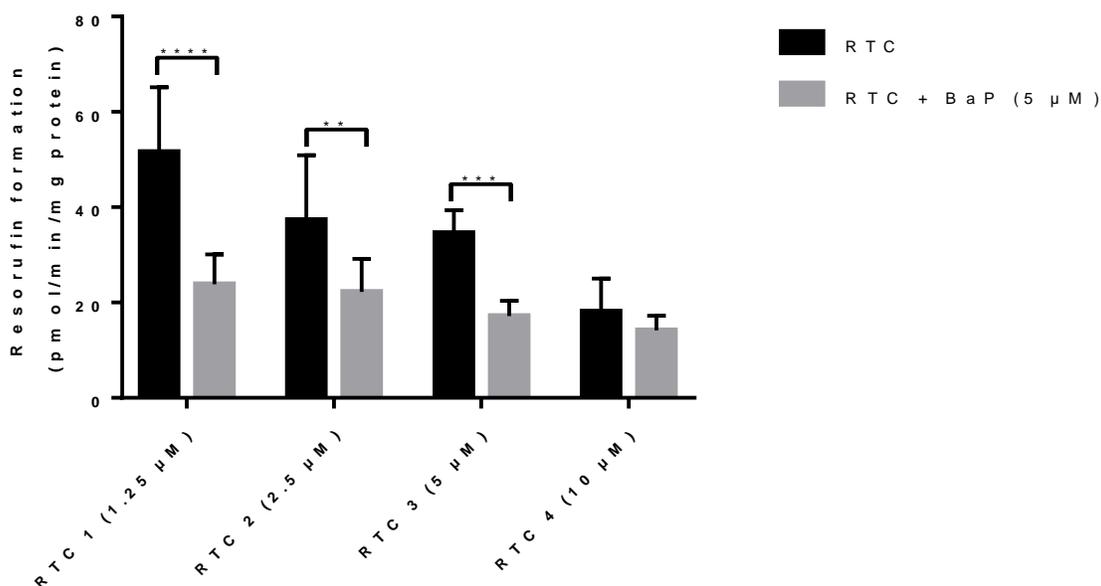
Figure 4. 3 CYP1A1 enzyme activity in different concentrations (1.25 µM, 2.5 µM, 5 µM, 10 µM) of RTC or RTC and 5 µM BaP treated H292 cells. **$p<0.005$, ***$p<0.0005$, ****$p<0.0001$.

4.4 Discussions

According to the results of the concentration-dependent induction of CYP1A1 by RTC study in H292 cells, there is a negative correlation with RTC concentrations from 1.25 to 10 µM and induction of CYP1A1 activity.. As RTC concentration increased, there was a decrease in the

induction of both RTC- and BaP-induced CYP1A1 enzyme activity, which suggests that a higher

concentration of RTC has a greater inhibitory effect on CYP1A1 than its inductive effect.

However, at the concentrations from 0.01 to 1 μM of RTC showed positive correlation with the

induction of CYP1A1 activity in Hepa-1c1c7 cells [18]. There are two possible explanations for

our result: (i) RTC is a competitive inhibitor of CYP1A1 enzyme. However, before we

performed the EROD assay, we washed the cells with fresh medium so we did not expect RTC to

remain in the cells, and compete with 7-ethoxyresorufin during the EROD assay. (ii) RTC is a

mechanism-based inactivator of CYP1A1 enzyme, thus, with increase concentration of RTC,

more CYP1A1 enzymes will be inactivated, resulting in a decrease in CYP1A1 enzyme activity

with increasing RTC concentration. This appears to be a more likely explanation so it was

investigated further.

Chapter 5: Investigating the Potential of Rutaecarpine on Mechanism-Based Inactivation of CYP1A1 Enzyme Activity in β-Naphthoflavone Induced Rat Liver Microsomes

5.1 Introduction

5.1.1 Mechanism-based inactivator (MBI)

A mechanism-based inactivator is also known as a mechanism-based inhibitor or suicide inactivator. The definition of MBI can be described as an unreactive compound whose structure resembles that of either the substrate or product of the target enzyme and which undergoes a catalytic transformation by the enzyme to a species that, before release from the active site, inactivates the enzyme [26]. According to this definition, there are two criteria for which an MBI should satisfy. First, the inactivation of MBI is irreversible. Second, a catalytic step is required to convert the inactivator to the ultimate inactivating species at the active site, which suggests the MBI process needs the presence of nicotinamide adenine dinucleotide phosphate (NADPH) to support the catalytic process. Therefore, a NADPH-dependent inactivation study could be used to investigate the potential of MBI.

5.1.2 Time-dependent inactivation (TDI)

Typically, TDI is produced by irreversible covalent or quasi-irreversible, non-covalent, tight binding of a chemically reactive intermediate to an enzyme that catalyzes its formation, resulting in loss of enzyme function. TDI is defined as a compound that causes an increase of inactivation when it is pre-incubated with an enzyme before the addition of the substrate. TDI could be regarded as a necessary criteria for MBI since it indicates an irreversible inhibition process. Hence, TDI is a good tool for the study of MBI.

5.2 Material and Methodology

5.2.1 Materials

β-nicotinamide adenine dinucleotide phosphate (β-NADPH) was purchased from Chem-Impex International, Inc (Wood Dale, IL, USA). β-naphthoflavone (βNF)-induced rat liver microsome was purchased from Sekisui XenoTech, LLC (Kansas City, KS, USA). Alfa Aesar™ 1-Ethynylpyrene was purchased from Fisher Scientific (Pittsburgh, Pennsylvania, USA).

5.2.2 NADPH- and time-dependent inactivation study

1-ethynylpyrene (1-EP), a potent suicide inhibitor of CYP1A1 enzyme [27-29], was used as a positive control in this study. To ensure that 7-ethoxyresorufin in the EROD assay is a proper probe for CYP1A1, β-NF-induced rat liver microsome (RLM) was used to test the enzyme activity [28-30]. The enzyme assay components for pre-incubation included 100 µM RTC or 120 µM 1-EP, 1mM NADPH, 0.1 M KPO_4 buffer (pH 7.4) and 12.5 mg/mL β-NF-induced RLM. All the enzyme assay components except NADPH were equilibrated at 25 °C water bath in a pre-incubation tube. The reaction was initiated by adding NADPH and the final volume of pre-incubation components was made to be 100 µL. As soon as all of the components of the pre-incubation tube were added, a stopwatch was started to mark time zero. Once the solution has been mixed, 10 µL aliquots were removed at 7 time points (30 sec, 1 min 30 sec, 2 min 30 sec, 5 min, 10 min, 20 min, 30 min) and were diluted 10-fold into the EROD assay mixture containing 25 µM 7-ethoxyresorufin and 1mM NADPH and 0.1 M KPO_4 buffer (pH 7.4) pre-equilibrated at 25 °C water bath for 10 minutes, after which the EROD assay was carried out. Quenching reaction was performed after all reactions were finished by adding 100 µL MeOH and spinning the tubes at 1000 g for 5 min. 150 µL supernatant from each tube was

transferred into a 96-well plate and fluorescence was read at excitation wavelength of 544 nm

and an emission wavelength of 590 nm.

5.3 Results

The CYP1A1 enzyme activity with RTC pre-incubation of β-NF-induced RLM was

significantly ($p<0.0005$) lower than that of no treatment (Figure 5.1). In other words, the EROD

assay data of β-NF-induced RLM with different pre-incubation treatments suggested that RTC is

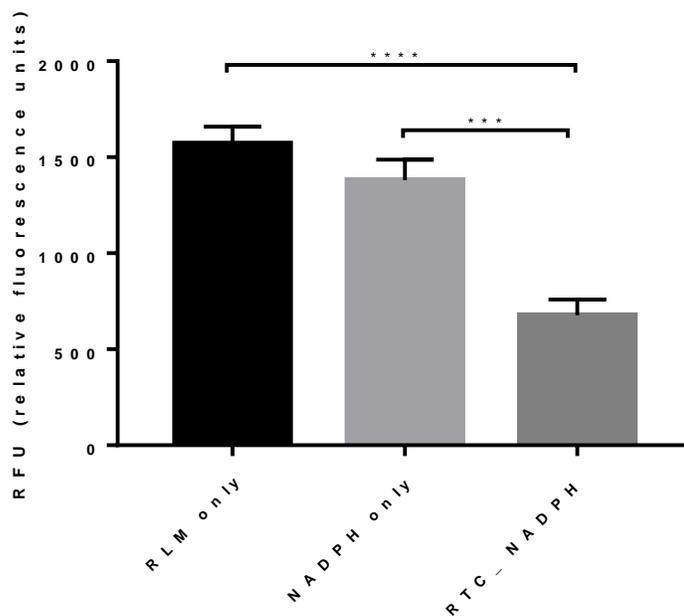an inhibitor of CYP1A1 enzyme, as reported earlier [13, 19].

Figure 5. 1 EROD activity of β-NF-induced RLM with different pre-incubation treatments (RLM
only: no treatment and without NADPH, NADPH only: no treatment with NADPH,
RTC_NADPH: 100 μM RTC with NADPH). ***$p<0.0005$, ****$p<0.0001$.

The results of NADPH- and time-dependent inactivation of CYP1A1 studies were shown in Figure 5.2. CYP1A1 activity at 30 sec was intentionally set as 100 % so that the inhibitory effect contributed by the competitive mechanism would be eliminated. The results of negative control groups, β-NF-induced RLM pre-incubated with 1-EP without NADPH or NADPH alone or nothing showed no significant differences. 1-EP as the positive control showed the percentage of remaining CYP1A1 enzyme activity decreased as the pre-incubation time increased under the presence of NADPH. However, there was no similar observed trend in RTC treatment groups, which suggested that RTC did not show the NADPH- and time-dependent inactivation of CYP1A1, and it might not be a mechanism-based inactivator of CYP1A1 as was previously proposed.

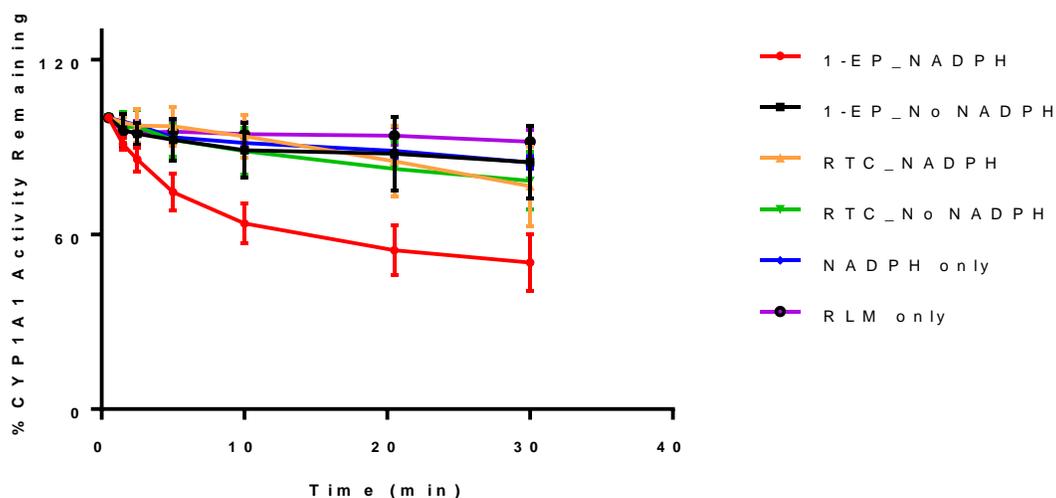Figure 5. 2 NADPH- and time-dependent inactivation of CYP1A1 by 1-EP or RTC using β-NF-induced RLM.

5.4 Discussions

The data from CYP1A1 enzyme activity studies shown above show that at the concentration tested (100 μM), RTC did not exhibit MBI characteristics. To further investigate if RTC is a mechanism based inactivator of CYP1A1, more concentrations of RTC for NADPH- and time-dependent study need to be tested.

Conclusion

In H292 and Hep3B cells, both BaP and RTC induced CYP1A1 activity. However, co-administration of RTC and BaP induced less CYP1A1 enzyme activity than only BaP alone. A negative correlation between the induction of CYP1A1 enzyme activity by RTC and concentrations of RTC was found in H292 cells. An increase of RTC concentration produced a reduction of CYP1A1 or BaP-induced CYP1A1 enzyme activity. Furthermore, RTC exhibited a protective effect on BaP-induced DNA Damage. However, since RTC did not show mechanism-based inactivation effect on CYP1A1 enzyme activity in βNF-induced RLM, we have yet to explain the previous results.

In general, our data suggested a crucial role of RTC in regulating the CYP1A1 enzyme activity of lung cancer cells and hepatocytes, revealing that RTC might be a hopeful target for the chemoprevention of lung cancer. And the study of the mechanisms of the potential protective effect of RTC on BaP-induced carcinogenicity is an important and necessary step to further identify chemopreventive candidates for human lung cancer.

Future Plan

Some limitations of the methods in this work can be improved by future studies. First, since we did not measure the concentrations of RTC inside the cells, we cannot know for sure whether or not RTC has been removed completely from the cell after RTC pretreatment and before EROD assay. If the washed cells still contain RTC then the residual RTC may compete with 7-ethoxyresorufin for binding to CYP1A1 active sitewhen we used the EROD assay to measure CYP1A1 enzyme activity. Therefore, future studies can be performed, utilizing a quantitative method like LC-MS/MS, to quantify the level of RTC inside cells after pretreatment to rule out the effect of intracellular RTC on enzyme activity. In addition, pre-treatment of RTC instead of co-treatment of RTC and BaP could be tried to mitigate the possible effect of residual RTC. Second, to derive a more rational mechanism of the inhibition of CYP1A1 by RTC, different concentrations of RTC need to be used for NADPH- and time-dependent study so that enzyme kinetic parameters can be collected to obtain more insight. Third, for *in vitro* studies, we only used two cell lines, more different types of cell lines can be used in future to get more insights. Fourth, in our studies, we only tested CYP1A1 enzyme activity. For future studies mRNA and protein expressions are also worth measuring. Therefore, RT-qPCR and Western blotting assay can be used to trace the regulation of the enzyme. Fifth, previous studies report RTC induces the activity and expression of Phase 2 carcinogen detoxifying enzymes, such as NAD(P)H:quinone reductase (QR) in Hepa-1c1c7 cells [31] which suggests that rutaecarpine has a chemopreventive effect through its ability to modulate carcinogen detoxification pathways. Therefore, the less DNA damage caused by BaP after co-administration of RTC might not only be due to less CYP1A1 enzyme activity. To further investigate the effect of RTC on the metabolism of BaP, the ultimate carcinogenic metabolite, BPDE, should be quantified by LC-

MS/MS method. Last, only *in vitro* experiments are not adequate to draw the conclusion. *In vivo* experiments need to be conducted in further studies.

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